UNIVERSITÄT BERN

Graduate School for Cellular and Biomedical Sciences

University of Bern

The importance of pneumococcal capsule in innate immunity and biofilm formation

PhD Thesis submitted by

Eliane Küng

from **Heiligenschwendi (BE)**

Thesis advisors

Prof. Dr. Kathrin Mühlemann †
Dr. Markus Hilty
Dr. Lucy J. Hathaway

Institute for Infectious Diseases
Faculty of Medicine, University of Bern

Accepted by the Faculty of Medicine, the Faculty of Science, and the Vetsuisse Faculty of the University of Bern at the request of the Graduate School for Cellular and Biomedical Sciences

Bern, Dean of the Faculty of Medicine

Bern, Dean of the Faculty of Science

Bern, Dean of the Vetsuisse Faculty Bern

Table of contents

List of abbreviations

aliA	ami-like gene encoding peptide binding protein of an ABC transporter, gene immediately downstream of capsule operon
ATP	adenosine triphosphate
BHI	Brain Heart Infusion
bp	base pair
BSA	bovine serum albumin
CCC	child care center
CcpA	catabolite control protein A
CD	Cluster of Differentiation
CDM	Chemically defined medium
CF	cystic fibrosis
CFU	colony forming unit
Com	competence protein
cpsA	first gene in pneumococcal capsule operon
CSBA	Columbia sheep blood agar
CSP	Competence-stimulating peptide
CXCL8	Interleukin 8
dexB	glucan 1,6-alpha-glucosidase, gene immediately preceding capsule operon
DNA	deoxyribonucleic acid

ECM	extracellular matrix
eDNA	extracellular DNA
EPS	exopolysaccharide matrix
FBS	fetal bovine serum
FITC	Fluorescein isothiocyanate
ICAM	Intracellular adhesion molecule
IFN	Interferon
iHBEC	immortalized human bronchial epithelial cells
IL	Interleukin
LPS	Lipopolysaccharide
LytA	N-acetylmuramoyl-l-alanine amidase (autolysin)
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PLY	pneumolysin
PRR	pattern-recognition receptor
Psp	pneumococcal surface protein
RNA	ribonucleic acid
RSV	Respiratory Syncytial Virus
SCV	Small colony variant
TGF	Transforming growth factor

TLR	Toll-like receptor
TNF	Tumor necrosis factor
UndPP	undecaprenyl lipid carrier
WHO	World Health Organization
wzx	flippase, involved in capsule biosynthesis
wzy	polymerase, involved in capsule biosynthesis

Overview and summary of the thesis

Chapter 1 gives an overview about *Streptococcus pneumoniae*, its role as a human pathogen and its virulence factors. Additionally, biofilm development and its relevance in clinics are introduced, and the innate immune response to pneumococcus as well as bacterial-viral interactions in the upper respiratory tract are also discussed.

Chapter 2 emphasizes the three main topics of this thesis: the role of capsule and pneumolysin in the immune response in the respiratory tract, biofilm formation of *S. pneumoniae* serotypes and commensal streptococci *in vitro*, and host innate immune responses to RSV and *S. pneumoniae* during *in vitro* co-infections. Aims and hypotheses are provided here.

Chapter 3 is divided into two parts:

First, the release of the pro-inflammatory cytokines CXCL8 and IL-6 from the human pharyngeal epithelial cell line Detroit 562 and from human bronchial epithelial cells (iHBEC) is described in response to *S. pneumoniae*. Capsule was shown to suppress the release of both cytokines in both cell lines tested, but release was much less from iHBEC cells. During intranasal colonization of mice, suppression of CXCL8 release by the capsule was also observed *in vivo*, but the effect was only measured in the absence of pneumolysin. Long term, stable nasopharyngeal carriage in a mouse model resulted in the dissemination of nonencapsulated pneumococci into the lungs, whereas encapsulated strains remained in the nasopharynx. The *S. pneumoniae* capsule thus plays a role in modulation of the pro-inflammatory immune response in the respiratory tract.

Second, results on immunological cells and immune regulation in a long term, stable nasopharyngeal carriage mouse model are presented. Mice were infected with encapsulated or nonencapsulated pneumococcal strains, and after 1, 3, 8 and 15 days, were sacrificed to evaluate the numbers of CD45+ cells, neutrophils, macrophages, FoxP3+ regulatory T-cells and CD3+ T-cells in the nasal mucosa as well as the amount of secreted IL-10 in the

nasopharynx. Nasopharyngeal colonization which is effectively silent resulted in the stimulation of FoxP3+ regulatory T-cells and IL-10 release associated with immune homeostasis, whereas lung infiltration was required to increase the number of neutrophils and macrophages resulting in a stronger innate immune response in the nasal mucosa.

Chapter 4 contains results of mono- and co-stimulation using RSV and pneumococci or pneumococcal virulence factors on the human bronchial epithelial cell line BEAS-2B. An increase in CXCL8 and IL-6 levels was measured for mixed stimulations of RSV and pneumococcus when encapsulated bacteria were used. Increasing pneumolysin concentrations resulted in enhanced CXCL8 levels. Priming of bronchial epithelial cells with RSV opens the door for more severe pneumococcal infections.

Chapter 5 is composed of two parts:

The first part describes initial biofilm formation of serotypes 6B and 7F in a static model *in vitro*. Biofilms of both serotypes contained SCVs, but only serotype 6B increased in SCV formation between 16 and 65h of incubation. SCV stability was tested by passaging clones in complex medium, where SCV production is not associated with advantages in growth. Serotype 6B lost the SCV phenotype indicating a fast adaptation to a changing nutritional environment. Limitations of our *in vitro* model are discussed.

The second part is about initial biofilm formation of mixed culture growth of *S. pneumoniae* with commensal streptococci. Competition dominates this process. *S. oralis* and pneumococcus compete for nutrients, whereas mixed species growth of *S. mitis* or *S. pseudopneumoniae* with *S. pneumoniae* is mainly influenced by other factors.

In **Chapter 6** the findings of chapters 3, 4 and 5 are discussed and an outlook for further studies is provided.

Chapters 7, 8, 9, 10 and 11 contain the references, the acknowledgements, the *curriculum vitae*, the appendix and the declaration of originality.

1. General Introduction

1.1 *Streptococcus pneumoniae*

1.1.1 Description

Billroth observed chain-forming cocci in wounds in 1878 and cocci were first isolated by Georg Miller Sternberg and Louis Pasteur in 1881 [1]. One member of this genus that they had discovered, *Streptococcus,* is *Streptococcus pneumoniae,* the subject of this thesis.

Taxonomy level	Description
Kingdom	Cellular organisms
Superkingdom	Bacteria
Phylum	*Firmicutes*
Class	*Bacilli*
Order	*Lactobacillales*
Family	*Streptococcaceae*
Genus	*Streptococcus*
Species	*Streptococcus pneumoniae*

Table 1: Taxonomic classification of *Streptococcus pneumoniae*

S. pneumoniae (the pneumococcus) is a Gram-positive diplococcus [2, 3]. This strict human pathogen [4] uses the nasopharynx as major ecological reservoir [4, 5] and has the potential to cause serious diseases such as pneumonia, meningitis and sepsis as well as the common childhood condition of otitis media. Bacterial cells show a spherical or ovoid shape, are facultatively anaerobic, non-spore forming, homofermentative, catalase negative and require complex nutritional environments for growth. They have a fermentative metabolism which produces L(+) lactic acid as major product in glucose fermentation [6]. One single circular chromosome of about 2 million base pairs (bp) forms the pneumococcal genome with a guanine + cytosine content of 39.7% [7]. A 200 to 400 nm thick polysaccharide capsule covers the peptidoglycan cell wall in the majority of strains [8]. Each of the currently known 93 different serotypes [9, 10] of *S. pneumoniae* has capsular polysaccharides differing in structure and antigenicity [11]. First observed in 1928 by Griffith [12] and

described in more detail by Avery and co-workers in 1944 [13], the pneumococcus is naturally competent and able to take up DNA from the environment [14].

1.1.2 The genus streptococcus

One of the first methodologies to divide the streptococci into different groups was developed by Lancefield in 1933 using differences in precipitin reactions based on group-specific carbohydrates [15]. Today, the genus streptococcus containing about 40 species [6] is grouped into α-, β-, and γ-hemolytic species and these groups are sub-divided into six clusters: the salivarius, the mitis, the angiosus, the pyogenic, the mutans, and the bovis clusters[16]. The three first are also often termed viridans streptococci. Natural competence seems to occur mainly in the Mitis and the Angiosus groups [14]. The streptococcal species important for this study are *S. pneumoniae*, *S. mitis*, *S. oralis* as well as the recently described *S. pseudopneumoniae* [17] which belong to the Mitis cluster, whereas *S. danieliae* found in 2013 belongs to the Viridans streptococci [18]. There is a high degree of similarity between commensal streptococci and the pneumococcus which makes diagnostics more complicated [19].

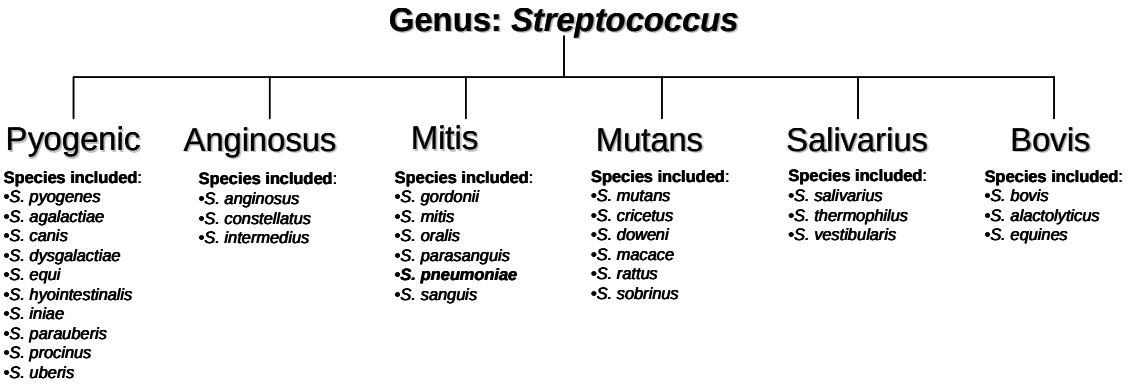


Figure 1: Clustering of the genus *Streptococcus* (modified from [6])

1.1.3 Pneumococcal evolution

The most important factors influencing the evolution of bacterial clones are recombination, mutations, selective forces as well as bacterial and host properties which affect colonization and transmission [20]. As the pneumococcus is naturally competent, it is able to incorporate DNA fragments from pneumococcal and non-pneumococcal bacterial species into its genome. For this process, homologous recombination is the most important tool [21]. An extreme diversity in pneumococcal populations is observed as a result of high levels of recombination after which variant genotypes can easily emerge. Selective forces for this process are the ecological environment, the innate and adaptive immune responses of the host as well as antibiotic pressure. Interestingly, transformation-competent cells are able to kill non-competent cells and the DNA released can then be taken up [20, 21].

1.1.4 Competence

Competence is defined as the process of DNA uptake from the surrounding medium to replace homologous regions in the host's genome which results in a permanent change in the cell's phenotype. In *S. pneumoniae* regulation of competence is performed via a quorum sensing system via the competence stimulating peptide (CSP) (Figure 1). For binding, uptake, and integration of foreign DNA, a unique set of genes is required. In naturally competent streptococci, the competence regulation operon *comCDE* is responsible for this process: ComC is a precursor of the competence-stimulating peptide CSP, processed and secreted by the ComAB transporter. ComD is a histidine kinase and *comE* encodes its cognate response regulator. Autophosphorylation of ComD leads to the transfer of a phosphoryl group to ComE which then leads to the activation of early genes *comAB*, *comCDE*, *comW* and *comX* [14]. *ComX* encodes a competence-specific σ-factor which is able to recognize a -10 σ^X-promoter. This promoter is found in front of the late *com* genes and thus leads to the transcription of genes responsible for DNA uptake and processing [22].

Competence is induced when a certain level of CSP in reached in the medium [14]. Autocatalytic accumulation of CSP is started when a critical threshold concentration is reached. Thus, competence induction in pneumococcal cultures is highly synchronic: The transcription of early *com* genes reaches a maximum after 5-7 minutes and late *com* genes after 10-12 minutes [22, 23].

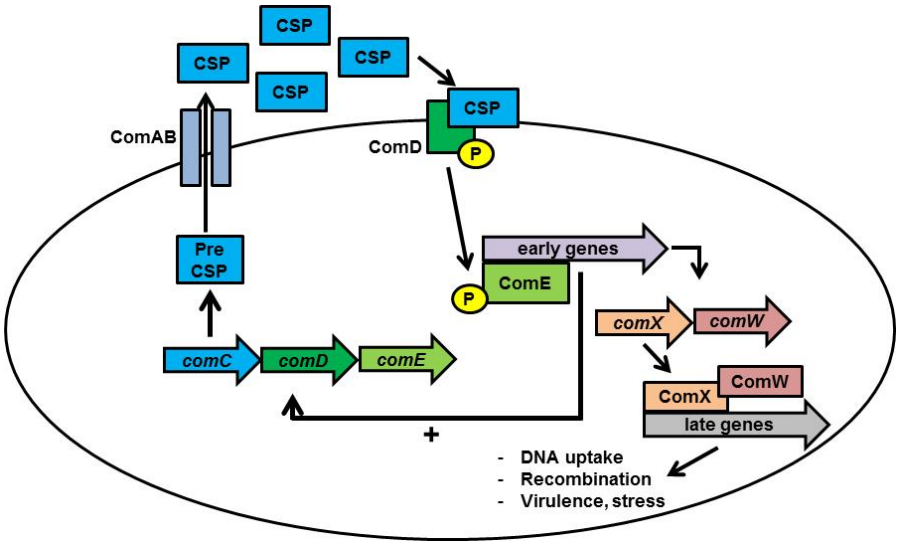


Figure 2: Regulation of competence in the pneumococcus. *comC* encodes pre-CSP which is then processed and secreted by the ComAB transporter. As a result, extracellular mature CSP accumulates. CSP then binds to its receptor ComD leading to autophosphorylation of ComD followed by transfer of the phosphoryl group to ComE. This leads to the activation of early genes and the auto-induction of a positive feedback loop increasing the extracellular CSP. Additionally, the alternative σ factor ComX as well as ComW are triggered. Proteolytic cleavage of ComX is prevented by ComW. The production of late genes involved in DNA uptake, recombination and virulence or stress is then induced (modified from [24]).

In all *S. pneumoniae* strains a functional *comCD* unit is found which means that competence is a very important, if not essential, tool for these bacteria [23]. Besides pneumococci, other streptococci are also naturally competent. Most of these species are present in the Mitis cluster [25]. Generally, CSP seems to be pherotype-specific but for a few species cross-induction was observed to occur. [14].

1.2 The pneumococcus as a pathogen

1.2.1 The human pathogen

Generally, pneumococci colonize the human nasopharynx asymptomatically by binding to the mucosal epithelial cells. However, if the bacteria spread to the sinuses or to the middle ear, if they are aspirated into the lungs or invade the bloodstream, infections are started [5]. More people die of *S. pneumoniae* infections than of the Human Immunodeficiency Virus (HIV) in the United States and mainly the very old and the very young are affected.

One of the major pediatric problems worldwide is otitis media where antibiotic treatment is a big challenge [26]. In special cases, chronicity was shown to be caused by pneumococci surviving intracellularly in middle ear mucosa epithelial cells where the bacteria cannot be eliminated by antibiotic therapies. This phenomenon then might lead to hearing loss and associated learning difficulties in young children [27].

1.2.1.1 Epidemiology

Child-care centres (CCC) cause an increased risk for children to suffer from infectious diseases due to the frequency of contacts in crowded conditions. In addition to the significant morbidity there is also an important economic problem due to work loss and medical care cost. The biggest problems in CCCs are the increased risk of young children carrying pneumococci, the high rates of viral infections in these children and the poor hygienic behaviour. Children attending CCCs are at a 6x higher risk of becoming pneumococcal carriers than children not attending CCCs and these institutions are important for the variability of pneumococcal serotypes in carriage in the population [28]. Thus, often young children are responsible for the introduction of new serotypes into a household [29].

Interestingly, there are differences in pneumococcal infections observed between different ethnicities. For example, the indigenous populations in Alaska, Canada, Greenland as well as the American Indians, African Americans in the USA, Australian Aborigines, Maoris of New Zealand and the Bedouins of Israel are more often affected by invasive pneumococcal diseases and there is evidence that genetic factors play a role [5, 30]. Socio-economic factors also play a role in infections as shown by the fact that *Moraxella catarrhalis*, *Staphylococcus aureus* and antibiotic resistant *S. pneumoniae* were more often carried by children from lower socio-economic schools in Belgium as vaccination is less common than in higher socio-economic schools [31]. Additionally, passive cigarette smoke also significantly increases the risk factor for invasive disease, mainly in children aged younger than 5 years [32]. Patients suffering from B and T cell defects, congenital asplenia, defects of the complement pathway, interleukin-1 receptor-associated kinase 4 (IRAK-4) deficiency or ectodermal dysplasia with immunodeficiency form the high-risk group for developing severe and invasive pneumococcal disease [33]. People suffering from sickle cell disease show a 30-600 fold higher risk of developing invasive pneumococcal disease than individuals not suffering from sickle cell disease [34]. An absence of spleen increases the risk of pneumococcal infections 10-50 times, mainly in the blood and the lungs [35] whereas children suffering from acute lymphoblatic leukaemia show, depending on their age, a 8-50 times increased risk for infectious pulmonary disease compared to the healthy population [36]. Organ transplantation is another risk factor for invasive pneumococcal disease, mainly if chronic graft versus host disease occurs [5].

Generally, mortality rates for bacteremic pneumococcal pneumoniae are <3% for children and 10-30% for adults. Fatal meningitis is observed in 16-37% of infected adults and in 1-2.6% of infected children [5]. Many factors have been shown to be associated with an increased risk to die from pneumococcal pneumonia such as, for example, underlying chronic disease, severity of illness, older age (> 65 years), immunosuppression [37], nursing home living, need for mechanical ventilation, chronic pulmonary disease [38], nosocomial

pneumococcal bacteremia, diabetes mellitus, congestive heart failure, malignancy, alcoholism [39] or parenteral nutrition [40].

1.2.1.2 Vaccination

Already one year after the first isolation of pneumococcus, immunizing experiments were performed. The first larger clinical trial dates back to 1911, but due to the lack of knowledge about the different pneumococcal serotypes the vaccinations failed. In the 1980s two polysaccharide-based pneumococcal vaccines were introduced, first a 14-valent pneumococcal vaccine, and 6 years later a 23-valent vaccine. Children younger than 2 years are not able to produce antibodies against polysaccharides as a T-cell independent immune response is induced and thus pneumococcal conjugate vaccines such as the 7-valent PCV7 (Prevnar ®, Pfizer Inc.; which covers serotypes 4, 6B, 9V, 14, 18C, 19F and 23F [41]) and the 13-valent PCV13 (Prevnar 13 ®, Pfizer Inc.; which covers serotypes 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F and 19A [41]) were introduced [42]. In Switzerland, PCV7 was introduced in 2006 and replaced by PCV13 in 2011 [43]. Interestingly, in nature capsular switching is possible via transformation and recombination when genes encoding one capsular type are taken up by pneumococci of a different serotype. Of concern is the possibility of vaccine-to-nonvaccine serotype switching as the rate of serotype replacement is significantly increased by vaccination [44]. Different types of polysaccharide-based vaccines are currently available or being tested (Table 2):

Type of vaccine	Advantages	Disadvantages
Capsular polysaccharides	Elicit antibodies similar to natural response, safe, easy to purify	Poorly immunogenic, no immunological memory
Capsular polysaccharide-protein conjugate	Elicit antibodies similar to natural response, T-lymphocyte dependent response in infants and children, safe	No T-lymphocyte dependent response in elderly, most opsonic IgG subclasses not produced, undesired immunity against carrier protein possible
Peptide mimotype of polysaccharide antigen	Biochemically defined, T-lymphocyte dependent response and immunological memory, focus response on protective antibodies	Poorly immunogenic without carrier molecule, no experience for efficacy in humans

Table 2: polysaccharide-based vaccines (modified from [45]).

Vaccine candidates
Pneumolysin (PLY)
Pneumococcal surface protein A (PspA)
Pneumococcal surface protein C (PspC)
Pneumococcal surface antigen A (PsaA)
Adhesins (targeting diferent infection stages)

Table 3: The most promising vaccine candidates against pneumococcus (modified from [46])

Currently, other pneumococcal components are also being tested as candidates for a non-serotype-specific vaccine. Table 3 lists the most promising candidates. All the different components have already been tested in mouse models resulting in an increased survival of infected animals [46].

Another approach to reduce pneumococcal infection is the principle of bacterial replacement. In oral microbiology for example, a reduction in dental caries was observed after ingestion of probiotic lactobacilli. In the nasopharynx of infants it was shown that an infection with *S. pneumoniae* results in a reduced microbial diversity compared to healthy children [47]The main idea of bacterial replacement is to add a non-pathogenic “effector” bacterium which is able to out-compete potential pathogens but a prerequisite for this process is the persistence of the effector in the normal flora which is often is difficult to obtain [48].

1.2.2 Pneumococcus in the human body

1.2.2.1 Nutritional environment

S. pneumoniae is able to use a huge variety of different sugars for growth such as pentose, cellobiose, galactose, galactitol, fucose, fructose, lactose, glucose, mannitol, glycerol, raffinose, mannose, maltosaccharides and trehalose [7]. All of them are directly involved in the glycolytic pathway. As a nitrogen and a carbon source, ten different amino acids and *N*-acetylglucosamine can be used. Many different ATP-dependent transporters have been identified and over 30% of all transporters are involved in sugar transport. Host glycoproteins as well as murein polysaccharides are used as major carbon sources but the pneumococci's own capsular polysaccharides (mainly mucins, hyaluronic acid and glycolipids) are important donors as well. ATP- and ion-driven transporters for amino acids, uracil, xanthine and polyamines were identified but there was only one single transporter found able to transfer di- and monocarboxylates [7].

1.2.2.2 Ecology

S. pneumoniae is generally a strict human pathogen but serotype 3 has been found as a natural pathogen in horses [11]. The major ecological reservoir of the pneumococcus is the nasopharynx with 30-50% of young children, 4-10% of all adults and 8% of all adolescents being carriers [5]. The evolutionary selective pressure for absolute pathogenicity factors is very low and thus *S. pneumoniae* colonizes the nasopharynx without causing an invasive disease in most cases. Nasopharyngeal colonization is a good way to spread to other hosts via aerosols and mucous exchange [4]. Nutrients, space, attachment as well as co-inhabitants are important ecological factors influencing bacterial presence in a microecosystem. Co-colonization is mainly influenced by the production of harmful substances and the induction of distinct immune responses. Competition of bacteria from the same species is mainly due to limited shared resources whereas competition between two different species is mediated by inhibitory agents and toxins or the immune system [49].

In changing environmental conditions bacteria are able to change the transcription of carbohydrate utilization genes and virulence factors. Studies in *Bacillus subtilis*, the model organism of Gram-positive bacteria, have shown that changes in environmental carbohydrate concentrations result in different expression levels of the catabolite control protein A (CcpA). CcpA is the direct link between the environmental carbohydrate levels and the transcriptional regulation of carbohydrate utilization genes. Even virulence genes were affected which results from the fact that CcpA is a global regulator of carbohydrate metabolism in *S. pneumoniae* with important effects on transcriptional regulator and virulence factor genes [50]. CodY, a nutritional repressor, is able to repress the transcription of more than 100 genes during exponential growth involved in different processes such as development of genetic competence, peptide uptake, branched-chain amino acid biosynthesis, motility, and sugar uptake [51].

In *B. subtilis* it was shown that during starvation the extracellular signals are able to induce more than one regulon and at least 400 genes change their expression program. Most of the glucose-starvation inducible proteins show similar expression kinetics: The induction period is short, then the gene expression is switched off but there still is a transient induction observed which results in the accumulation of proteins which are required during glucose starvation [52].

1.2.2.3 Bacterial colonization of the human body

Microbial colonization starts immediately after birth and a few weeks later the child's microbial flora has developed to become remarkably similar to that of the adult [53]. One of the most important features of the normal microbial flora is the formation of a barrier against infectious agents. For example, viridans streptococci are important to prevent *S. pneumoniae*, *S. pyogenes* or Gram-negative bacilli colonization. For this process, bacteriocin and short-chain fatty acid production are very important factors. The short chain fatty acids are metabolic end products of the normal flora which inhibit growth of potential

pathogens either by being directly toxic or by indirect inhibition due to lowering the local oxidation potentials [54].

1.2.2.4 Nasopharyngeal colonization

The human nasopharyngeal mucosal surface is nutritionally poor. Fluctuations in osmolarity, nutrient availability and pH have been measured. The temperature is about 34°C, pH-values range from 6.4 to 6.9, chloride from 82 to 108 mM, sodium from 82 to 91 mM and glucose from 3.9 to 5.8 mM. In nutritionally limited areas such as the nasopharynx, sessile growth in biofilms is a survival strategy [55].

The most important species colonizing the human nasopharynx are staphylococci (including *S. aureus*), streptococci (including *S. pneumoniae*), *Moraxella* (*Branhamella*) *catarrhalis*, *Neisseria* sp. and *Haemophilus* sp. In the human oropharynx viridans streptococci are important to suppress the spreading of *S. pyogenes*, *S. pneumoniae*, *N. meningitidis*, *S. aureus*, *Mycobacterium tuberculosis*, *Legionella pneumophila* and additional Gram-negative bacilli. In physiologically normal humans few Gram-negative bacilli are observed in the oropharynx but they increase greatly when illness occurs as the epithelial cells in ill humans are better targets for adherence. Reduction of oropharyngeal bacteria leads to the emergence of different pathogens. Obligate anaerobes were observed to produce much fewer infections than aerobic microorganisms [54]. Pneumococcus produces only small amounts of exotoxins and thus disease is mainly caused by bacterial replication and also the host's inflammatory response [56].

Nasopharyngeal colonization requires different steps such as availability of nutrients from the human tissues, tropism to specific host cells and evasion of the innate immune system [57]. Sugars present in the nasopharynx are important as nutrients but also for adhesion of the pneumococcus. Additionally, there is evidence that sugars may also serve as signals leading to enhanced virulence. Sialic acid is shown to increase the colonization and the spread of pneumococcus in the nasopharynx [58].

1.2.2.5 Colonization of the upper respiratory tract

Pathogens which are located in the nasopharynx can become able to invade the upper and the lower respiratory tract. Lack of competing bacteria has been shown to be associated with a higher rate of reinfection after otitis media treatment. In this context mainly α-streptococci are of importance as increased recurrences were measured when these bacteria were absent. Other species such as *Prevotella* or *Peptostreptococcus* have the same effects i.e. their presence reduced recurrence of otitis [59]. Additionally, pathogenic factors of one microbial species may affect other bacteria present in this environment, for example, supernatants of *S. pneumoniae* cultures have been shown to inhibit *H. influenzae* growth [60].

Performing 16S rRNA analysis of the whole microbiome showed shifts in the upper respiratory tract depending on age, vaccination, antibiotic use or respiratory tract infections. A lower microbial diversity was measured when *S. pneumoniae* was present and a negative correlation was observed between *Corynebacterium*, *Dolosigranulum* and *Streptococcus* [61]. The most prevalent phyla isolated from nasopharyngeal swabs are *Firmicutes* (73%), *Proteobacteria* (12.6%), *Bacterioidetes* (7%), and *Actinobacteria* (5.6%). *Streptococcus*, *Shigella*, *Acitenobacter*, and *Corynebacterium* spp. are commonly identified but also some environmentally linked *Leuconostoc*, *Lactococcus*, and *Weissella* spp. often can be found. Interestingly, nasopharyngeal communities seem to be less stable over time compared to oropharyngeal communities [62].

1.2.2.6 Pneumococcal adhesion to human epithelial cells

Some bacterial adherence is specific for different epithelial cell types and depends on species-specific surface antigens termed adhesins (lectins) which bind the bacteria to cell-specific receptors of the host. Carbohydrates in variable chain structures are responsible for the adhesion specificity [54]. *S. pneumoniae* can be internalized by mammalian cells as a result of cell-specific mechanisms [63]. PspC is the only pneumococcal protein identified as an adhesin and it is specific for human secretory component (SC), the extracellular domain

of polymeric immunoglobulin receptor A (PIgR). PspC is able to bind free SC and SIgA which results in phagocytosis prevention [63, 64]. Non-encapsulated pneumococci have an advantage over encapsulated in terms of binding to host cells in the respiratory tract [65] but encapsulated *S. pneumoniae* strains have an advantage in transiting from the luminal mucus, their initial site in the host, to the epithelial surface. Only the bacteria in close association with the epithelial surface are able to produce a stable colonization and thus escape from the mucus seems to be an important step in persistence but the capsule inhibits the adhesion to bacterial host cells. *S. pneumoniae* in close association with epithelial cells was shown to down-regulate its capsule. [66]. Encapsulated strains are resistant to opsonophagocytosis and so have an advantage in surviving in the bloodstream [65, 67].

Binding of the bacterium to the epithelium occurs via the cell-surface carbohydrates, e.g. N-acetyl-glucosamine on the respiratory epithelium [68]. Cell-wall associated surface proteins of the bacterium are important for this binding. Cell wall hydrolases (CWHs), for example LytB and LytC, which are found on the surface of pneumococci, cleave specific covalent bonds of the cell wall and they also modify bacterial peptidoglycan and as this is a main pathogen-associated molecular pattern, modifications are used to overcome host innate immunity. Hydrolases for peptidoglycan are important for attachment to hydrophobic surfaces and are important for bacterial pathogenesis and full virulence. LytB and LytC are involved in the attachment to the nasopharynx but some degree of colonization is still possible in the absence of both of these factors, indicating that there are additional proteins involved in this process. [69].

1.2.3 Pneumococcal virulence factors

The main pneumococcal virulence factors are listed in Table 3.

Pneumococcal virulence factor	Main role in colonization
Upper-airway colonization	
Capsule	Inhibiting effective opsonophagocytosis, preventing entrapment in the nasal mucus
ChoP	Binding of rPAF on epithelial surface of nasopharynx
CbpA (also known as PspC)	Binds to human secretory component on polymeric Ig receptor during first stage of translocation through epithelium
NanA, BgaA, StrH	Cleaving of terminal sugars from glycoconjugates, revealing receptors for adherence
Hyl	Breaking down of hyaluronan-containing extracellular matrix components
PavA	Binding to fibronectin
Enolase	Binds to plasminogen
Competition in upper airway	
Bacteriocin (pneumocin)	Small antimicrobial peptide targeting members of the same species
Respiratory-tract infection and pneumonia	
Ply	Cytolytic toxin activating complement. Important virulence factor for *in vivo* models. Wide range of effects at sub-lytic concentrations
PspA	C3 binding on pneumococcal surface prevented. Binds lactoferrin
LytA	Cell wall digestion resulting in Ply release
PsaA	Component of ABC transport system, resistance to oxidative stress
PiaA, PiuA	Component of ABC transport system
NanA, NanB	Reveals receptors for adherence by modification of the surface of competing bacteria within the same niche. Modification of host clearance glycoprotein functions
IgA protease	Cleaving of human IgA1

Table 4: The most important pneumococcal virulence factors and their main role in colonization are listed in this table. Modified from [11].

1.2.3.1 Capsule

The anti-phagocytic polysaccharide capsule is one of the main virulence factors as it forms a shield to prevent phagocytosis or killing in neutrophil extracellular traps [11, 70-72] and reduces the total load of complement, mainly C3b [73], on the bacterial surface [71] and thus gives an advantage in colonization for the bacterium [65]. As a result, non-encapsulated pneumococci are less virulent [74], but these strains are important pathogens producing superficial infections such as conjunctivitis [75, 76].

The *S. pneumoniae* capsule is 200-400 nm thick [8] and is attached to the outer surface of the cell wall peptidoglycan covalently [77]. Capsular polysaccharides are acidic in most cases and constructed of a core oligosaccharide next to the lipid part and an O-

polysaccharide which is based on repeating units [45]. Structural types of capsule vary between linear polymers composed of two or more monosaccharides to branched polysaccharides containing one to six monosaccharides in combination with additional side chains [77]. The sugars detected most frequently in pneumococcal capsules are listed in Table 2.

Sugar
α/β-D-glucose
α/β-D-galactose
α/β-L-rhamnose
N-acetyl-α/β-D-glucosamine
N-acetyl-α/β-D-galactosamine
N-acetyl-β-D-mannosamine
N-acetyl-α-L-fucosamine
α/β-D-glucuronic acid

Table 5: Sugars detected most frequently in pneumococcal capsules (from [78]).

One of the common things between the different capsule types is that none of them shows a net positive charge [65]. Differences in the thickness of encapsulation result in colonization of different niches of the human body. Thicker capsules reduce entrapment in the mucus [79], and, due to the reduced opsonophagocytosis, survival in the blood stream is increased [80]. In contrast, thinner capsules show an advantage in adhering to host tissue due to increased exposure of adhesion molecules [81].

Genetic organization of capsule operon

Clusters of up to 20 different tightly linked genes are required for serotype-specific capsular polysaccharide synthesis [65]. Most serotypes encode their polysaccharide capsule in a gene cluster which is located between genes *dexB* and *aliA* [82]. Figure 3 shows serotypes 6B and 7F as examples. One exception to this rule are serotypes 37 [44] and 3 [83]. The pneumococcal capsular locus shows an average size of 20,174 bp [10]. The first four genes of the operon, *cpsA-D* (also termed *wzg*, *wzh*, *wzd* and *wze*), are quite conserved and affect the capsule expression level [11] while further downstream the serotype-specific genes are located. The first gene of the capsule operon is *cpsA* which is the most conserved

and might have a role in capsule the regulation of capsule expression. Generally, higher *cpsA* expression is observed in serotypes causing invasive diseases than in serotypes with a higher prevalence for colonization resulting in a correlation between *cpsA* expression and invasiveness. A new finding showed a connection between the upregulation of *cpsA* and the production of essential components for survival and growth in difficult and stressful environments such as the nasopharynx [82]. In the central region of the cps locus, genes encoding for specific glycosyl transferases have been identified which are responsible for the serotype-specific oligosaccharide-repeat unit on a lipid carrier, for example a flippase transporting the repeat unit to the external face of the membrane and a polymer responsible for linking both units. At the end of the locus genes encoding proteins for the synthesis of activated sugar precursors have been identified [11]. Interestingly, capsular switching via transformation and recombination is possible when genes encoding one capsule type are replaced by those of another [44].

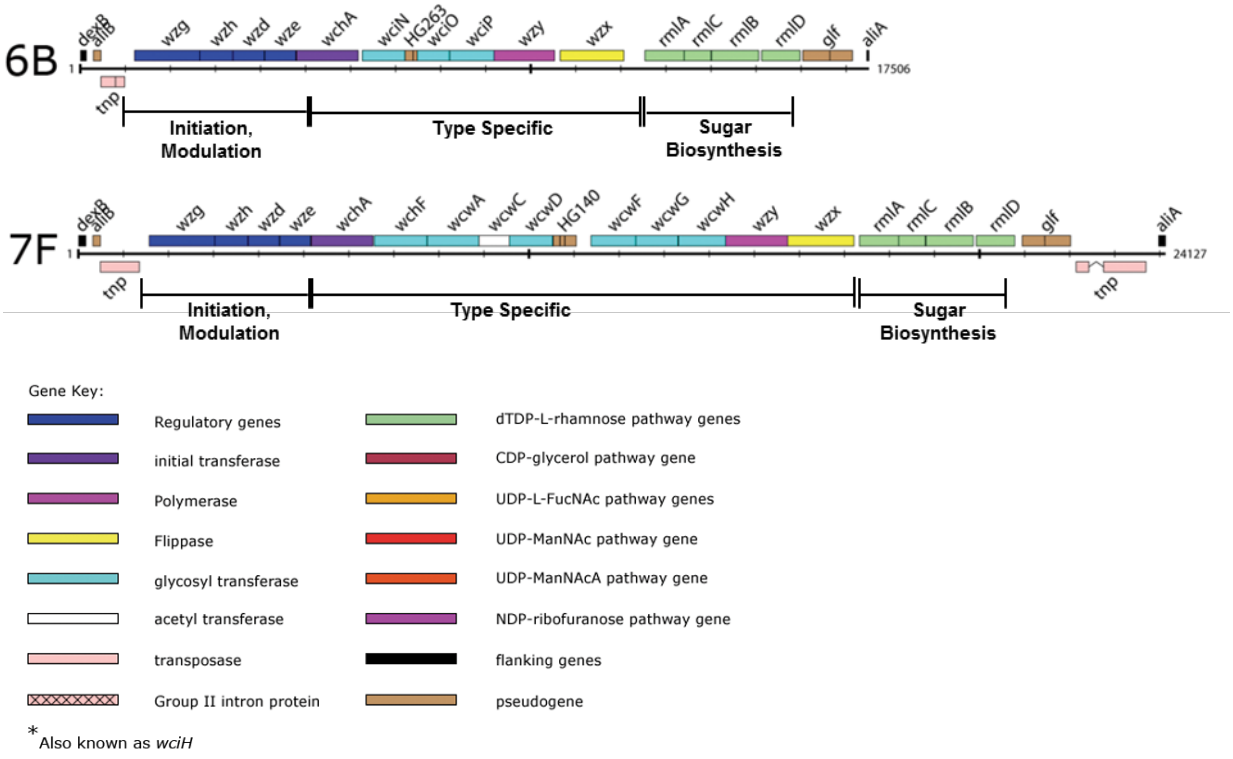


Figure 3: Capsule biosynthesis genes of pneumococcal serotypes 6B and 7F (modified from [10]).

Biosynthesis of pneumococcal polysaccharide capsule

For 91 serotypes [84], capsular synthesis occurs via the Wzx/Wzy-dependent pathway [10] (see Figure 4), only serotypes 3 and 37 use a processive transferase for capsule synthesis [83]. In the Wzy-dependent pathway, first the polysaccharide repeat unit is synthesized on an undecaprenyl lipid carrier (UndPP), then the oligosaccharide is translocated across the membrane by the help of a Wzx flippase. On the outer side of the membrane the oligosaccharide is then polymerized with the help of Wzy to produce a UndPP-attached polymer [85].

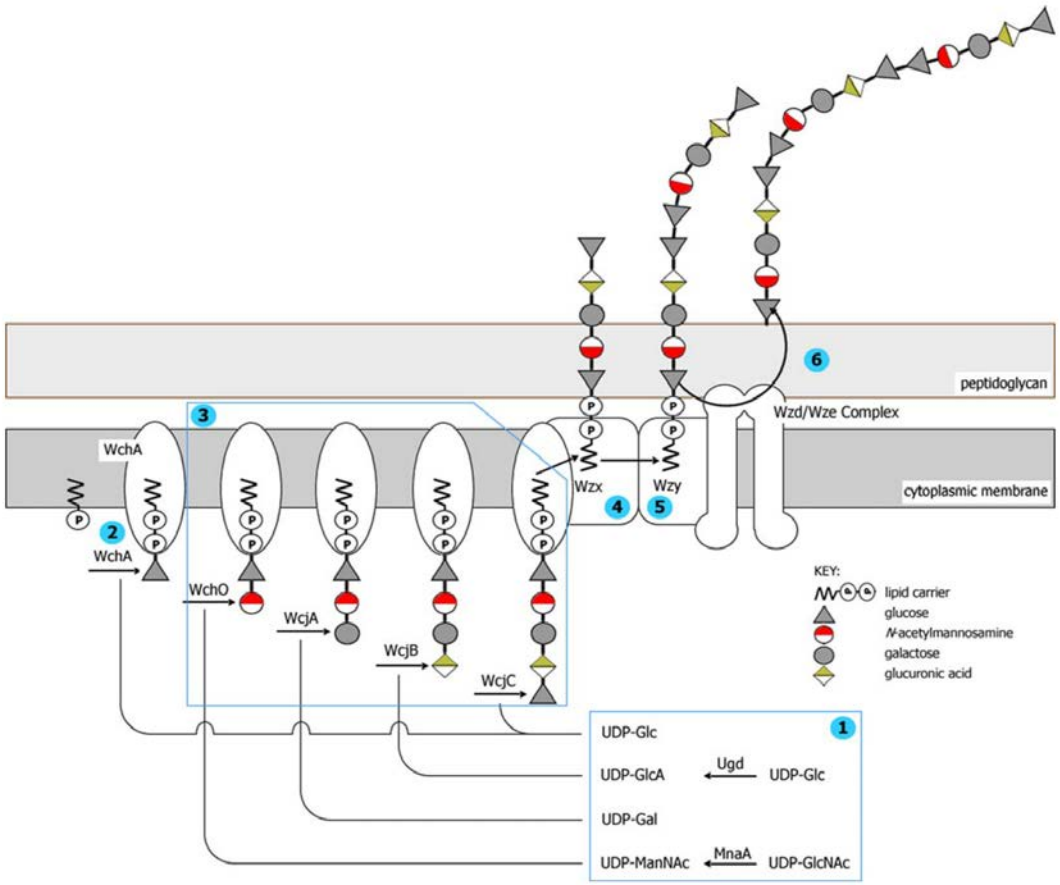


Figure 4: Representation of the Wzx/Wzy-dependent pathway for biosynthesis of CPS 9A [10]:
Pictured is a hypothetical model for capsule biosynthesis in *S. pneumoniae* based on a mixture of experimental evidence and speculation.

1) Non-housekeeping nucleotide sugar biosynthesis
2) The initial transferase (WchA) in this case links the initial sugar as a sugar phosphate (Glc-P) to a membrane-associated lipid carrier (widely assumed to be undecaprenyl phosphate).
3) Glycosyl transferases sequentially link further sugars to generate repeat unit.
4) Wzx flippase transports the repeat unit across the cytoplasmic membrane.
5) Wzy polymerase links individual repeat units to form lipid-linked CPS.
6) Wzd/Wze complex translocates mature CPS to the cell surface and may be responsible for the attachment to peptidoglycan. The complex of WchA, Wzy, Wzx, Wzd and Wze shown in the membrane is based on that in Figure 2 of Whitfield and Paiment for the related *Escherichia coli* Type 1 capsule.

Regulation of capsular polysaccharide production

No control element for transcription of the pneumococcal capsular genes associated with the pneumococcal σ^{70} cps promoter has been identified so far [11] but it is suggested that there are differences in the level of expression between transparent and opaque serotypes as there was a reduction of CpsD measured by Western Blotting in transparent variants [86]. The proteins derived from the genes *cpsA-D* were shown to be involved in capsular production [11]. *cpsA*-deficient mutants showed reduced capsular levels but virulence was not affected in a murine *in vivo* model [87]. The manganese-dependent phosphotyrosine-protein phosphatase CpsB, the polysaccharide co-polymerase related membrane protein CpsC and the autophosphorylating protein-tyrosine kinase CpsD act together as regulatory system for capsule production [83].

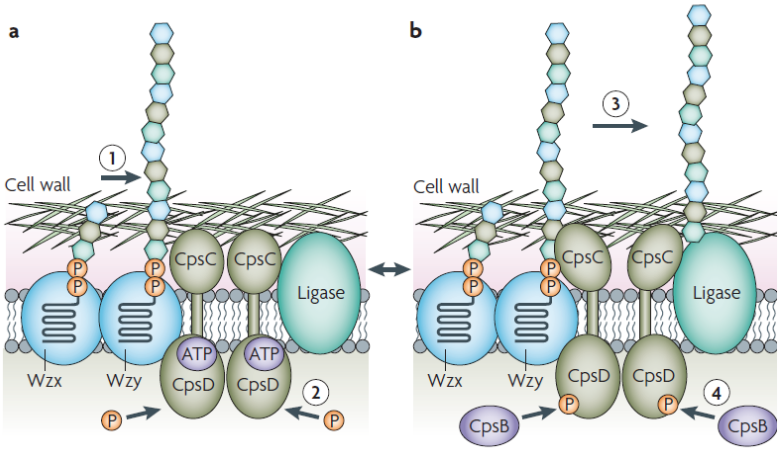


Figure 5: Model showing the regulation of capsular polysaccharide (CPS) production by tyrosine phosphorylation of CpsD. a) CpsC, CpsD and ATP interact to promote CPS biosynthesis by the polysaccharide polymerase (step 1). CpsD autophosphorylates, which causes a change in protein interactions and slows CPS biosynthesis (step 2). b) The CPS polymer is then transferred to the putative CPS cell-wall ligase, and is ligated to the cell wall (step 3). Finally, CspB dephosphorylates CspD, thereby allowing the cycle to be repeated (step 4) [11].

1.2.3.2 Pneumolysin

Beside the capsule, *S. pneumoniae* is also able to produce pneumolysin (Ply), a toxic intracellular protein of 471 amino acids and 53 kDa in size [88] which belongs to the family of cholesterol-binding cytolysins and which is produced during the late log phase of growth [89]. This toxin is able to form oligomers which then form pores into the epithelial membranes to trigger pro-inflammatory signaling and activates the classical pathway of complement [74]. Additionally, it is able to stimulate the production of TNF-α, IL-1β, nitric oxide, IL-8, prostaglandins and leukotrienes. Inhibition of non-specific defenses such as the respiratory cilial beat [71, 90] or phagocytosis and lymphocyte function are other main activities. Ply also causes damage to the ependymal cilia of the brain and thus is responsible for pathogenesis of meningitis as well as for hearing losses due to ultrastructural damage to the cochlea [71, 89]. Ply attachment to the cell membrane requires cholesterol [88] and thus pneumolysin is lytic to all cells containing cholesterol in their membrane [91]. Pneumolysin is able to bind to membrane cholesterol, 20-80 toxin molecules are associated within the membrane to form transmembrane pores of 30-35 nm in diameter. As a result, cytolysis by leakage of cytosolic proteins occurs [90].

Ply is found in almost all pneumococcal isolates. Its amino-acid sequence is well conserved and the toxin was shown to be essential for the pneumococcal survival in the murine upper and lower respiratory tract [91-93]. Ply-deficient pneumococci showed a delayed infiltration of the lungs in a murine model [89].

Ply interacts with epithelial cells forming pores in the cell membrane. Large molecular weight osmoles are allowed to enter the cell and signal transduction, osmosensing, is started resulting in the activation of p38 mitogen-activated protein kinase (MAPK9) and the nuclear factor κ-B (NF-κB). Interaction with Toll-like receptor (TLR)-4 and its adaptor protein MyD88 is another possible pathway to activate NF-κB. As a result, cytokine and chemokine production is activated which results in neutrophil activation (for interleukin-8 (IL-8 / CXCL8)) which increases bacterial killing. Neutrophils additionally release reactive oxygen species and proteases which cause tissue injury [88] (see Figure 2 and Table 4).

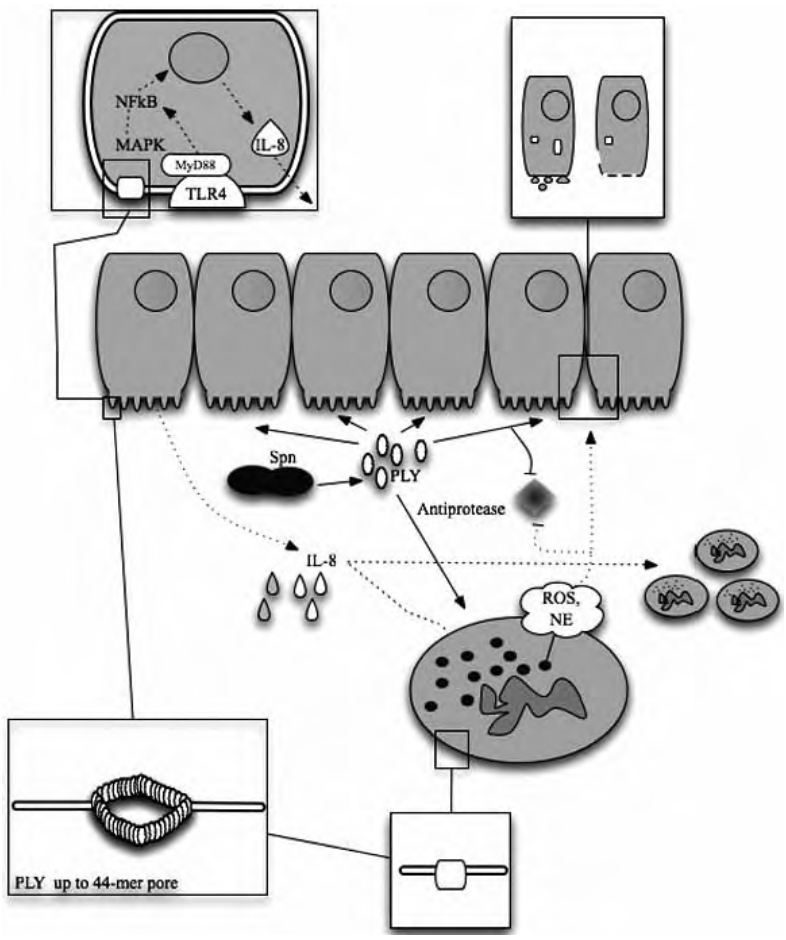


Figure 6: Interaction of pneumolysin with epithelial cells causing pore formation results in NF-κB activation, cytokine/chemokine production and tissue injury. From [88].

Function	Mechanism of alteration	Consequence
Membrane integrity	Lytic concentration: pore formation	Cell cytotoxicity, mitochondrial swelling, membrane blebbing.
	sub-lytic concentration: pore formation	Osmosensing, in epithelial cells: activation of p38 mitogen-activated protein kinase and innate responses
Cell signalling	GTPases: direct / indirect activation of GTPases, protein kinases and phospholipases	Roles in host responses, inflammatory responses and tissue injury
Gene transcription	Activation / expression of transcription factors	Large number of genes (mainly involved in host responses, cell adherence / survival): Altered transcription
	Epigenetic regulation via Histone H3 dephosphorylation	Downregulation of genes involved in host defense
Cytokine production	TLR45-stimulated or TLR-independent (e.g. by osmosensing)	Pro-inflammatory cytokines (e.g. including TNFα, IL-1β and IL-6): Increased production
Generation of microbicidal molecules	Expression of reactive oxygen species, nitric oxide and proteases by neutrophils and macrophages increased	Role in microbial killing / tissue injury
Cell adhesion	Adhesion molecules (e.g. intercellular adhesion molecule 1): Transcriptional upregulation	Potential enhancement of inflammatory response
Apoptosis	Cell death induction (cytotoxic effects of toxin or due to host-mediated response)	Tissue damage / host responses enhancing bacterial killing

Table 6: Major functions / consequences of pneumolysin on eukaryotic cells (modified from [88]).

1.3 Innate immune response to pneumococcus

Complement activation, phagocytosis and the inflammatory response are the principal mechanism of innate immunity induced by extracellular bacteria [94]. During the innate immunity to *S. pneumoniae*, the classical pathway with binding of IgM to bacteria is important [95].

1.3.1 Mucosal immunity

The first line of defense against *S. pneumoniae* infections are intact mucosal surfaces as well as mucosal immune responses [96]. Additional components of the first non-specific barrier are antibacterial peptides, ciliated cells and the simultaneous action of mucus [97]. The most important factor preventing pneumococcal carriage is secretory IgA. This process of mucosal immunity is relatively immature in young children [96].

1.3.2 Recognition of pathogen

The first step in innate immunity is recognition of pathogens by their conserved pathogen-associated molecular patterns (PAMPs) [98]. PAMPs are recognized by pattern recognition receptors which mainly regulate the production of inflammatory mediators such as IL-1β, TNF-α, IFN-α/-β or IL-6 [99]. One family of receptors which recognize PAMPs are the Toll-like receptors (TLRs) which are expressed on innate immune cells. Pneumococcal cell wall components (lipoteichoic acid and lipoproteins [99]) are recognized by TLR-2 [98]. Murine macrophage responses to pneumolysin were shown to be dependent on TLR-4 [100]. Pneumolysin mainly stimulates processing of IL-1β via caspase-1. Additionally, pneumolysin pore formation seems to be a prerequisite for IL-1β release [101]. Pneumococci are autolytic bacteria releasing DNA containing unmethylated CpG motifs which has shown to be recognized by TLR-9 within endosomes [99, 102]. Activation of TLRs

leads to the expression of Nuclear Factor κB (NF-κB) which is essential for neutrophil recruitment (NF-κB is necessary and sufficient for this process), cytokine expression and bacterial killing in pneumococcal pneumonia. Another important pathway is the tumor necrosis factor α (TNF-α) cascade activated in alveolar macrophages and the airway epithelium. TNF-α in combination with IL-1 is a critical cytokine for early response as they are responsible for the induction of the nuclear translocation of NF-κB [103].

1.3.3 Cellular reactions

For co-ordination of the innate immune response and the phagocytosis of bacteria, dendritic cells and alveolar macrophages are of main importance [104]. After streptococcal invasion of the lungs a massive local inflammatory response is measured with massive immigration of granulocytes, neutrophils and activated monocytes/macrophages [105]. After the infiltration of neutrophils into the tissue, they become the major cell types for phagocytosis. Alveolar macrophages are still present, but no longer of main importance [103]. For pneumococcal clearance, neutrophils are of essential importance as they are able to phagocytose, to adhere to blood vessels as well as to react to chemotaxis [106]. Chemotaxis and as a result the motility of neutrophils are stimulated by IL-8 which is induced by TNF-α and IL-1 [103]. Another important cytokine is IL-6 which is able to delay neutrophil apoptosis and enhances their reactive cytotoxicity. As a result, the resolution of neutrophil-mediated inflammation is delayed [107]. IL-6 also plays an important role in adaptive immunity as it stimulates B-cells, induced T-cell proliferation as well as the development of antigen-specific cytotoxic T-lymphocytes [103]. Already early in the development of pneumonia T-cells increase in the peribronchiolar region. For an optimal response a certain level of CD4+ T-cells is required [101]. Anti-inflammatory cytokines are then important to regulate the inflammation and to prevent further tissue damage. Regulatory T-cells (Tregs, CD4+Foxp3+ cells) were shown to play an important role to mediate resistance to pneumococcal pneumonia [108] and are known as suppressors of immune responses [109].

Tregs are required to keep the inflammatory reaction in balance and to prevent tissue damage and dissemination of bacteria into the blood. IL-10 plays an important role in immune homeostasis by suppressing the immunological reactivity to limit infection-related tissue damages [108, 110].

A chronology of pneumococcal infection using an inoculum of 10^7 CFU counts is shown in Table 11. Lower inocula of pneumococci resulted in a plateau of 10^7 CFUs/g lung tissue 48h after infection. Inflammatory response then still increased and reached a maximum 84h after infection as at this time point, pneumococci started to auto-lyse and to release large amounts of toxin [111].

Infection (10^7 CFUs)	Step 1 (0-4h)	Step 2(2-24h)	Step 3(24-48h)	Step 4 (48-72h)	Step5 (72-96h)
Bronchoalveolar lavage	TNF, IL-6	Neutrophils, TNF, IL-6, IL-1	Downregulation of TNF, IL-1	Monocytes, lymphocytes	
Tissue	TNF, IL-6, IL-1	Neutrophils, TNF, IL-6, IL-1	Downregulation of TNF, IL-1; bacteria in tissue		
Serum levels	IL-6	IL-1	Bacteraemia	Reduction in leukocytes; bacteraemia; TNF, IL-6	
Histopathology	Inflammatory cells	Inflammatory cells	Low intensity of inflammatory cells, tissue injury regeneration	High intensity of inflammatory cells, tissue injury regeneration	Severe air-space disorganisation, diffuse tissue damage, regeneration

Table 9: Chronology of pneumococcal infection in the mouse model (modified from [103]).

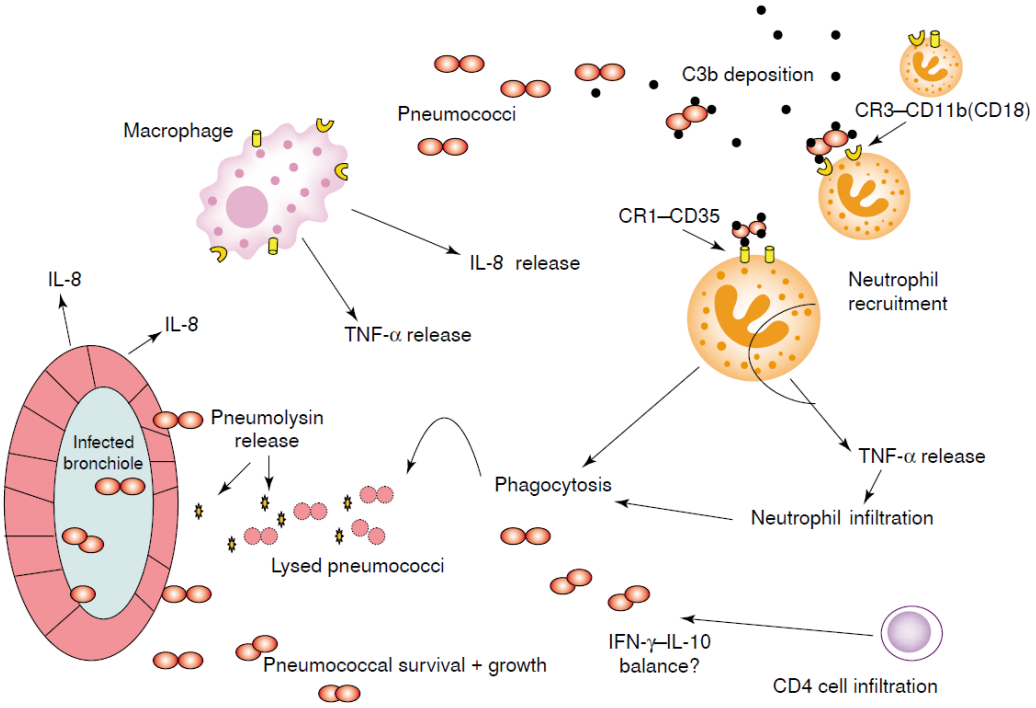


Figure 8: Summary of some aspects of the host immune response to pneumococcal infection during bronchopneumonia. Pneumococci infect bronchiolar airspaces, subsequently invading bronchoepithelial cells. This eventually leads to activation of these cells and the subsequent release of chemokines, such as IL-8. Alveolar macrophages also release IL-8 as well as TNF-α on pneumococcal infiltration. The release of IL-8 and TNF-α attracts neutrophils to areas of pneumococcal invasion. Neutrophils subsequently phagocytose pneumococci through complement C3 deposition and its associated receptor (opsonophagocytosis). Cytokines, such as TNF-α, are also released by neutrophils, which leads to increased neutrophil infiltration into infected areas. CD4 T-cell infiltration into infected tissue also occurs with increased release of IFN-γ and IL-10. Lysed pneumococcal populations release pneumolysin into tissue surroundings, which have a wide range of cytotoxic and inhibitory effects on host tissue and immune cells. The release of pneumolysin increases the chances of pneumococcal survival and growth *in vivo* [95].

1.4 Pneumococcal colonization and viruses

Local viral infections, particularly in the upper respiratory tract, predispose for invasive bacterial diseases [112] and often increase the severity of the disease. Epidemiological evidence for this phenomenon was already gained during the 1918, 1957 and 1968/69 influenza pandemics where complications were often associated with bacterial superinfections [113].

One of the best studied examples of bacterial-viral interactions is that of influenza and parainfluenza viruses which are able to cleave sialic acids on eukaryotic cells by the action of their neuraminidases resulting in increased resulting in increased binding possibilities and adherence of *S. pneumoniae* and *H. influenzae* [114]. Sialic acid signaling additionally increases the production of co-transcribed transporters and metabolic enzymes. A local increase of free sialic acid is produced by influenza viruses what then serves as a signal for increasing the amount of pneumococci on the epithelium. A passive transfer of *S. pneumoniae* to the lower respiratory tract then results [58].

1.4.1 Bacterial-viral interactions in the upper respiratory tract

Viruses are able to disrupt the respiratory mucosal epithelium by inducing cell death and thus facilitating adherence and translocation of bacteria [60]. This may be because the basement membrane of the epithelial layer is exposed, increasing for example the binding of *S. pneumoniae* to the now freely accessible fibronectin [115]. Other species such as *M. catarrhalis* show binding to extracellular matrix proteins [116] indicating the benefit to the bacteria of disrupted epithelia. Another important factor is the upregulation of adherence proteins such as platelet-activating factor receptor (PAFr), intracellular adhesion molecule 1 (ICAM-1), carcinoembryonic adhesion molecule-1 (CEACAM-1) [114] or the outer membrane protein P5-homologous fimbriae (P5 fimbriae) [117]. Viral factors such as neuraminidases (influenza virus) or protein G (RSV) also increase bacterial adhesion to host epithelial tissue. Increased bacterial colonization can also be the result of changes in the

immune system caused by viruses such as enhanced neutrophil apoptosis, impairing neutrophil function or affecting biological activity and production of cytokines, e.g. downregulation of TNF-α production. The equilibrium of microbiota can be destroyed by microbial interactions which might result in viral invasion and transmission [118]. Retroviruses are even able escape immune clearance by the help of their microbial environment [119].

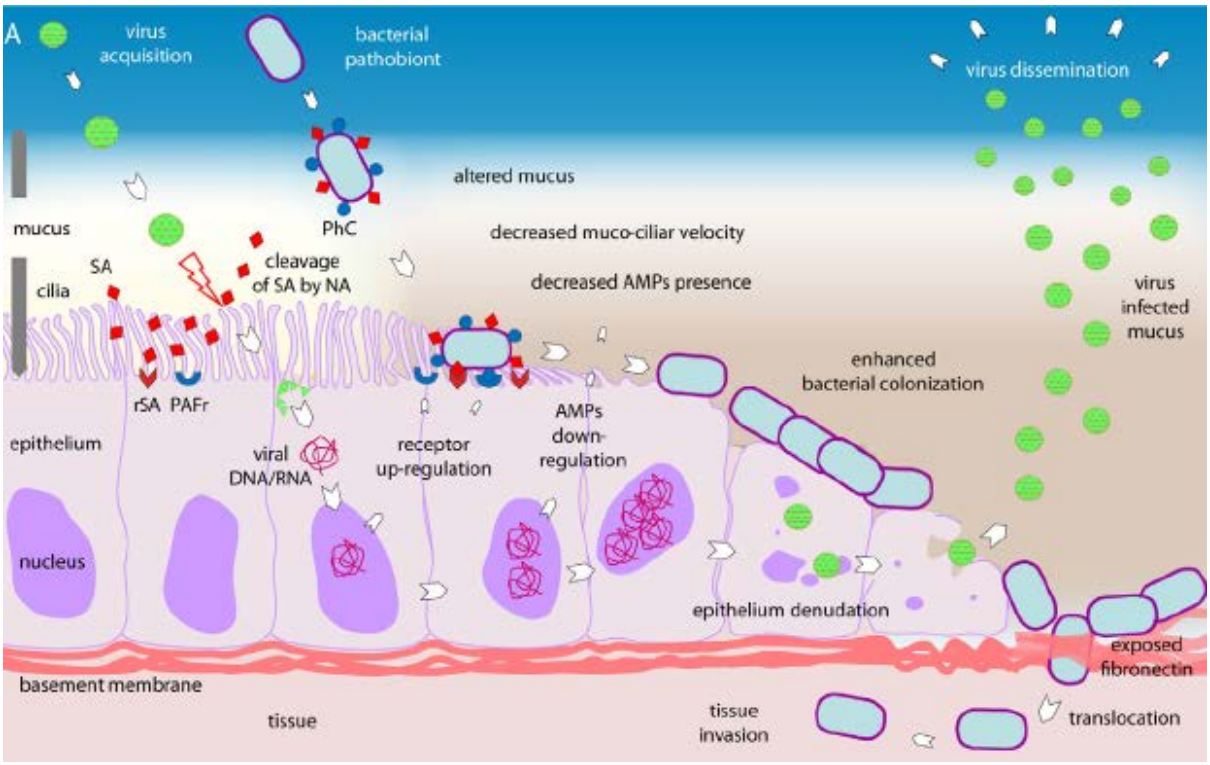


Figure 9: Viral-bacterial interactions. Viral-bacterial interaction on the respiratory epithelial surface. Viral presence is thought to predispose the respiratory niche to bacterial colonization by different mechanisms. First, viruses may render the epithelium more susceptible to bacterial colonization by altering the mucosal surfaces. Ciliae may be damaged, leading to decreased mucociliar function of the respiratory epithelium. Additionally, due to viral-induced damage and loss of integrity of the epithelium layer, bacterial colonization may be enhanced and translocation may be increased. Virus-infected cells may decrease the expression of antimicrobial peptides, as shown for β-defensins, thereby affecting the natural defense of the host epithelium. Viral neuraminidase (NA) activity is able to cleave sialic acids residues, thereby giving access to bacterial receptors that were covered by these residues. Finally, viruses may induce bacterial colonization and replication both directly and indirectly, the latter by inducing upregulation of various receptors required for bacterial adherence, including PAFr, CAECAM-1, P5F, ICAM-1 and G-protein.

PAFr, platelet activating factor receptor; ICAM-1, intracellular adhesion molecule 1; P5 fimbriae, outer membrane protein P%-homologous fimbriae; CAECAM-1, carcinoembryonic adhesion molecule-1; PhC, phosphorylcholine; SA, sialic acids; rSA, receptor for sialic acids; NA, neuraminidase; mRNA messenger RNA; AMPs, antimicrobial peptides (from [118]).

1.4.2 Respiratory Syncytial Virus and bacterial co-infections

Respiratory Syncytial Virus (RSV), a non-segmented, negative-strand RNA virus of the genus *Pneumovirus* [120] is one of the leading causes of lower respiratory tract infections in infants and children causing cold-like symptoms in most healthy adults and children [121, 122] causing worldwide 64 million infections and 160,000 deaths per year [123]. Generally, only airway luminal ciliated epithelial cells and in some special cases non-ciliated cells are affected. A loss of ciliated cells results from RSV infections, additionally excess mucus production or occlusion of the airways by sloughed cells. Immune cells, mainly macrophages, neutrophils and T-cells, massively infiltrate the airways and some pro-inflammatory cytokines are positively correlated with disease severity (see Table 5) [124].

Cytokine or chemokine	RSV-infected infants	Ranking
CXCL8	> 1000 pg/ml	+++
CXCL10	> 1000 pg/ml	+++
IL-6	100-1000 pg/ml	++
CCL5	100-1000 pg/ml	++
CCL2	100-1000 pg/ml	++
CCL3	100-1000 pg/ml	++
IL-1β	< 100 – 1000 pg/ml	+/++
TNF-α	< 100 pg/ml	+
IL-10	< 100 pg/ml	+
IFN-α, IFN-β		-

Table 10: Cytokine and chemokine levels in aspirates from RSV-infected infants (modified from [124]).

RSV infections of human epithelial cells result in a significant increase of bacterial binding [125, 126] and decreased bactericidal functions were measured in murine alveolar macrophages [127]. Experiments with the human monocytic leukaemia cell line THP-1 showed a reduced binding capacity of cells infected with RSV which resulted in lower amounts of bacterial killing. Additionally, the TNF-α bioactivity from cells infected with bacteria was reduced resulting in prolonged bacterial infection of patients [128]. Bacteria are also able to bind to RSV G protein which is expressed on respiratory epithelial cells increasing the binding capacity of pathogens to the host cells [129].

1.5 Biofilm formation

1.5.1 Introduction

More than 99% of all bacteria found on Earth grow in biofilms. As organic compounds are found in higher concentrations on surfaces than in liquid medium, bacteria tend to grow on surfaces particularly those which are hydrophobic and nonpolar to which it is easier to attach [130], However, biofilms are not only observed attached to a host surface but they are also able to form in the surrounding mucus layer of fluid [131]. Thus, more than 60% of all human bacterial infections and up to 80% of all chronic infections are related to bacterial biofilms [132]. Bacteria in this growth phase are also up to one thousand times more resistant to antibiotics than when they are grown planktonically [133] and thus biofilm formation is a key factor for bacteria to survive in different environments [130, 134]. The most important trigger for bacterial assembly into biofilms is nutrient stress [135]. The formation of microcolonies is enhanced by predation stress on bacteria [136].

1.5.2 Biofilm development

Biofilms are complex three-dimensional structures formed by behavior coordination of bacteria and are defined as structured communities of bacterial cells which are enclosed in self-produced polymeric matrix [132, 137]. In biofilms, gradients of nutrients and oxygen availability are created and thus a heterogenic bacterial population is observed as each bacterium is adapted to the environment in which it is found [131], and thus, metabolic variation between biofilm bacteria themselves is measured during biofilm development. As much as 50% of the proteome can be differentially produced between planktonic and biofilm growth [134].

In the beginning, proteins and polysaccharides are bound to the surface in a liquid environment and form a so-called “conditioning film” to which the microbes can then attach.

Hydrophobic and rough materials are the easiest for microbes to attach to but they are also able to attach to most other surfaces. [133]. At first, bacteria are associated in a loose or transient state with the surface [134]. This process is reversible as it is only based on electrostatic attraction and not on chemical bonds [130] and some bacteria still leave the surface to continue planktonic growth. Attachment seems to be responsible for the formation synthesis of extracellular matrix substances in which the bacteria then become embedded [138]. In a second step, attachment requires bacterial surface protein interaction and becomes irreversible [130]. Bacteria start to form microcolonies, grow and mature. There is evidence that the nutrient source influences clonal growth and rearrangements of cells. Quorum sensing seems to be very important for the maturation process: When a critical density of cells is reached the quorum sensing system is activated to start the transcription of specific genes [138]. The formation of extracellular polymeric substances then leads to the third step of biofilm formation. In this process, polysaccharides, nucleic acids and proteins are involved to form the extracellular polymeric substance [130]. The last step of biofilm development is the formation of a mature biofilm structure. Open water channels are of importance in this step as they are able to form a primitive circulatory system to form homeostatic conditions within the biofilm. The late stage of biofilm formation which is characterized by cell-to-cell interactions, cell adhesion, the formation of a multi-layered architecture and microcolony formation is positively influenced by competence-stimulating peptide (CSP) addition [139]. In a mature biofilm 70-95% of the space will be occupied by the EPS matrix and only 5-25% by bacterial cells [130]. In the last stage, bacteria become able to return to transient motility again [134] and colonize other surfaces [137]. After 24 hours, biofilms form an extracellular polymeric matrix (EPM) and after 48h, the EPM makes up the majority of the biofilm – only 27% of all cells are still viable [140].

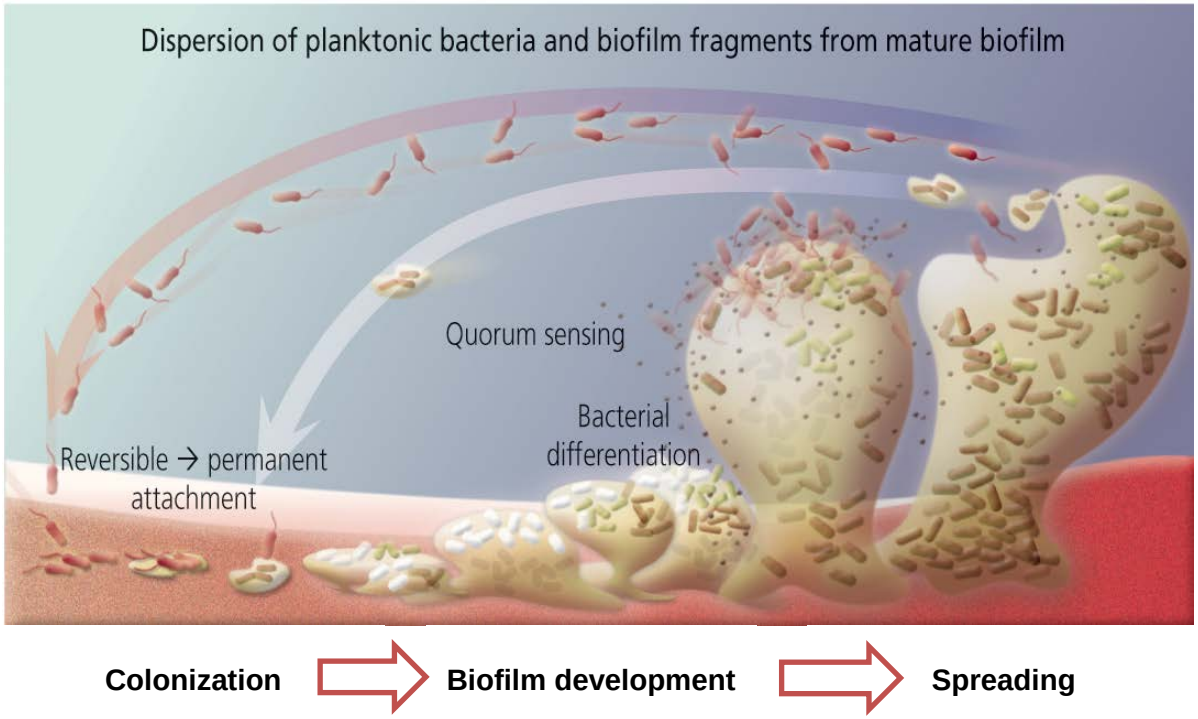


Figure 7: (modified from [141]): **Life cycle of biofilms** . First, bacteria adhere reversibly to a surface. In a second step, attachment becomes permanent and biofilms start to grow. The bigger the biofilm gets, the more specialized to their microenviroment the bacteria get. From a mature biofilm, bacteria can detach and spread to colonize new surfaces.

1.5.2.1 Exopolysaccharide matrix

For the three-dimensional structure of a biofilm, the exopolysaccharide matrix (EPS) is of main importance to hold bacterial cells on the surface and to provide binding sites enabling continuous accumulation of bacterial cells. Additionally, the EPS is also responsible for structural stability and vertical growth of the biofilm. During the early phase of biofilm formation (2-8h), the EPS is continuously formed. In *Streptococcus mutans* biofilms an EPS increase of more than 30x was measured in this early phase. After 20 to 30 hours, the EPS was observed to spread vertically and also occupying upper biofilm parts [142].

1.5.2.2 Extracellular DNA in biofilms

In 2002 it was shown for the first time that the biofilm extracellular material responsible for structural stability was DNA and not only exopolysaccharide and that DNaseI treatment had a strong inhibitory effect on biofilm formation [143], mainly in young biofilms [144]. There are differences in the amount of extracellular DNA (eDNA) production between different species, but even low eDNA-producers have significant influences on the amount of eDNA in multispecies biofilms [145]. For biofilms of the Gram-positive soil bacterium *Bacillus cereus* it was shown that most of the bacteria in the biofilm are embedded in a DNA-containing matrix and as a consequence, most of the eDNA was cell associated and not freely present in the culture broth [146]. DNA-rich environments like biofilms are cation limited as eDNA binds cations, mainly Mg^{2+}. As a result, modification of LPS and induction of resistance genes to antimicrobials were measured in *Pseudomonas aeruginosa* [147]. In a first step, eDNA is important for attachment, aggregation as well as stabilization and maturation of a biofilm. In a second step, exopolysaccharides (EPS) are accumulated and mature biofilms developed [148]. In *P. aeruginosa* biofilms eDNA presence was shown to be different depending on the biofilm age: after 2 days eDNA was observed as a grid-like structure on the substratum and in the microcolonies, whereas 4-day old biofilms contained the highest concentration of eDNA in the outer parts of the stalks [149]. As a result of increased biofilm formation, an accumulation of DNA at infection sites is propagated. In cystic fibrosis (CF) patients, for example, a clearly increased DNA concentration [150] in lung secretions as well as reduced Mg^{2+}-levels were measured.

The most important effect of CSP is on cell survival: In the absence of CSP only 1% of all *S. mutans* in a biofilm are dead but after adding 5 µM of CSP more than 50% of the cells died. Cells dying in a biofilm make it possible for planktonic cells to attach to the structure. Thus, biofilm dispersal and differentiation is increased. Extracellular DNA, which is released after cell death, is the most important factor for intercellular connection in biofilms as well as for adhesion matrix formation [139]. eDNA, polysaccharides and proteins are

important components of the extracellular matrix whereas eDNA appears to be a critical element for structural stability and protection against antimicrobial agents [151]. Fragmented DNA is not able to increase biofilm mass production which means that DNA plays structural role for biofilm architecture development [151].

1.5.3 Pneumococcal biofilms

The most important event in the pathogenesis of pneumococcal infection is the switch from planktonic life to biofilm production. [152]. Only bacteria in planktonic state of growth were able to induce bacteremia and death in mice. In contrast, biofilm bacteria only showed low levels in mice lungs and were not detectable in the blood. All of the biofilm-infected mice were able to clear the infection successfully. But planktonic bacteria die much faster in mice than biofilm ones – the latter are able to remain alive for a long time. Mutants which lost the capacity of biofilm formation were shown to be out-competed in the nasopharynx of mice. Thus, no biofilms are required for invasive diseases but they are required for long-term colonization of the nasopharynx and as a result, for transmission of pneumococci from one host to another [140]. Patients suffering from chronic rhinosinusitis were shown to suffer from mixed biofilms also containing pneumococci on adenoid tissue and mucosal epithelial cells [153]. Colonized mice show pneumococcal biofilms on their nasal septa [154] and mutants which lost the capacity of biofilm formation were shown to be out-competed in the nasopharynx of mice [140]. But it was shown that pneumococci isolated from biofilms are not able to cause invasive diseases. This might be due to the reduced production of capsular polysaccharides (CPS), pneumolysin, choline binding protein A (CbpA), phosphorylcholine (ChoP) and pneumococcal serine-rich repeat protein (PsrP). There is evidence that biofilms are of main importance for long-term quiescent colonization [140]. Interestingly, pneumolysin is increased in pneumococcal biofilms compared to planktonic cultures and knockout mutants showed a reduced ability to form biofilms, mainly in the early phase (8-10h postinoculation) [155].

1.5.3.1 Gene expression and mutations in biofilms

In pneumococcal biofilms, capsule expression seems to be down-regulated up to 10-fold giving evidence for a variable modulation during adherence and sessile growth [156, 157] and different types of colony morphologies are observed *in vitro*. Additionally to the normal-shaped pneumococci, more aggregative, hyperadherent, small, non-mucoid colonies are present. [158]. Interestingly, selection for small colony variants (SCVs) was shown for *S. pneumoniae* serotypes 3, 8 and 19 biofilms. SCVs are smaller in size, able to autoaggregate, less mucoid and deficient in capsule production. Under biofilm conditions SCVs show a selective advantage in growth. A random tandem duplication within the *cps3D* and *cap8E* genes was shown to be responsible for colony variation [159-161].

Comparing protein expression of biofilm and planktonic growth of *S. uberis* showed that in biofilms proteins important for adhesion, glutamine transport, internalization and sugar metabolisms are increased. A relatively large number of proteins, mainly involved in cell wall synthesis, cell division and metabolism, are down-regulated. A decrease in metabolic activity and thus in growth rate results. This process is increased as the biofilm ages (comparison of biofilms after 8 and 36h of growth) [162]. In *Pseudomonas aeruginosa* biofilms, only 40% of the proteins tested were similar to the ones found in planktonic growth, 60% were expressed over time. But on DNA microarray technology only 1% of all genes were differentially expressed in planktonic and biofilm growth [144]. Genetic variation in biofilms is arises through mutation or recombination. Differences in gene expression often often due to stochastic responses of the individual bacteria [134]. Biofilm bacteria occur in a quiescent state. As a result, 32 genes encoding ribosomal proteins or translation initiation and elongation factors showed a reduced level during pneumococcal biofilm growth. Additionally, a reduction in 8 genes for the ATP synthase machinery was measured. Fatty acid metabolism and phospholipid biosynthesis also show lower levels. 8 genes of the capsule expressing CPS cassette were reduced as well. However, during biofilm growth, the

virulence factor pneumococcal serine-rich repeat protein (PsrP) was enhanced. PsrP is used as a host cell and intra-species bacterial adhesin which contributes to biofilm formation [140].

Gene or component / Species	Function	Reference
brpA / *Streptococcus mutans*	Surface-associated protein, involved in autolysis, stress tolerance and cell division regulation. Mutant: biofilm accumulation defects, low capacity of surface binding, longer chains, weakened acid tolerance.	Wen et al. 2006 [163]
recA / *Streptococcus mutans*	Sensor for stress response (e.g.) to low pH and thus crucial protein for biofilm formation and regulation	Inagaki et al. 2009 [164]
ciaH / *Streptococcus mutans*	Regulation of biofilm formation, mainly in sucrose	Inagaki et al. 2009 [164]
ComDE / *Streptococcus mutans*	Quorum sensing signalling system, responding to peptide pheromone by inducing genetic competence	Li et al. 2002 [165]
hk11 / *Streptococcus mutans*	Membrane-associated protein, pH sensor, important determinant of biofilm formation	Li et al. 2002 [165]
cps locus / *Streptococcus pneumoniae*	Synthesis of capsule. If impaired, enhanced biofilm formation observed	Qin et al. 2013 [166]
cps3D / *Streptococcus pneumoniae*	Capsule operon gene; capsule synthesis. If impaired, enhanced biofilm formation	Allegrucci et al. 2007 [159]
cap8E / *Streptococcus pneumoniae*	Capsule operon gene; capsule synthesis. If impaired, enhanced biofilm formation	Waite et al. 2003 [160]
lytA / *Streptococcus pneumoniae*	Cell wall hydrolase. If impaired, 20% reduction in biofilm formation	Moscoso et al. 2006 [55]
lytB / *Streptococcus pneumoniae*	Cell wall hydrolase. If impaired, 45% reduction in biofilm formation.	Moscoso et al. 2006 [55]
lytC / *Streptococcus pneumoniae*	Cell wall hydrolase. If impaired, 25% reduction in biofilm formation	Moscoso et al. 2006 [55]
Peptidoglycane breaks / *Lactococcus lactis*	Positive correlation between peptidoglycane breaks and biofilm formation	Mercier et al. 2002 [167]
eDNA / *Streptococcus pneumoniae*	Spontaneously released into environment, important component. Significantly reduced biofilm formation when DNase I is added.	Moscoso et al. 2006 [55]
cbpA / *Streptococcus pneumoniae*	Critical for colonization Mutant: reduced binding opportunities	Blanchette-Cain et al. 2013 [168]
luxS / *Streptococcus pneumoniae*	Important for biofilm formation in vitro; important in quorum sensing system. Mutant: blocking of bacterial communication, biofilm phenotype cannot be formed	Vidal et al 2013 [169]
pspR / *Streptococcus pneumoniae*	Adhesin for intraspecies aggregation; biofilm formation increased by homodimerization of pneumococci; binding to cytokeratin 10 on host cells Mutant: dramatically reduced biofilm formation *in vivo*	Blanchette-Cain et al. 2013 [168]
spxB / *Streptococcus pneumoniae*	Hydrogen peroxide production; trigger of autolysis Mutant: reduced biofilm formation	Blanchette-Cain et al. 2013 [168]

Table 7: Genes and components involved in increased biofilm formation.

1.5.4 Biofilm formation in clinics

In more than half of all infectious diseases commensal bacteria are involved and they often grown in biofilms [138]. Due to their reduced metabolic state, these bacteria only cause very little systemic response and are able to persist for longer time periods [170]. Chronic otitis media as well as chronic rhinosinusitis both are linked to biofilm formation. On the epithelium of the middle-ear biofilm aggregates of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were observed [131, 170]. But also 54% of all tonsils removed from children showed biofilm formation [171] with biofilms mainly occurring within crypts of tonsils. In some cases, the biofilms grew to a thickness of several millimetres, and Gram-positive cocci were the predominant species [172]. There is evidence that pneumococcal biofilms are an important factor for chronic middle ear effusion. Isolates taken from the middle ear are better in biofilm formation when they are cultured compared to blood isolates [156]. An additional factor responsible for chronic otitis media are intracellularly persisting bacteria [27].

Infection or disease	Common biofilm bacterial speices
Dental caries	Acidogenic Gram-positive cocci (e.g. *Streptococcus*)
Periodonitis	Gram-negative anaerobic oral bacteria
Otitis media	Nontypable strains of *Haemophilus influenzae*
Musculoskeletal infections	Gram-positive cocci (e.g. staphylococci)
Necrotizing fasciitis	Group A streptococci
Bilary tract infection	Enteric bacteria (e.g. *Escherichia coli*)
Osteomyelitis	Various bacterial and fungal species – often mixed
Bacterial prostatitis	*E. coli* and other Gram-negative bacteria
Native valve endocarditis	Viridans group streptococci
Cystic fibrosis pneumonia	*Pseudomonas aeruginosa* and *Burkholderia cepacia*
Meloidosis	*Pseudomonas pseudomallei*
Nosocomial infections	
ICU (Intensive care unit) pneumonia	Gram-negative rods
Sutures	*Staphylococcus epidermidis* and *S. aureus*
Exit sites	*S. epidermidis* and *S. aureus*
Arteriovenous shunts	*S. epidermidis* and *S. aureus*
Schleral buckles	Gram-positive cocci
Contact lens	*P. aeruginosa* and Gram-positive cocci
Urinary catheter cystitis	*E. coli* and other Gram-negative rods
Peritoneal dialysis peritonitis	A variety of bacteria and fungi
IUDs (Intra uterine Device)	*Actinomyces israelii* and many others
Endotracheal tubes	A variety of bacteria and fungi
Hickman catheters	*S. epidermidis* and *C. albicans*
Central venous catheters	*S. epidermidis* and others
Mechanical heart valves	*S. aureus* and *S. epidermidi*
Vascular grafts	Gram-positive cocci
Biliary stent blockage	A variety of enteric bacteria and fungi
Orthopedic devices	*S. aureus* and *S. epidermidis*
Penile prostheses	*S. aureus* and *S. epidermidis*

Table 8: Infectious diseases produced by biofilm forming bacteria (from [138]

2. Aims of this thesis

Three main areas were of interest in this thesis.

2.1 The role of capsule and pneumolysin in the immune response in the respiratory tract

2.1.1 The pneumococcal polysaccharide capsule and pneumolysin differentially affect CXCL8 and IL-6 release form cells of the respiratory tract

This is a study of the role of pneumococcal capsule in pro-inflammatory cytokine induction using human pharyngeal and bronchial epithelial cells as well as in a murine model of nasopharyngeal colonization.

Hypotheses:

a) Pneumococcal capsule plays a role in the regulation of innate immune responses in the respiratory tract
b) Pneumolysin stimulates the release of the pro-inflammatory cytokines CXCL8 and IL-6 from respiratory tract cells *in vitro* and *in vivo*.

Aims:

a) To determine the effect of capsule on release of CXCL8 and IL-6 from respiratory tract cells using isogenic pneumococci mutants with and without capsule
b) To determine the effect of pneumolysin on release of CXCL8 and IL-6 from respiratory tract epithelial cells using isogenic mutants.
c) To determine whether any effects observed *in vitro* are also seen *in vivo* in a murine model of intranasal colonization.
d) To determine whether encapsulation affects dissemination of the pneumococci in the murine model.

Strategy: To test the effect of an *S. pneumoniae* serotype 2 strain (D39) and its isogenic mutants D39*cps*-, D39*ply*- and D39*cps-ply*- as well as a naturally nonencapsulated clinical isolate (110.58) and its isogenic encapsulated mutant 110.58::D39*cps* on CXCL8 and IL-6 release from the human pharyngeal epithelial cell line Detroit 562 and the human bronchial epithelial cell line iHBEC. Also, to study CXCL8 release induced by these strains and bacterial dissemination in a murine model of nasopharyngeal colonization.

2.1.2 Immunological cells and immune regulation

The focus of this study is on comparing encapsulated and non-encapsulated pneumococcal strains with the same background with respect to invasiveness and immunological response in a murine nasopharyngeal colonization model.

Hypotheses:

a) IL-10 secreted by FoxP3+ regulatory T-cells is induced by nasopharyngeal colonization in a murine model and depends on pneumococcal strain
b) Nasopharyngeal colonization increases FoxP3+ regulatory T-cell numbers in the nasal mucosa.
c) Lung infiltration results in increased levels of CD45+ cells, macrophages and neutrophils in the nasal mucosa.

Aims:

a) To test whether dissemination of pneumococci to the lungs is associated with changes in numbers of CD45+ cells (leukocytes), F4/80+ cells (macrophages) and Gr-1+ cells (neutrophils).
b) To test the effect of nasopharyngeal colonization on numbers of FoxP3+ regulatory T-cells, CD3+ T-cells and levels of IL-10 in the nasal mucosa.

Strategy: To test the effect of the pneumococcal strains described in Aim 1 plus strain 106.66 (serotype 6B) on immune cell induction in a murine nasopharyngeal colonization model by quanitifying CD45+ cells, F4/80+ cells Gr-1+ cells, CD3+ T-cells and FoxP3+ regulatory T-cells in the nasal mucosa and the amount of secreted IL-10 in the nasopharynx.

2.2 Investigating host innate immune responses to Respiratory Syncytial Virus and *Streptococcus pneumoniae*: co-stimulation *in vitro* model of human airway epithelial cells

This study is focused on pneumococcal/RSV co-infections on bronchial epithelial cells *in vitro* to gain new information on the influence of co-stimulation on innate immune responses.

Hypotheses:

a) RSV and pneumococcus act synergistically on CXCL8 and IL-6 induction in respiratory epithelial cells
b) CXCL8 release is affected by the presence pneumolysin and capsule in the pneumococcus.
c) The synergistic effect differs according to whether epithelial cells are exposed first to the bacteria or to the virus.

Aims: To test whether:

a) Co-stimulation of the human bronchial epithelial cell line BEAS-2B with RSV and pneumococcus results in increased CXCL8 and IL-6 levels.
b) Differences in IL-6 and CXCL8 response occur when bronchial cells are either primed with RSV or with pneumococcus or whether both pathogens are added together.
c) Encapsulated pneumococci result in different levels of IL-6 and CXCL8 secretion than non-encapsulated.

d) A pneumolysin-secreting strain increases CXCL8 levels in co-stimulation with RSV compared to a non-pore forming pneumolysin mutant.
e) Increasing free pneumolysin concentrations results in increased CXCL8 secretion in pneumolysin/RSV co-stimulations.

Strategy: Test mono- and co-stimulation with RSV and pneumococcus on the human bronchial epithelial cell line BEAS-2B *in vitro*. Pneumococcal strain D39 and its isogenic mutants PLN-A (expressing non-pore forming pneumolysin) and R36A (non-encapsulated) will be used to investigate the role of pneumolysin and capsule on CXCL8 and IL-6 release in co-stimulations with RSV. Different concentrations of free pneumolysin and a PdB (non-pore forming pneumolysin) will be used to investigate their effect on CXCL8 and IL-6 release.

2.3 Biofilm formation of *S. pneumoniae* and commensal streptococci

2.3.1 Differences of initial biofilm formation among pneumococcal serotypes

The goal of this study is to investigate differences in initial biofilm forming behaviour among typical invasive compared to colonizing pneumococcal serotypes

Hypotheses:

a) Pneumococcal serotypes differ in their ability to initial biofilm formation
b) Serotypes differ in their propensity to form small colony variants (SCV) in biofilms
c) Serotypes differ in their ability to adapt to a change in the nutritional environment

Aims:

a) To compare the initial attachment of different pneumococcal serotypes under different growth conditions (carbon sources).
b) To compare biofilm quantity and structural composition between serotypes.

c) To compare adaptation to different nutritional environments between serotypes by comparing their ability to adjust the proportion of SCVs (over time).

Strategy: To investigate the impact of carbon source on biofilm formation, chemically defined medium (CDM) supplemented with either 5 mM glucose or 5 mM galactose will be used in a static biofilm model. Pneumococcal serotypes 6B and 7F, which differ significantly in their *in vitro* growth behaviour, will be used to study the impact of capsule type on biofilm formation. Differences in numbers of living cells in biofilms and SCVs will be analyzed over time. To study their adaptation to a change in the nutrient environment SCVs of both serotypes will be grown in different media and FITC-Dextran imaging used to determine whether SCVs remained stable after growth in a complex medium. Whole genome sequencing of a wild-type and a SCV 7F clone will be performed to understand how SCVs are produced.

2.3.2 Interactions between commensal streptococcal strains and *S. pneumoniae* serotypes

The main focus of this study will be on nutrient competition and the influence of commensal streptococcal strains on pneumococcal biofilm formation and *vice versa*.

Hypotheses:

a) Competition for nutrients occurs in mixed cultures of *S. pneumoniae* with commensal streptococci *S. pseudopneumoniae*, *S. mitis* or *S. oralis*.
b) Commensal streptococci and pneumococcus influence one another in initial biofilm formation.

Aims:

a) To compare initial biofilm formation of *S. oralis*, *S. pseudopneumoniae*, *S. mitis* and *S. pneumoniae* in monoculture
b) To determine the impact of carbon source on initial monocultural biofilm formation of *S. oralis*, *S. pseudopneumoniae*, *S. mitis* and *S. pneumoniae*

c) To study initial biofilm formation in mixed cultures of *S. pneumoniae* with *S. oralis*, *S. mitis* or *S. pseudopneumoniae* and to determine differences to monocultural growth
d) To study the effect of carbon source competition on *S. pneumoniae* with *S. oralis*, *S. mitis* or *S. pseudopneumoniae*

Strategy: A chemically defined medium (CDM) supplemented with either 5 mM glucose or 5 mM galactose will be used and monoculture as well as mixed culture biofilm growth of *S. pneumoniae* with *S. oralis*, *S. mitis* and *S. pneumoniae* strains will be analyzed in planktonic growth as well as using a static biofilm model.

3. The role of pneumococcal capsule and pneumolysin in the immune response in the respiratory tract

3.1 Submitted manuscript

The pneumococcal polysaccharide capsule and pneumolysin differentially affect CXCL8 and IL-6 release from cells of the upper and lower respiratory tract

Eliane Küng, William R. Coward, Daniel R. Neill, Hesham A. Malak, Kathrin Mühlemann, Aras Kadioglu, Markus Hilty and Lucy J. Hathaway

The pneumococcal polysaccharide capsule and pneumolysin differentially affect CXCL8 and IL-6 release from cells of the upper and lower respiratory tract

Eliane Küng[1,2], William R. Coward[3], Daniel R. Neill[4],Hesham A. Malak[4], Kathrin Mühlemann[1,†], Aras Kadioglu[4*], Markus Hilty[1,5*] and Lucy J. Hathaway[1*#]

[1]Institute for Infectious Diseases, University of Bern, Switzerland

[2]Graduate School for Cellular and Biomedical Sciences, University of Bern, Switzerland

[3]Nottingham Respiratory Biomedical Research Unit, Clinical Sciences Building, Nottingham City Campus, Nottingham, UK

[4]Clinical Infection, Microbiology and Immunology, Institute of Infection & Global Health, University of Liverpool, Liverpool, UK;

[5]Department of Infectious Diseases, University Hospital, Bern, Switzerland

† Deceased

*These authors contributed equally.

[#]Corresponding author:

Dr. Lucy J. Hathaway

Institute for Infectious Diseases, University of Bern

Friedbühlstrasse 51, Postfach 61

3010 Bern, Switzerland

Phone: +41 31 632 32 51

E-mail: lucy.hathaway@ifik.unibe.ch

Abstract

The polysaccharide capsule and pneumolysin toxin are major virulence factors of the human bacterial pathogen *Streptococcus pneumoniae*. Colonization of the nasopharynx is asymptomatic but invasion of the lungs can result in invasive pneumonia. Here we show that the capsule suppresses the release of the pro-inflammatory cytokines CXCL8 (IL-8) and IL-6 from the human pharyngeal epithelial cell line Detroit 562. Release of both cytokines was much less from human bronchial epithelial cells (iHBEC) but levels were also affected by capsule. Pneumolysin stimulates CXCL8 release from both cell lines. Suppression of CXCL8 release by the capsule was also observed *in vivo* during intranasal colonization of mice but was only discernable in the absence of pneumolysin.

When pneumococci were administered intranasally to mice in a model of long term, stable nasopharyngeal carriage, encapsulated *S. pneumoniae* remained in the nasopharynx whereas the nonencapsulated pneumococci disseminated into the lungs.

Pneumococcal capsule plays a role not only in protection from phagocytosis but also in modulation of the pro-inflammatory immune response in the respiratory tract.

Introduction

Two of the main virulence factors of *Streptococcus pneumoniae* are the polysaccharide capsule that surrounds most *S. pneumoniae* strains and the toxin pneumolysin [1]. It has been shown that pneumolysin can stimulate the innate immune response including release of the inflammatory cytokine CXCL8 from the host's airway epithelial cells [2-4].

The pneumococcal capsule is mainly composed of polysaccharides, with each capsule type having a different composition and linkage of the sugars and other components [5]. *S. pneumoniae* is classified into over 90 different serotypes on the basis of antibody reactions with the capsule [6-9]. Some serotypes frequently colonize the human nasopharynx asymptomatically whereas others are more associated with invasive diseases such as pneumonia, sepsis or meningitis, but are found less frequently in the nasopharynx because they colonize for a shorter duration [10-12].

Epithelial cells express pattern-recognition receptors (PRRs) that are required to signal the presence of pathogens and to recruit and activate professional antigen presenting cells such as macrophages or dendritic cells [13]. Numerous pro-inflammatory chemokines and cytokines are secreted such as CXCL8, IL-6, IL-1β, granulocyte-macrophage colony stimulating factor (GM-CSF), transforming growth factor (TGF) α and –β [14]. Secretion of cytokines tends to be a brief, self-limited event with synthesis beginning with gene transcription and mRNA having a short half-life [15].

Toll-like receptors (TLRs) 2-6 are expressed on airway epithelial cells. TLR2 is the principal receptor for recognition of bacterial components (e.g. lipoprotein, lipoteichoic acid, peptidoglycan, GPI anchor) and some viral envelope proteins [16]. TLR signaling in epithelial cells is not only important for microbial defence but also for mucosal homeostasis which is determined by the magnitude of signaling [13]. CXCL8 plays a major role in the initial control of respiratory tract infection due to its chemotactic activity for neutrophils and monocytes [17] and can be secreted by all cells which have TLRs [18].

In the current study we tested the role of the pneumococcal capsule in pro-inflammatory cytokine induction using human pharyngeal and bronchial epithelial cells and in a murine model of nasopharyngeal colonization. We also looked at the effect of the capsule on the ability of the bacteria to disseminate into the lungs following nasopharyngeal colonization.

Materials and Methods

Bacteria

The bacterial wild type and mutants strains used are listed in Table 1. The capsule mutants were constructed according to the protocols described previously [9,19]. D39 strain was kindly provided by Professor Jeffrey Weiser (University of Pennsylvania, USA). The pneumolysin mutant was a kind gift from Professor Jeremy Brown (University College London, UK) [20]. For the construction of D39*cps*$^{-}$*ply*$^{-}$ mutant, the D39*cps*$^{-}$ mutant was used and mutant construction performed according to the method described previously [21]. Briefly, the up- and downstream flanking regions of the pneumolysin-gene were amplified using iProof polymerase (Bio-Rad, Switzerland) using the following primers: upstream forward primer KO_Ply_us_F 5'-GATTGATAATACCAGCACTC-3', upstream reverse primer KO_Ply_us_R 5'-GGTAGAGGATAAGGTAG-3', downstream forward primer KO_Ply_ds_F 5'-ATCGTAATTCATAGCTAG-3' and downstream reverse primer KO_Ply_ds_R. PCR conditions were: 98°C for 30 sec, 35 cycles of 98°C for 10 sec, 55°C for 15 sec, 72°C for 20 sec and then 72°C for 10 min. PCR, using the same conditions, was also performed to amplify a spectinomycin cassette from the plasmid. The primers (us_Ply_Spec_F1 5'-CTAGCTATGAATTACGACTAGTGGATCCCCCGTTTGA-3' and Spec_ds_Ply_R1 5'-CTACCTTATCCTCTACCATAGTTCCCTTCAAGAGCGATACC-3') were designed to create overhangs which allowed fusion of the three PCR products as described elsewhere [22]. The fusion reaction, using Phusion High Fidelity Polymerase (Fisher Scientific, Switzerland), was: 98°C for 1 min, 10 cycles of 98°C for 10 sec, 50°C for 15 sec, 72°C for 1 min then the primers (KO_Ply_us_F and KO_Ply_ds_R) were added followed by 25 cycles of 98°C for 10 sec, 62°C for 15 sec, 72°C for 2 min 30 sec and then 72°C for 10 min. The amplified construct was then isolated from a 1% agarose gel. After transformation, clones were selected on CSBA plates supplemented with 200 µg/ml spectinomycin [23]. Incubation was performed under anaerobic conditions. Knockout of the pneumolysin gene was confirmed by PCR and by sequencing using primers KO_Ply_us_F and KO_Ply_ds_R using the following

conditions: 98°C for 30 sec, 35 cycles of 98°C for 10 sec, 55°C for 15 sec, 72°C for 1 min 15 sec followed by 72°C for 10 min [24].

Bacteria were plated on Columbia sheep blood agar (CSBA) plates and incubated overnight at 37˚C and 5% CO_2. Liquid cultures of bacteria were prepared using either 5 ml of Chemically Defined Medium (CDM) [25] supplemented with 50 mM of filter-sterilized sucrose or 5 ml of Brain Heart Infusion (BHI) broth. Bacteria were grown to mid-log phase, meaning to OD_{600} of 0.1 to 0.2 in CDM and to OD_{600nm} of 0.5 to 0.8 in BHI, and then counted in a Neubauer chamber. The bacteria were pelleted, washed twice with pyrogen-free PBS then resuspended in 1 ml Eagle's minimum essential medium (MEM; Invitrogen, Basel, Switzerland) without FCS and warmed in a water bath to 37˚C.

Epithelial cell culture, exposure to pneumococcus and cytokine assays

The human pharyngeal epithelial cell line Detroit 562 (ATCC CCL 138) was cultured as published earlier in MEM supplemented with 10% of heat-inactivated fetal calf serum (FCS), 2mM of L-glutamine (Invitrogen, Basel, Switzerland), 1% sodium bicarbonate (Invitrogen, Basel, Switzerland), 1x MEM non-essential amino acid solution (Sigma, St. Louis, MO, USA), 1mM sodium pyruvate (Sigma, St. Louis, MO, USA), 100 µg/ml streptomycin and 100 U/ml penicillin at 37˚C in 5% CO_2 [26]. Cells were grown in 24-well plates to a confluent cell layer (≈$3x10^5$ cells per well). MEM containing the bacteria at three CFU concentrations (1, 1.5 and 2 x 10^6) were added to the Detroit 562 cells and the plates centrifuged at 173 g for 5 min at 25˚C. After 24 h of incubation at 37˚C and 5% CO_2 supernatants from the wells were collected in Eppendorf tubes, spun down at 132 g for 3 min at room temperature, and then stored at -80˚C until further use.

Immortalised human bronchial epithelial cells (iHBEC) were kindly provided by Professor Jerry W. Shay, (University of Texas, Dallas, USA). The iHBECs were grown in Keratinocyte serum-free media (Invitrogen) supplemented with epidermal growth factor and bovine pituitary extract. All cells were grown and experimented upon in humidified 5% CO_2, 95% humidity air at 37°C, in the absence of antibiotics. Confluent iHBECs monolayers were

grown in 24 well plates and incubated in serum-free media for 18h prior to investigation. Unstimulated cells were used as controls. Cells were cultured in 24 well cell culture plates for 24h with medium or live *S. pneumoniae*. After incubation, culture supernatants were collected, centrifuged at 10 000 g for 5 minutes to remove cellular debris and filter sterilized and stored at -80°C until assayed.

The amounts of CXCL8 and IL-6 were measured by ELISA (R&D systems ELISA kits, Abingdon, United Kingdom).

Mouse model

Ethics statement

All animal experiments were performed at the University of Liverpool and with prior approval from the UK Home Office and the University Ethics Committee.

Age-matched 8-12 week old female MF1 mice (Charles River, UK) were intranasally infected with 1 x 10^5 colony forming units of *S. pneumoniae* in 10 µl PBS as previously described [27]. Mice were sacrificed at pre-determined time points post-infection and organs removed for assessment of bacterial numbers and ELISA analysis. Nasopharynx and lungs were homogenized in PBS and serially diluted onto blood agar for enumeration of bacterial numbers by the Miles and Misra method. Homogenates were retained for use in ELISA.

Statistics

Student *t* test was used to assess the significance of the results.

Results and Discussion

We investigated the effect of capsule on CXCL8 and IL-6 induction in cells of the upper and lower respiratory tract using wild type *S. pneumoniae* serotype 2 (strain D39) and its capsule-deleted mutant. In addition, we compared the naturally occurring nonencapsulated clinical isolate 110.58 with its mutant in which the capsule of D39 has been inserted. Furthermore, D39 mutants in which pneumolysin alone was deleted or both pneumolysin and capsule were deleted were tested to investigate the role of capsule in the presence or absence of toxin (see Table 1 for bacterial wild type and mutant strains).

Deletion of capsule from D39 caused a small, but non-significant, increase of CXCL8 from the upper respiratory tract cells and insertion of the D39 capsule into strain 110.58 significantly decreased CXCL8 levels ($p = 0.04$) (Figure 1A). Deletion of pneumolysin significantly decreased CXCL8 compared to wild type control ($p = 0.0009$) but CXCL8 levels increased when the capsule was additionally deleted when compared to pneumolysin deletion alone ($p = 0.0036$). The same pattern was seen for IL-6 but the cytokine concentrations were lower and not significantly different between cells exposed to different strains (Figure 1B). For the bronchial epithelial cells, deletion of capsule in D39 caused a decrease in CXCL8 ($p < 0.0001$) but insertion of D39 capsule in strain 110.58 also caused a decrease in CXCL8 ($p < 0.0001$) (Figure 1C). These responses were in contrast to upper respiratory cells indicating a clear niche difference in host responses to pneumococcal capsule. Deletion of pneumolysin in D39 decreased CXCL8 levels in keeping with the response of upper respiratory cells ($p < 0.0001$), however CXCL8 was only slightly increased upon the additional deletion of the capsule ($p < 0.0001$) once again indicating a capsule-dependent difference in upper versus lower respiratory tract responses. IL-6 values were low with no difference between D39 and its capsule-deficient mutant but with levels reduced by insertion of the capsule into strain 110.58 ($p = 0.0002$). Deletion of pneumolysin increased IL-6 level ($p < 0.0001$) but additionally deleting the capsule reduced the level ($p < 0.0001$) (Figure 1D).

Overall, capsule restricted the release of CXCL8 from respiratory tract epithelial cells in contrast to pneumolysin, which stimulated the release of CXCL8.

No significant difference in cytotoxicity or haemolytic activity was observed between the encapsulated or nonencapsulated pneumococci (data not shown).

Having found that the capsule plays an important role in regulating CXCL8 induction *in vitro*, we next tested the influence of the capsule in a mouse model of nasopharyngeal carriage. Figure 2 shows that no difference in CXCL8 levels were detected between encapsulated or nonencapsulated strains in the nasopharynx and that deletion of pneumolysin did not have a measurable effect either. Importantly however, when the capsule was additionally deleted in the pneumolysin mutant, CXCL8 level increased ($p = 0.05$) supporting the *in vitro* finding that capsule suppresses CXCL8 release but that this effect may be masked by the presence of pneumolysin.

Interestingly, *in vivo* bacterial kinetics showed that, while the presence or absence of capsule did not appear to affect the ability of the pneumococcus to colonize the nasopharynx, only nonencapsulated pneumococci disseminated from the nasopharynx to the lungs (Figure 3) suggesting that the absence of capsule is important in facilitating pneumococcal movement from the nasopharynx to the lungs. Although nonencapsulated strains are expected to adhere to epithelial cells more efficiently than encapsulated strains, we did not detect higher numbers of nonencapsulated strains than encapsulated colonizing the nasopharynx.

The capsule is an important virulence factor as the thick polysaccharide layer helps the bacteria to escape opsonization and phagocytosis [28]. Encapsulation of the pneumococcus protects from complement activation. IgG and c-Adenosyl-monophosphate receptor protein (CRP) binding to the bacterial surface are reduced and thus activation of the classical pathway is impaired. Additionally, the degradation of C3b to iC3b is decreased by the capsule and phagocytosis by Fcγ receptor occurs less frequently [29]. Therefore, innate immunity initiated by encapsulated bacteria that causes macrophages and neutrophils to enter the nasopharynx as a result of chemotactic activity of CXCL8 will then not lead to

efficient opsonophagocytosis of the bacteria due to the polysaccharide capsule. Here we suggest that restriction of the initial step of CXCL8 release from the epithelial cells by the polysaccharide capsule may also contribute to bacterial survival.

There has been a recent study which also investigated the innate immune response due to *Streptococcus pneumoniae* in epithelial cells and which did not find a clear difference between wildtype strains and their capsule knock out mutants in terms of CXCL8 induction [30]. However, this group used microarrays to characterize the *in vitro* transcriptional response whereas here we have detected CXCL8 itself both *in vitro* and in a mouse model of nasopharyngeal colonization. Another group, like us, found greater CXCL8 production in response to nonencapsulated pneumococci than their encapsulated parent strains [31].

In conclusion, we find that the pneumococcal capsule plays an important role in regulation of innate immunity by reducing CXCL8 release from upper respiratory tract cells and also by restricting pneumococcal dissemination into the lower respiratory tract, where the pneumococcus would normal elicit a strong pro-inflammatory response leading to its clearance. We would argue that this is in keeping with the natural role of the pneumococcus as a commensal of the upper respiratory tract, whereby its primary function is to establish colonization with limited or no host inflammation to sustain its longer term survival in the nasopharynx without host mediated clearance.

Acknowledgement

We thank Dr Hans Jürg Engel for advice on genetic constructions.

References

1. Kadioglu A, Weiser J, Paton J, Andrew P (2008) The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. Nature Reviews Microbiology 6: 288-301.

2. Ratner AJ, Lysenko ES, Paul MN, Weiser JN (2005) Synergistic proinflammatory responses induced by polymicrobial colonization of epithelial surfaces. Proc Natl Acad Sci U S A 102: 3429-3434.

3. Dogan S, Zhang Q, Pridmore AC, Mitchell TJ, Finn A, et al. (2011) Pneumolysin-induced CXCL8 production by nasopharyngeal epithelial cells is dependent on calcium flux and MAPK activation via Toll-like receptor 4. Microbes Infect 13: 65-75.

4. McNeela E, Burke A, Neill D, Baxter C, Fernandes V, et al. (2010) Pneumolysin activates the NLRP3 inflammasome and promotes proinflammatory cytokines independently of TLR4. PLoS Pathog 6: e1001191.

5. Ogunniyi AD, Giammarinaro P, Paton JC (2002) The genes encoding virulence-associated proteins and the capsule of Streptococcus pneumoniae are upregulated and differentially expressed in vivo. Microbiology 148: 2045-2053.

6. van Cuyck H, Pichon B, Leroy P, Granger-Farbos A, Underwood A, et al. (2012) Multiple-locus variable-number tandem-repeat analysis of Streptococcus pneumoniae and comparison with multiple loci sequence typing. BMC Microbiol 12: 241.

7. Isaacman DJ, McIntosh ED, Reinert RR (2010) Burden of invasive pneumococcal disease and serotype distribution among Streptococcus pneumoniae isolates in young children in Europe: impact of the 7-valent pneumococcal conjugate vaccine and considerations for future conjugate vaccines. Int J Infect Dis 14: e197-209.

8. Hathaway LJ, Battig P, Muhlemann K (2007) In vitro expression of the first capsule gene of Streptococcus pneumoniae, cpsA, is associated with serotype-specific colonization prevalence and invasiveness. Microbiology 153: 2465-2471.

9. Hathaway LJ, Brugger SD, Morand B, Bangert M, Rotzetter JU, et al. (2012) Capsule Type of Streptococcus pneumoniae Determines Growth Phenotype. PLoS Pathog 8: e1002574.

10. Brueggemann AB, Peto TE, Crook DW, Butler JC, Kristinsson KG, et al. (2004) Temporal and geographic stability of the serogroup-specific invasive disease potential of *Streptococcus pneumoniae* in children. J Infect Dis 190: 1203-1211.

11. Kronenberg A, Zucs P, Droz S, Muhlemann K (2006) Distribution and invasiveness of Streptococcus pneumoniae serotypes in Switzerland, a country with low antibiotic selection pressure, from 2001 to 2004. J Clin Microbiol 44: 2032-2038.

12. Weinberger DM, Trzcinski K, Lu YJ, Bogaert D, Brandes A, et al. (2009) Pneumococcal capsular polysaccharide structure predicts serotype prevalence. PLoS Pathog 5: e1000476.

13. Mayer AK, Dalpke AH (2007) Regulation of local immunity by airway epithelial cells. Arch Immunol Ther Exp (Warsz) 55: 353-362.

14. Gomez MI, Prince A (2008) Airway epithelial cell signaling in response to bacterial pathogens. Pediatr Pulmonol 43: 11-19.

15. Abbas A L, AH and Pillai, S (2007) Cellular and Molecular Immunology. 6th Edition ed. Elsevier, Philadelphia.

16. Kato A, Schleimer RP (2007) Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. Curr Opin Immunol 19: 711-720.

17. Rastogi D, Ratner AJ, Prince A (2001) Host-bacterial interactions in the initiation of inflammation. Paediatr Respir Rev 2: 245-252.

18. Yoon BN, Choi NG, Lee HS, Cho KS, Roh HJ (2010) Induction of interleukin-8 from nasal epithelial cells during bacterial infection: the role of IL-8 for neutrophil recruitment in chronic rhinosinusitis. Mediators Inflamm 2010: 813610.

19. Battig P, Muhlemann K (2007) Capsule genes of *Streptococcus pneumoniae* influence growth in vitro. FEMS Immunol Med Microbiol 50: 324-329.

20. Yuste J, Botto M, Paton J, Holden D, Brown J (2005) Additive inhibition of complement deposition by pneumolysin and PspA facilitates *Streptococcus pneumoniae* septicemia. J Immunol 175: 1813-1819.

21. Engel H, Gutierrez-Fernandez J, Fluckiger C, Martinez-Ripoll M, Muhlemann K, et al. (2013) Heteroresistance to fosfomycin is predominant in Streptococcus pneumoniae and depends on the murA1 gene. Antimicrob Agents Chemother 57: 2801-2808.

22. Shevchuk NA, Bryksin AV, Nusinovich YA, Cabello FC, Sutherland M, et al. (2004) Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. Nucleic Acids Res 32: e19.

23. Meier PS, Utz S, Aebi S, Muhlemann K (2003) Low-level resistance to rifampin in Streptococcus pneumoniae. Antimicrob Agents Chemother 47: 863-868.

24. Brugger SD, Hathaway LJ, Muhlemann K (2009) Detection of Streptococcus pneumoniae strain cocolonization in the nasopharynx. J Clin Microbiol 47: 1750-1756.

25. van de Rijn I, Kessler RE (1980) Growth characteristics of group A streptococci in a new chemically defined medium. Infect Immun 27: 444-448.

26. Luer S, Troller R, Jetter M, Spaniol V, Aebi C (2011) Topical curcumin can inhibit deleterious effects of upper respiratory tract bacteria on human oropharyngeal cells in vitro: potential role for patients with cancer therapy induced mucositis? Support Care Cancer 19: 799-806.

27. Richards L, Ferreira D, Miyaji E, Andrew P, Kadioglu A (2010) The immunising effect of pneumococcal nasopharyngeal colonisation; protection against future colonisation and fatal invasive disease. Immunobiology 215: 251-263.

28. Weiser J (2010) The pneumococcus: why a commensal misbehaves. J Mol Med 88: 97-102.

29. Paterson GK, Orihuela CJ (2010) Pneumococci: immunology of the innate host response. Respirology 15: 1057-1063.

30. Bootsma H, Egmont-Petersen M, Hermans P (2007) Analysis of the in vitro transcriptional response of human pharyngeal epithelial cells to adherent *Streptococcus pneumoniae*: Evidence for a distinct response to encapsulated strains. infect Immun 75: 5489-5499.

31. Marriott H, Gascoyne K, Gowda R, Geary I, Nicklin M, et al. (2012) Interleukin-1beta regulates CXCL8 release and influences disease outcome in response to *Streptococcus pneumoniae*, defining intercellular cooperation between pulmonary epithelial cells and macrophages. Infect Immun 80: 1140-1149.

32. Hathaway L, Brugger S, Morand B, Bangert M, Rotzetter J, et al. (2012) Capsule type of *Streptococcus pneumoniae* determines growth phenotype. PLoS Pathog 8: e1002574.

33. Hathaway L, Meier PS, Battig P, Aebi S, Muhlemann K (2004) A homologue of *aliB* is found in the capsule region of nonencapsulated *Streptococcus pneumoniae*. Journal of Bacteriology 186: 3721-3729.

316 **Table 1**

317

Strain	Description	Capsule	Pneumolysin
D39	Wild type serotype 2	+	+
D39*cps*$^-$	Mutant lacking capsule [32]	-	+
D39*ply*$^-$	Mutant lacking pneumolysin [20]	+	-
D39*cps*$^-$*ply*$^-$	Mutant lacking both capsule and pneumolysin (current study)	-	-
110.58	Wild type nonencapsulated [33]	-	+
110.58::D39*cps*	Mutant with serotype 2 capsule [19]	+	+

318

319

320 **Figure legends**

321 **Figure 1: Effect of capsule and pneumolysin on CXCL8 and IL-6 induction in human**
322 **nasopharyngeal and bronchial epithelial cells.** Detroit 562 nasopharyngeal epithelial cells
323 (A and B) and bronchial epithelial cells (C and D) were assessed for CXCL8 (A and C) and
324 IL-6 (B and D) release after exposure to wild type or mutant pneumococcal strains. All
325 experiments were performed in triplicate at each of three CFU concentrations (1, 1.5 and 2 x
326 10^6) and the results pooled for each strain. Error bars indicate SEM. * indicates significant
327 difference from value of the parent strain.

328

329 **Figure 2: Effect of capsule and pneumolysin on CXCL8 induction in the mouse**
330 **nasopharynx.** CXCL8 detected in nasopharyngeal homogenate of mice three days after
331 exposure to wild type or mutant pneumococci expressed as a percentage of the value
332 obtained with the wild type strain. Error bars indicate SEM. * indicates significant difference
333 from value of the parent strain.

334

335

336 **Figure 3: Capsule did not affect colonization of the nasopharynx but only**
337 **nonencapsulated strains reached the lungs.** Each symbol represents the CFU from the
338 nasopharynx or lungs of an individual mouse on days 0, 1, 3, 8 and 15 after intranasal
339 inoculation. Horizontal bars indicate means.

Figure 1

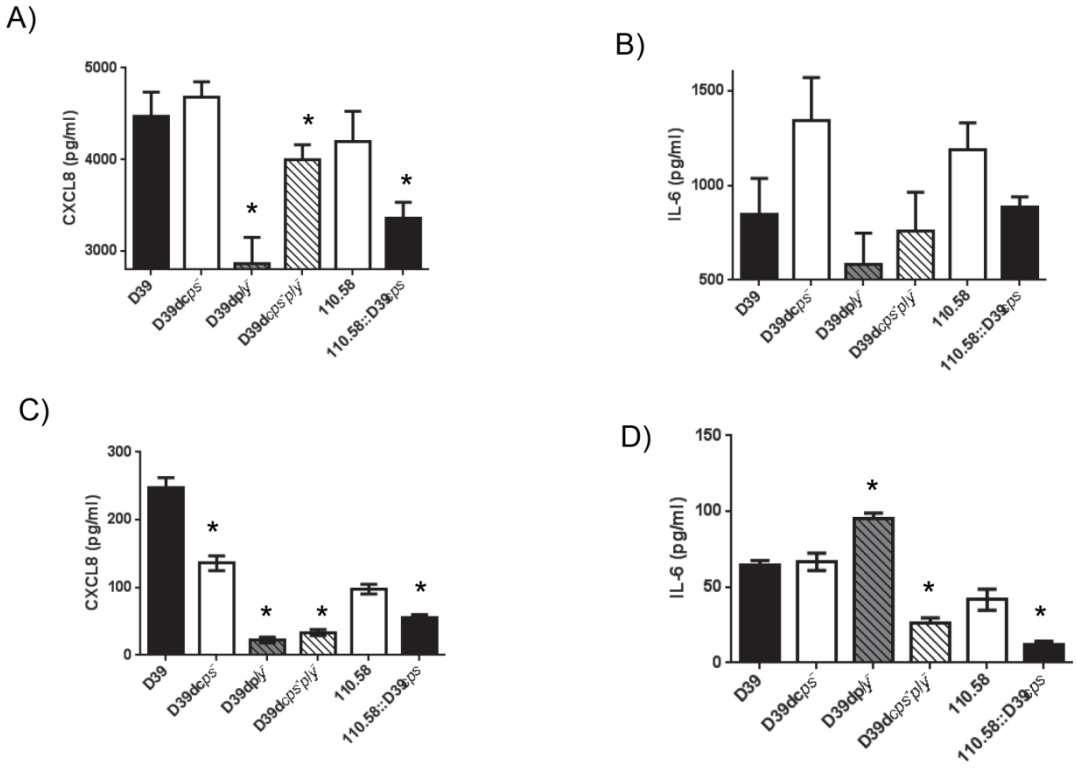

A)
CXCL8 (pg/ml)
5000
4000
3000
B)
IL-6 (pg/ml)
1500
1000
500
C)
CXCL8 (pg/ml)
300
200
100
0
D)
IL-6 (pg/ml)
150
100
50
0
D39
D39dcps-
D39dply-
D39dcps-ply-
110.58
110.58::D39cps

Figure 2

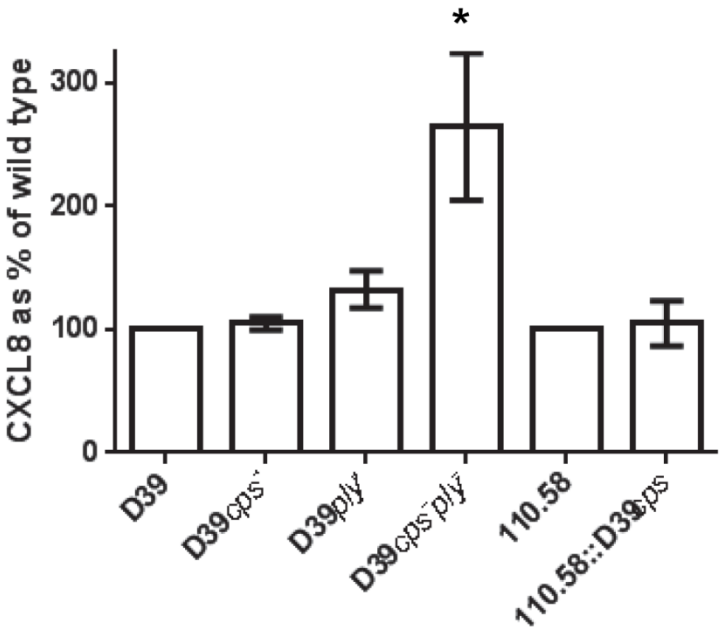
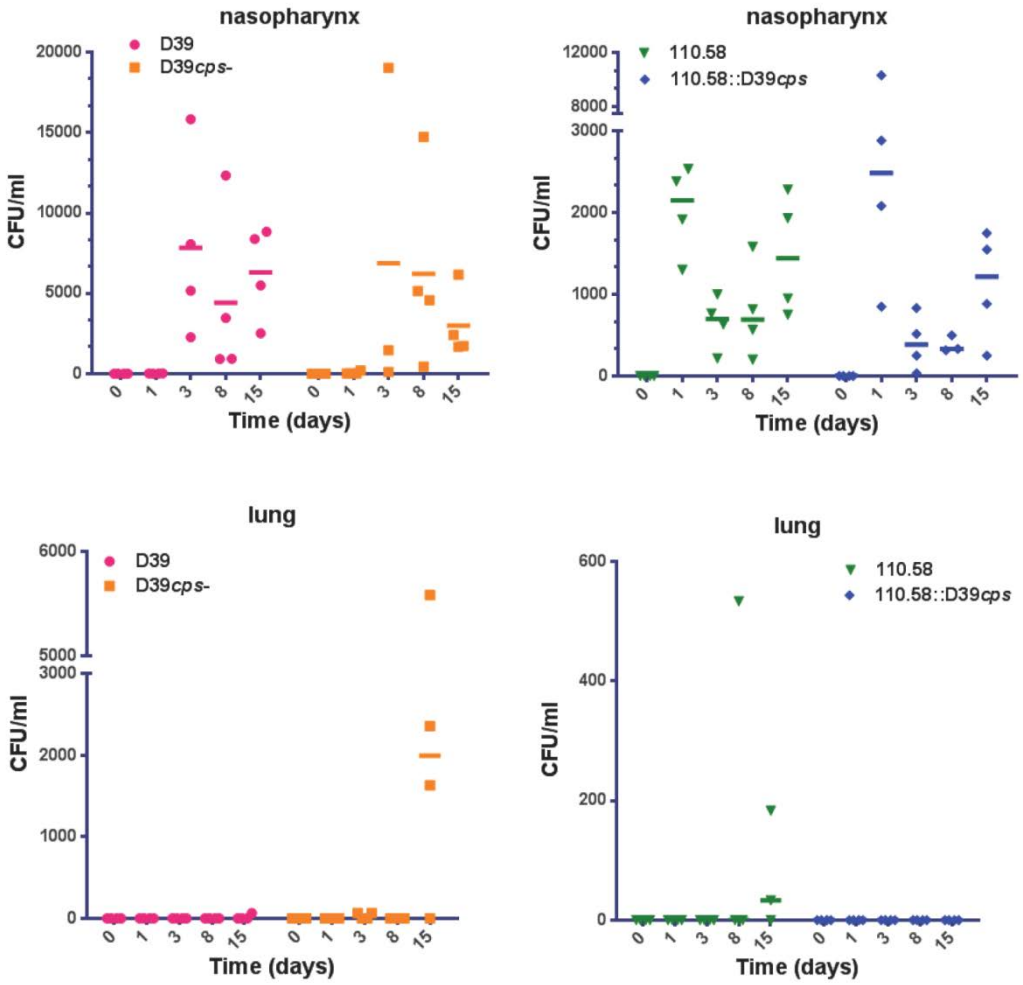

*
CXCL8 as % of wild type
300
200
100
0
D39
D39cps⁻
D39ply⁻
D39cps⁻ply⁻
110.58
110.58::D39cps

Figure 3


nasopharynx
D39
D39cps-
CFU/ml
Time (days)
nasopharynx
110.58
110.58::D39cps
CFU/ml
Time (days)
lung
D39
D39cps-
CFU/ml
Time (days)
lung
110.58
110.58::D39cps
CFU/ml
Time (days)

3.2 Immunological cells and immune regulation

Immunological cells and immune regulation in a mouse model of pneumococcal nasopharyngeal carriage

Immunological cells and immune regulation

Abstract

Immune responses against encapsulated and non-encapsulated pneumococcal strains were tested in a murine carriage model. Leukocytes, neutrophils, macrophages, regulatory T-cells and Interleukin-10 response were measured over 15 days of infection. Pneumococcal colonization of the mouse nasopharynx mainly remains asymptomatic. The encapsulated *S. pneumoniae* strains D39 (serotype 2) and the mutant strain 110.58::D39*cap* (serotype 2), which remained in the murine nasopharynx, resulted in an increase in FoxP3+ regulatory T-cells and IL-10 in the nasal mucosa. However, the strains 106.66 (serotype 6B), 110.58 (non-encapsulated) and the mutant strain D39*cps*- (non-encapsulated), which infiltrated the lungs, resulted in a stronger immune response with increased numbers of neutrophils, macrophages and leukocytes measured at 8 and 15 days after infection in the nasal mucosa. For strain 106.66, where infiltration of the lungs was recorded on day 8, a reduction in bacterial numbers in the lungs as well as in the nasopharynx was measured on day 15 indicating that immune cells stimulated in the lungs also attained access to the nasopharynx and that clearance of the infection had begun at both sites.

Introduction

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive, strictly human pathogen. Two of the main pneumococcal virulence factors are pneumolysin (Ply) and the anti-phagocytic capsule, which most *S. pneumoniae* strains possess [1]. Ply is the major toxin of *S. pneumoniae* and its targets cholesterol-containing membranes of eukaryotic cells. Ply has been demonstrated to be necessary and sufficient for airway epithelium activation [2]. This process occurs independently of TLR4 as membrane pores are created by Ply which leads to an increased spread of pneumococcal components to the cytoplasm of the host epithelial cells. Inside the cells these components then are capable of stimulating innate

immune responses [2]. The pores created by pneumolysin might allow an increased release of CXCL8 [3].

Colonization of the human nasopharynx by *S. pneumoniae* generally occurs asymptomatically and transiently [4, 5]. Colonization might be followed by invasive disease leading to sepsis, meningitis, pneumonia, or non-invasive mucosal infection such as acute otitis media [6]. Children younger than six years, elderly and immunosuppressed people are at increased risk [7]. Regulatory T-cells, including FoxP3+ cells, were shown to play an important role in preventing immunopathological effects, but they are also responsible for the inhibition of protective immunity which might result in prolonged infections and chronicity [8, 9]. Children colonized by pneumococcus showed significantly higher numbers of regulatory T-cells in their adenoids, but not in the peripheral blood, compared to non-colonized children. Regulatory T-cells are increased in number by local colonization with *S. pneumoniae* which results in delayed clearance and/or nasopharyngeal carriage [8]. In the lungs FoxP3+ regulatory T cells secrete IL-10 which results in a reduction of immunological reactivity during infection and limits the tissue damage [10]. In the early postnatal period, pneumococcal nasopharyngeal carriage resulted in an increase of IFN-γ and IL-10 response resulting in protection against pneumococcal infections but increasing the high rate of pneumococcal persistence in this age group [11]. In a recent study, specificity of regulatory T-cells for pneumococcal pneumolysin in tonsillar populations was shown indicating a role for these cells in the suppression of CD4+ T-cell responses to *S. pneumoniae.* Interestingly, the amount of pneumococcal-specific regulatory T-cells was much lower in the peripheral blood than in the tonsils [12].

As soon as *S. pneumoniae* leaves the nasopharynx and invades the lungs, local inflammatory responses are observed resulting in a massive infiltration of neutrophils and monocytes/macrophages [13] Neutrophils are one of the primary mediators of early defense [10]. Interestingly, also T-cells seem to play an important role in the earliest stages of pneumococcal immunity as bacterial growth in the lungs coincides with T-cell infiltrations into the lungs and a subsequent decrease in pneumococcal growth [14]. Thus, neutrophil

infiltration is followed by an increase of macrophages and lymphocytes. In a next step, induction of apoptosis of neutrophils and other recruited cells is started where the surface membranes stay intact and macrophages can clear these cells without spreading potential injurious contents. In a last step, macrophage apoptosis is induced [13, 15].

Material and Methods

Bacterial strains and growth conditions

The *S. pneumoniae* strains used to test immunological response in a stable nasopharyngeal carriage model are listed in Table 1.

Brain Heart Infusion (BHI) broth (Becton and Dickinson and Company, le Pont de Claix, France) alone own or supplemented with 20% Fetal Calf serum (FCS) (Biochrom KG, Berlin, Germany) was used for growth. Bacteria were plated onto Columbia Sheep Blood Agar (CSBA) plates and incubated overnight at 37°C in a microaerophilic milieu. As a quality control, optochin sensitivity was tested by placing an optochin disc onto the plate. The next day, a sweep of colonies was inoculated into 10 ml BHI broth and incubated statically for 16-18h at 37°C until OD_{500} reached 1.4. The tubes were then centrifuged at 1500xg for 15 minutes at room temperature, and the supernatant was discarded using a sterile Pasteur pipette. The pellet was resuspended in 1 ml BHI broth (80% v/v BHI broth and 20% v/v filtered FCS). 700 µl of the re-suspended pellet was added to 10 ml fresh BHI serum broth and incubated statically at 37°C for 5h. The cultures were then divided into 500 µl aliquots and frozen at -80°C. After aliquots had been at -80°C for longer than 24h, the viability was tested and absence of contamination checked by plated out the bacteria on CSBA plates. To measure colony forming unit (CFU) counts of frozen aliquots, serial dilutions were performed and plated out onto CSBA plates. After overnight incubation at 37°C under microaerophilic conditions, CFUs were counted. To inoculate animals, CFU counts were adjusted to $2x10^5$ CFUs/10µl using phosphate buffered saline (PBS).

Mouse strains

As a model of pneumococcal carriage outbred, female MF1 mice (Charles River, UK) were used at an age of 8-12 weeks.

Carriage model

The murine carriage model has been published previously [16]. In brief, mild anesthesia was used (2.5% v/v Isofluorane USP (Isocare) over oxygen (1.4-1.6 litres/min) to inoculate the required CFU counts into the mice. 10 µl of sterile PBS containing $2x10^5$ *S. pneumoniae* CFU counts were distributed between both nostrils. Groups of 4 mice were sacrificed on days 1, 3, 8 and 15 after inoculation by cervical dislocation. Nasopharyngeal tissue, cervical lymph nodes, nasal mucosa and the lungs then were dissected and placed into PBS (3 ml for nasopharynx, 5 ml for lungs, 500 µl for cervical lymph nodes and nasal mucosa). [17]

Colony forming unit counts in lung and nasopharynx

At the various timepoints after bacterial inoculation, the viable counts of bacteria in the lungs and the nasopharynx were determined. After dissection, nasopharyngeal and lung samples were disrupted with an Ultra-Turrax T8 homogeniser (IKA) and CFU counts were determined by serial dilutions in 1x PBS and plating on blood agar plates containing 5% (v/v) defibrinated horse blood (Oxoid) [14].

Flow cytometry

Mouse tissue suspensions from nasal mucosa and cervical lymph nodes were incubated with purified anti-Fc receptor blocking antibody (anti-CD16/CD32) before addition of specific antibodies. A combination of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, PE-Cy7- and allophycocyanin (APC)-conjugated monoclonal antibodies was used to stain cell surface markers. Intracellular staining for FoxP3 with monoclonal antibodies was performed according to the manufacturer's instructions (eBioscience). Analysis was performed using a Becton Dickinson FACScalibur flow cytometer running CellQuest acquisition and analyzed using FlowJo software (version 8.8.3, Tree Star).

Statistics

A two-tailed unpaired Student T test or one-way ANOVA was used, as appropriate, for data analysis. p-values lower than *0.05* were considered significant. GraphPad Prism 6 was used for statistical analysis.

Results

Immunological cells in the nasal mucosa

CD45+ cells represent all immunological cells present in the nasal mucosa. For most strains an increase in the number of CD45+ cells between day 1 and day 15 was observed, for strain 106.66 between day 1 and day 8 ($p<0.05$), for D39*cps*- between day 8 and day 15 ($p<0.05$) and for 110.58 between day 3 and day 8 ($p<0.05$). Interestingly, the increase of CD45+ cells correlated with lung infiltration (Supplementary Figure S1). For strains D39 and 110.58::D39cps, which generally remained in the nasopharynx and did not reach the lungs, no significant increase in CD45+ cells was measured (Figure 1A). Strain D39 infiltrated the lungs in one out of four mice on day 15. An increased CD45+ recruitment was observed for this mouse only and not for those three where the pneumococci remained in the nasopharynx (Supplementary Figure S2). Thus in Figure 1 the apparent increase in CD45+ cells in the nasal mucosa on day 15 for strain D39 is solely due to the infiltration of cells in the one mouse in which D39 reached the lungs.

An increasing number of macrophages (F4/80+ cells) was measured for the strains infiltrating the lungs (Supplementary Figure S3): Infection with strain 106.66 resulted in a non-significant ($p=0.3$) increase from 27 cells (range: 18.6-37.6) on day 3 to 396 cells (range: 29.3-1371) on day 15. Cell numbers were significantly increased from day 8 to day 15 for D39*cps*- ($p<0.05$) and from day 3 to day 8 for 110.58 ($p<0.05$) (Figure 1B). Cell numbers did not increase after infection with 110.58::D39cps or D39, both of which did not reach the lungs (Figure 1B and Supplementary Figure S3).

Neutrophil (Gr-1+ cells) infiltration of the nasal mucosa is also show to be dependent on lung CFU counts (Supplementary Figure S3). A significant increase was measured between day 1 and day 8 ($p<0.05$) for strain 106.66, between day 8 and day 15 ($p<0.05$) for

strain D39*cps*-, and a non-significant trend (*p=0.08*) for an increase of Gr-1+ cells between day 1 and day 8 after infecting the mice with strain 110.58. Infection with strains 110.58::D39*cps* and D39, which remained in the nasopharynx, and did not infiltrate the lungs, showed in no increase (Figure 1C and Supplementary Figure S4).

A significant increase of CD3+ T-cells is measured between day 1 and day 8 for infection with strain 106.66 (*p<0.01*), for strain D39 between day 1 and day 3 *(p<0.05*), for strain D39*cps*- between day 1 and day 3 (p<0.05), for strain 110.58 between day 1 and day 15 (*p<0.05*). Interestingly, CD3+ T-cell numbers remained on the same level throughout the experiment for 110.58::D39*cps* with a mean of 376.3 cells (range: 165-709.3) on day 1 (Figure 1D and Supplementary Figure S5).

For FoxP3+ regulatory T-cells infiltration of the nasal mucosa a dependency on nasopharyngeal CFU counts was observed (Figure 1E and Supplementary Figure S6). Infection with strain 106.66 resulted in a significant (*p<0.05*) increase between day 1 and 3, for D39*cps*- an increase between days 1 and 3 (*p<0.05*) as well as between days 8 and 15 (*p<0.05*) was measured, strain 110.58 infection resulted in a significant increase between days 1 and 8 (*p<0.05*), whereas a trend (*p=0.39*) towards increased FoxP3+ cells was observed for strain D39 with 66 cells (range: 5.3-168) on day 1 and 105 cells (range: 55.33-144) on day 3. T-cell numbers remained on the level of day 1 with a mean of 18 cells (range: 5.3-34.7) for 110.58::D39*cps*. As results for strains with 110.58 genetic background were shown to induce FoxP3+ cells infiltration on similar levels, whereas infiltration after infection with strains with a D39 genetic background resulted in higher FoxP3+ cell counts in the nasopharynx, genetic background also might have an influence on FoxP3+ recruitment.

Cytokine Interleukin 10 (IL-10)

We observed a trend towards increased IL-10 expression in mice after nasopharyngeal colonization compared to the untreated controls (Supplementary Figure S7). A significant increase was observed for strain D39 from day 0 to day 3 (*p<0.05*) and for 110.58 (p<0.05) whereas the significant increase occurred already from day 0 to day 1 for strain 110.58::D39*cps* (*p<0.0001*). The increase for strain 106.66 between day 0 and day 8

was not significant (*p=0.18*) and strain D39*cps*- also did not result in a significant increase (*p=0.54*) between day 0 and day 8, but the mean of absolute cell numbers increased from 774.3 on day 0 to 925.6 (range: 439-1478) on day 8 (Figure 1F). Strains with a 110.58 genetic background induced significantly (*p<0.05*) more IL-10 than strains with a D39 background on all timepoints tested.

Discussion

For the stimulation of FoxP3+ cells and the IL-10 response only nasopharyngeal colonization was required. However, pneumococcal strains 110.58, 106.66 and D39*cps-,* which were able to infiltrate the lungs, also caused an increase in neutrophils and macrophages in the nasopharynx compared to strains 110.58::D39*cps* and D39 which remained in the nasopharynx. Thus, the IL-10 response is dependent on the presence of pneumococci in the nasopharynx and no lung infiltration is required for secretion of this cytokine. Induction of IL-10 secretion was observed for all strains tested. This is evidence for successful carriage as IL-10 secretion is induced by carriage [8, 12]. Nasopharyngeal colonization is effectively silent and induces little or no immune response except of IL-10 response and the stimulation of FoxP3+ regulatory T cells both of which are responsible for immune homeostasis rather than for the induction of a strong immune response [10]. This allows the bacteria to persist in the niche. Once the bacteria reach the lung, a strong inflammatory response is initiated and this clears the bacteria from both the nasopharynx and the lung.

Interestingly, nonencapsulated strains infiltrated the lungs more easily than their encapsulated isogenic mutants. Generally, nonencapsulated pneumococci have a disadvantage in invading from the nasopharynx to other sites of the human body as they are more susceptible to opsonophagocytosis than encapsulated strains [18]. The presence of a capsule is not required for nasophayngeal colonization as nonencapsulated pneumococci are often isolated from this part of the body. Interestingly, also pneumococcal lineages are known which lack capsule, but show a clear ability to cause disease [19]. In cases of

conjunctivitis, nonencapsulated strains were even shown to have an advantage over encapsulated pneumococci [20]. Our results indicate an advantage for nonencapsulated *S. pneumoniae* strains in infiltrating the lungs (Figure 2).

For the IL-10 response mainly the genetic background was important: strain 110.58 and its isogenic mutant expressing a serotype 2 capsule 110.58::D39*cps* were able to induce a higher IL-10 response in the nasal mucosa compared to strains with a D39 or 106.66 genetic background.

References

1. Weiser JN: **The pneumococcus: why a commensal misbehaves**. *J Mol Med (Berl)* 2010, **88**(2):97-102.
2. Ratner AJ, Lysenko ES, Paul MN, Weiser JN: **Synergistic proinflammatory responses induced by polymicrobial colonization of epithelial surfaces**. *Proc Natl Acad Sci U S A* 2005, **102**(9):3429-3434.
3. Dogan S, Zhang Q, Pridmore AC, Mitchell TJ, Finn A, Murdoch C: **Pneumolysin-induced CXCL8 production by nasopharyngeal epithelial cells is dependent on calcium flux and MAPK activation via Toll-like receptor 4**. *Microbes Infect* 2011, **13**(1):65-75.
4. Obert C, Sublett J, Kaushal D, Hinojosa E, Barton T, Tuomanen EI, Orihuela CJ: **Identification of a Candidate Streptococcus pneumoniae core genome and regions of diversity correlated with invasive pneumococcal disease**. *Infect Immun* 2006, **74**(8):4766-4777.
5. Verhagen LM, Luesink M, Warris A, de Groot R, Hermans PW: **Bacterial respiratory pathogens in children with inherited immune and airway disorders: nasopharyngeal carriage and disease risk**. *Pediatr Infect Dis J* 2013, **32**(4):399-404.

6. Brugger SD, Hathaway LJ, Muhlemann K: **Detection of Streptococcus pneumoniae strain cocolonization in the nasopharynx**. *J Clin Microbiol* 2009, **47**(6):1750-1756.

7. Lynch JP, 3rd, Zhanel GG: **Streptococcus pneumoniae: epidemiology, risk factors, and strategies for prevention**. *Semin Respir Crit Care Med* 2009, **30**(2):189-209.

8. Zhang Q, Leong SC, McNamara PS, Mubarak A, Malley R, Finn A: **Characterisation of regulatory T cells in nasal associated lymphoid tissue in children: relationships with pneumococcal colonization**. *PLoS Pathog* 2011, **7**(8):e1002175.

9. Rouse BT, Sarangi PP, Suvas S: **Regulatory T cells in virus infections**. *Immunological reviews* 2006, **212**:272-286.

10. Neill DR, Fernandes VE, Wisby L, Haynes AR, Ferreira DM, Laher A, Strickland N, Gordon SB, Denny P, Kadioglu A *et al*: **T regulatory cells control susceptibility to invasive pneumococcal pneumonia in mice**. *PLoS Pathog* 2012, **8**(4):e1002660.

11. van den Biggelaar AH, Pomat WS, Phuanukoonnon S, Michael A, Aho C, Nadal-Sims MA, Devitt CJ, Jacoby PA, Hales BJ, Smith WA *et al*: **Effect of early carriage of Streptococcus pneumoniae on the development of pneumococcal protein-specific cellular immune responses in infancy**. *Pediatr Infect Dis J* 2012, **31**(3):243-248.

12. Pido-Lopez J, Kwok WW, Mitchell TJ, Heyderman RS, Williams NA: **Acquisition of pneumococci specific effector and regulatory Cd4+ T cells localising within human upper respiratory-tract mucosal lymphoid tissue**. *PLoS Pathog* 2011, **7**(12):e1002396.

13. Haslett C: **Granulocyte apoptosis and its role in the resolution and control of lung inflammation**. *American journal of respiratory and critical care medicine* 1999, **160**(5 Pt 2):S5-11.

14. Kadioglu A, Gingles NA, Grattan K, Kerr A, Mitchell TJ, Andrew PW: **Host cellular immune response to pneumococcal lung infection in mice**. *Infect Immun* 2000, **68**(2):492-501.

15. Marriott HM, Dockrell DH: **The role of the macrophage in lung disease mediated by bacteria**. *Experimental lung research* 2007, **33**(10):493-505.

16. Hathaway LJ, Brugger SD, Morand B, Bangert M, Rotzetter JU, Hauser C, Graber WA, Gore S, Kadioglu A, Muhlemann K: **Capsule type of Streptococcus pneumoniae determines growth phenotype**. *PLoS Pathog* 2012, **8**(3):e1002574.

17. Kadioglu A, Taylor S, Iannelli F, Pozzi G, Mitchell TJ, Andrew PW: **Upper and lower respiratory tract infection by Streptococcus pneumoniae is affected by pneumolysin deficiency and differences in capsule type**. *Infect Immun* 2002, **70**(6):2886-2890.

18. Hyams C, Camberlein E, Cohen JM, Bax K, Brown JS: **The Streptococcus pneumoniae capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms**. *Infect Immun* 2010, **78**(2):704-715.

19. Hanage WP, Kaijalainen T, Saukkoriipi A, Rickcord JL, Spratt BG: **A successful, diverse disease-associated lineage of nontypeable pneumococci that has lost the capsular biosynthesis locus**. *J Clin Microbiol* 2006, **44**(3):743-749.

20. Marimon JM, Ercibengoa M, Garcia-Arenzana JM, Alonso M, Perez-Trallero E: **Streptococcus pneumoniae ocular infections, prominent role of unencapsulated isolates in conjunctivitis**. *Clin Microbiol Infect* 2013, **19**(7):E298-305.

Strain	Serotype	Remarks
D39	2	Lab strain, International Reference
D39*cps*-	Non-encapsulated	Mutant of D39; capsule operon (*cpsA*-*O*) replaced with Janus-cassette
110.58	Non-encapsulated	Swiss clinical strain, naturally non-encapsulated
110.58::D39*cps*	2	Mutant of 110.58; capsule operon (*cpsA*-*O*) of strain D39 inserted
106.66	6B	Swiss clinical strain

Table 1: *S. pneumoniae* strains used for *in vivo* studies.

Figure 1

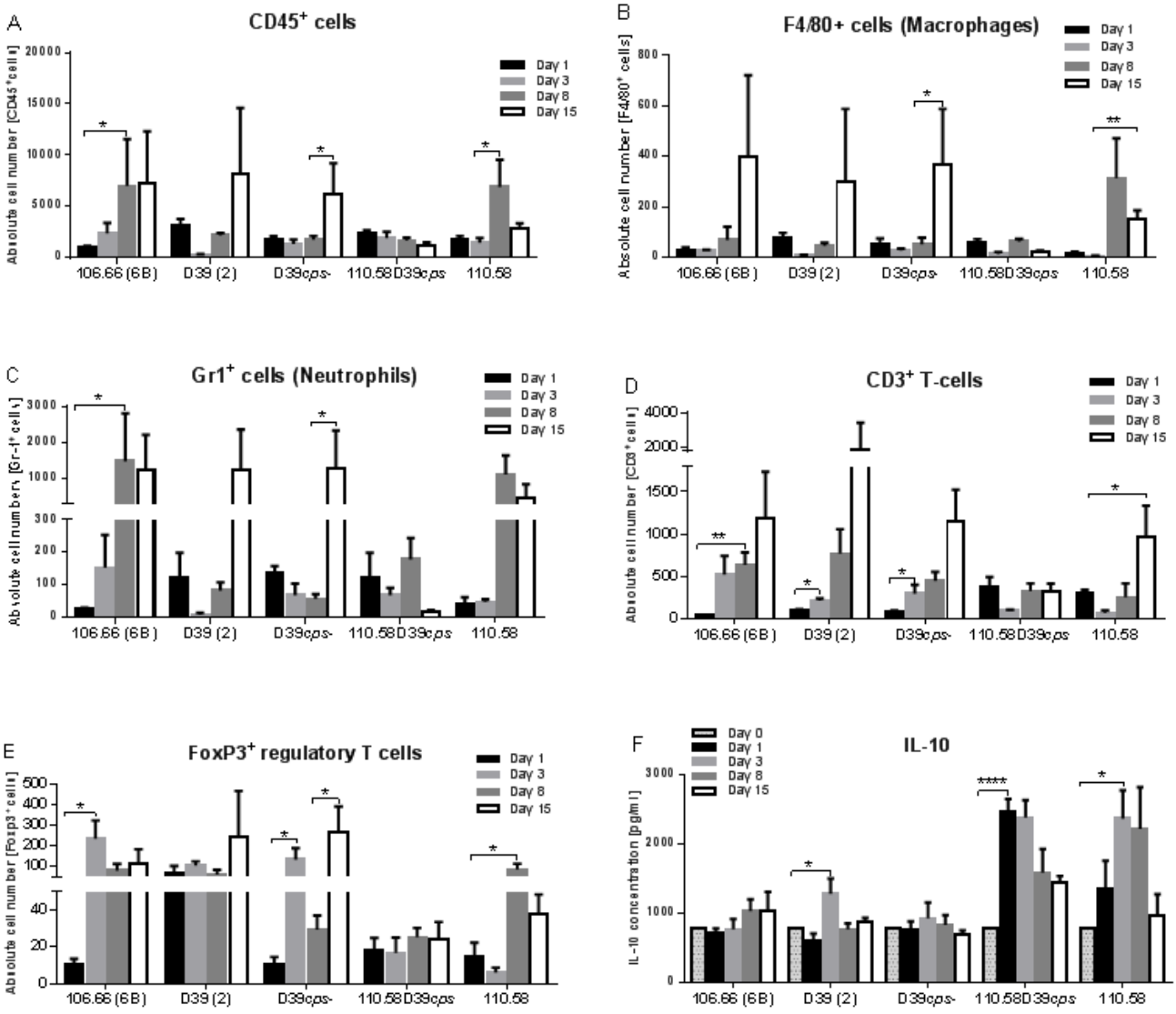


Figure 1: Induction of immune response by pneumococcal strains 1 to 15 days after infection. 106.66 (serotype 6B), D39 (serotype 2), mutant strain D39*cps*- (non-encapsulated); mutant strain 110.58D39*cps* (serotype 2) and strains 110.58 (non-encapsulated) in the murine nasopharynx. A) CD45+-cells. B) Macrophages (F4/80+ cells) C) Gr1+ cells (neutrophils) D) CD3+ T cells E) FoxP3+ Regulatory T-cells F) IL-10 secretion. * *p<0.05*, ** *p<0.01*, *** *p<0.001*, **** *p<0.0001*. n=4 mice per time point.

Figure 2

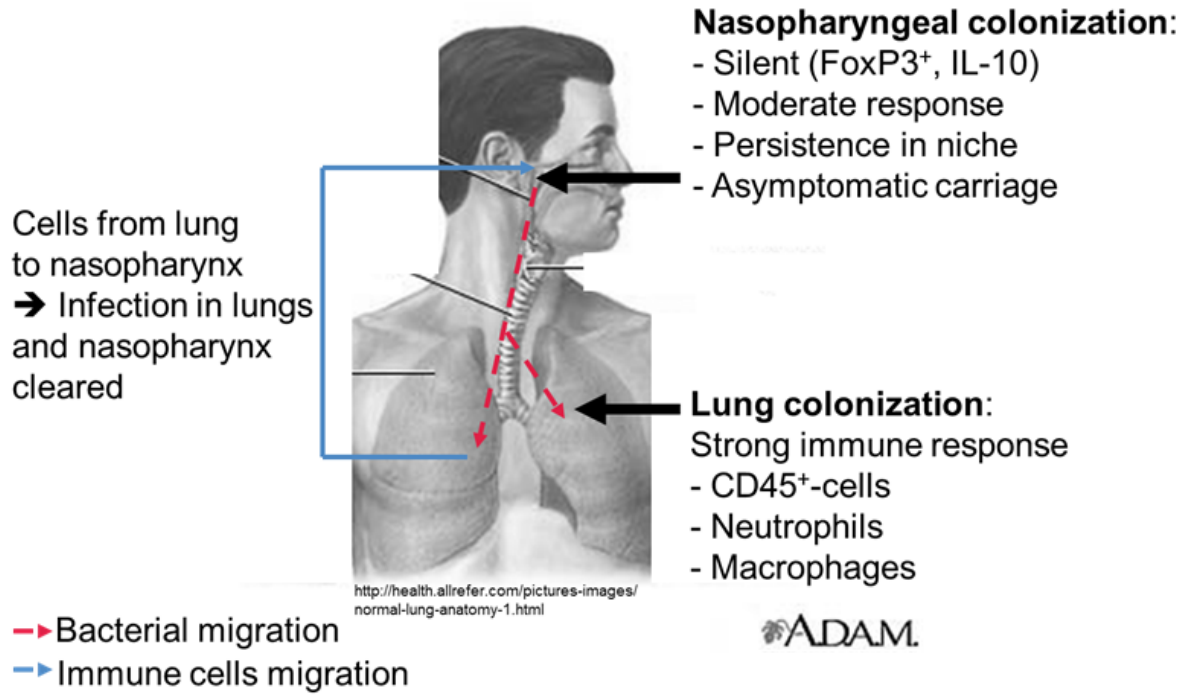
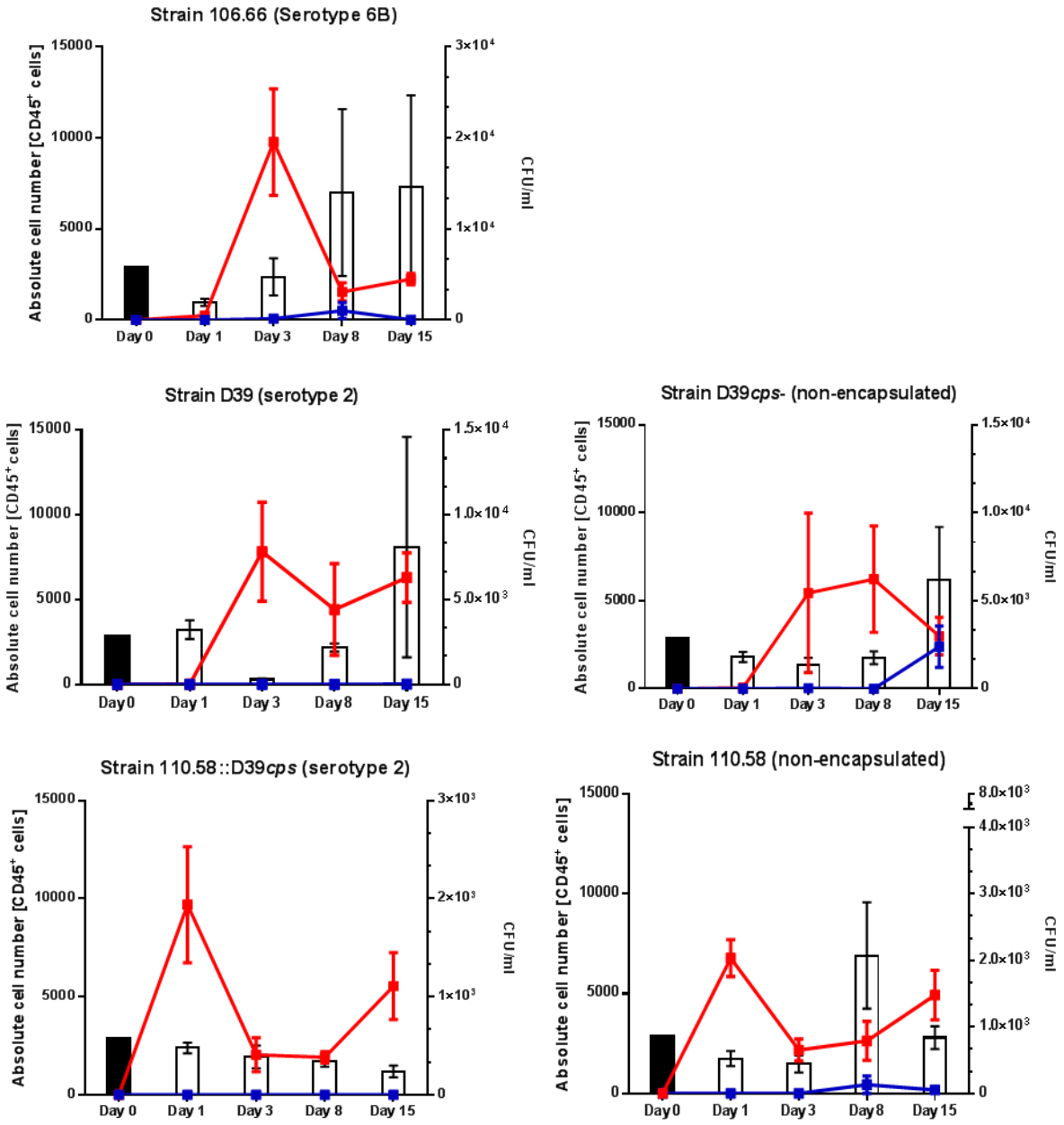
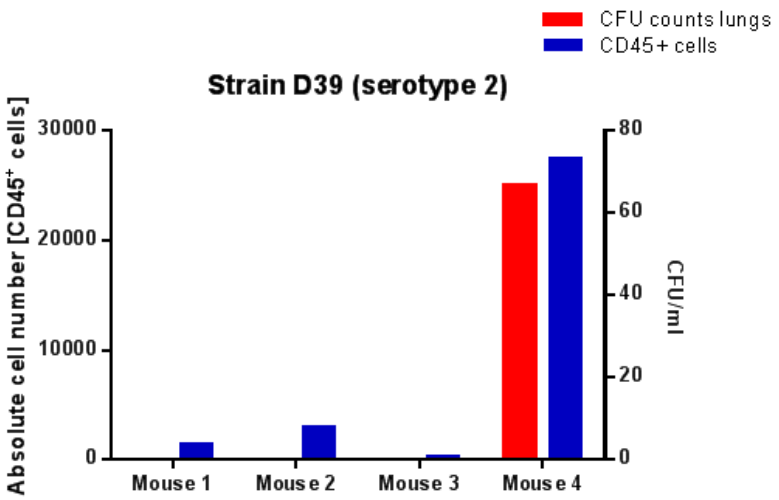


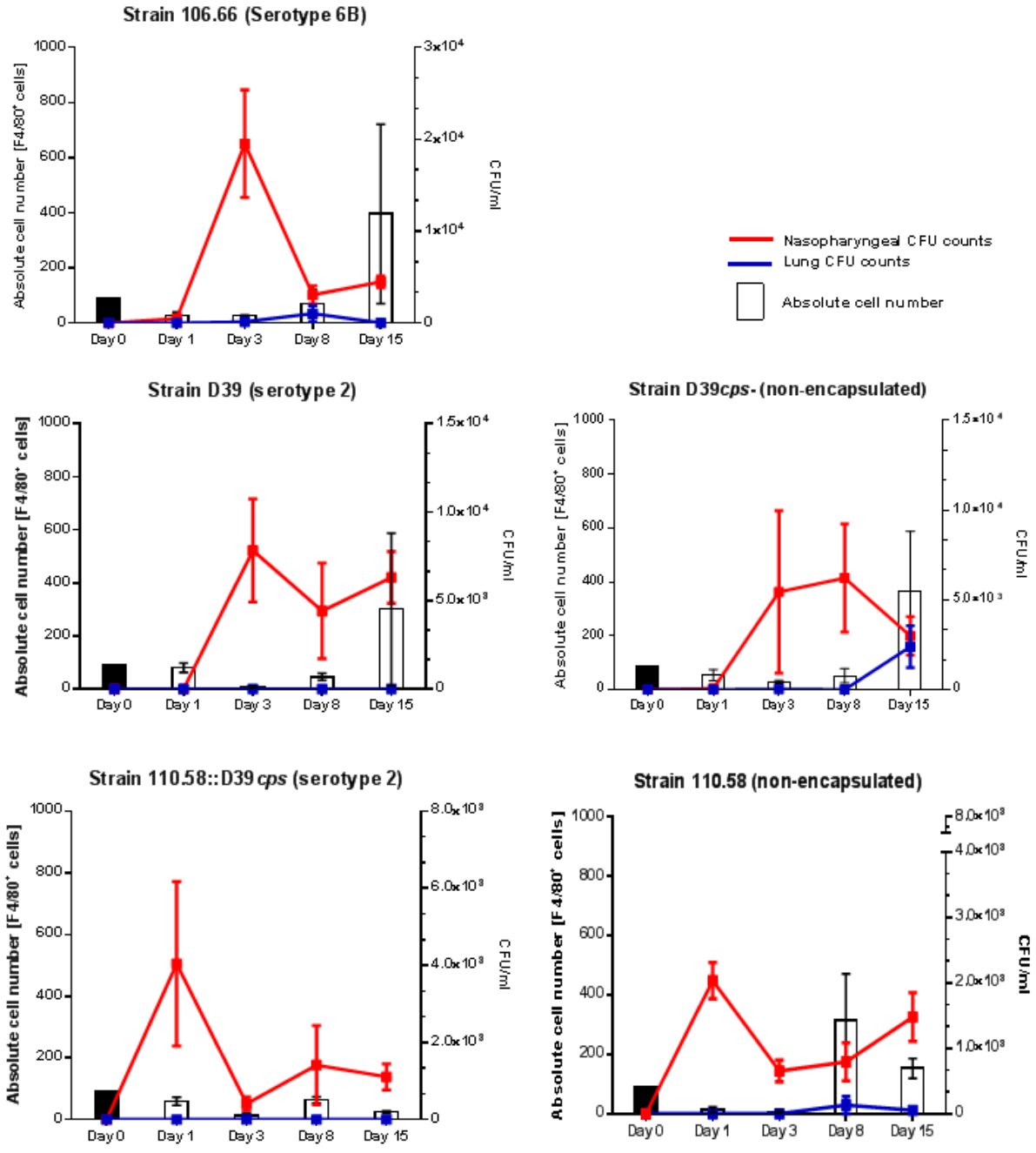
Figure 2: Summary of immune response to pneumococci residing in the nasopharynx and/or infiltrating the lungs. Pneumococcal strains colonizing only the nasopharynx induce a silent immune response where mainly FoxP3+ regulatory T-cells and Interleukin 10 (IL-10) are increased. This results in a moderate immune response, persistence in the niche and asymptomatic carriage. In contrast, *S. pneumoniae* strains invading from the nasopharynx and infiltrating the lungs result in a stronger immune response where also leukocytes (CD45+ cells), neutrophils and macrophages are increased. These immune cells are then able to migrate from the lungs also to the nasopharynx and the infections in the lungs as well as in the nasopharynx are cleared.



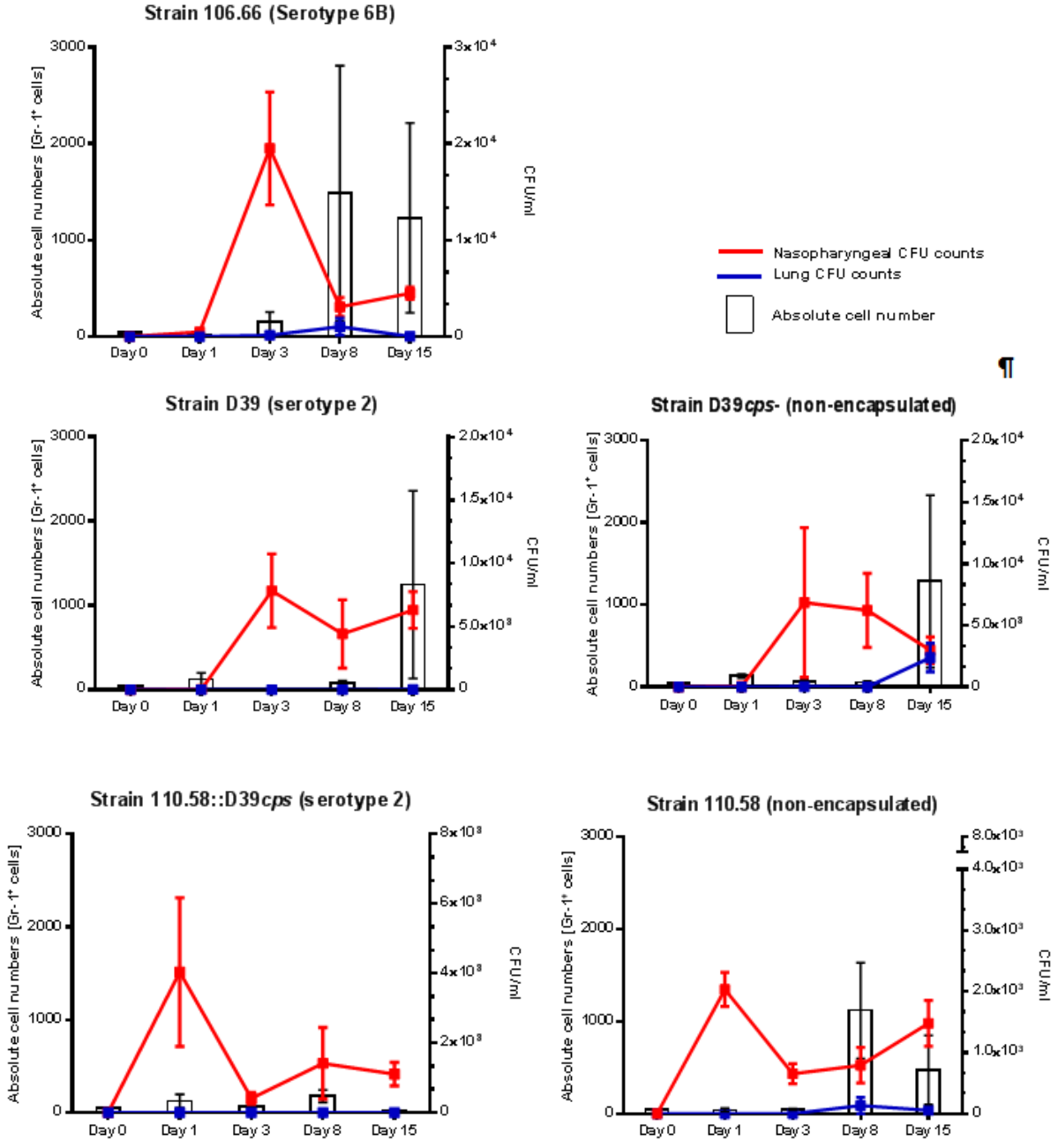
Supplementary Figure S1: Absolute numbers of CD45-positive cells measured in nasal mucosa for all different strains tested. Black bar: Reference for absolute cell count on day 0 from different experiments. n = 4. Error bars: Standard error of the mean.



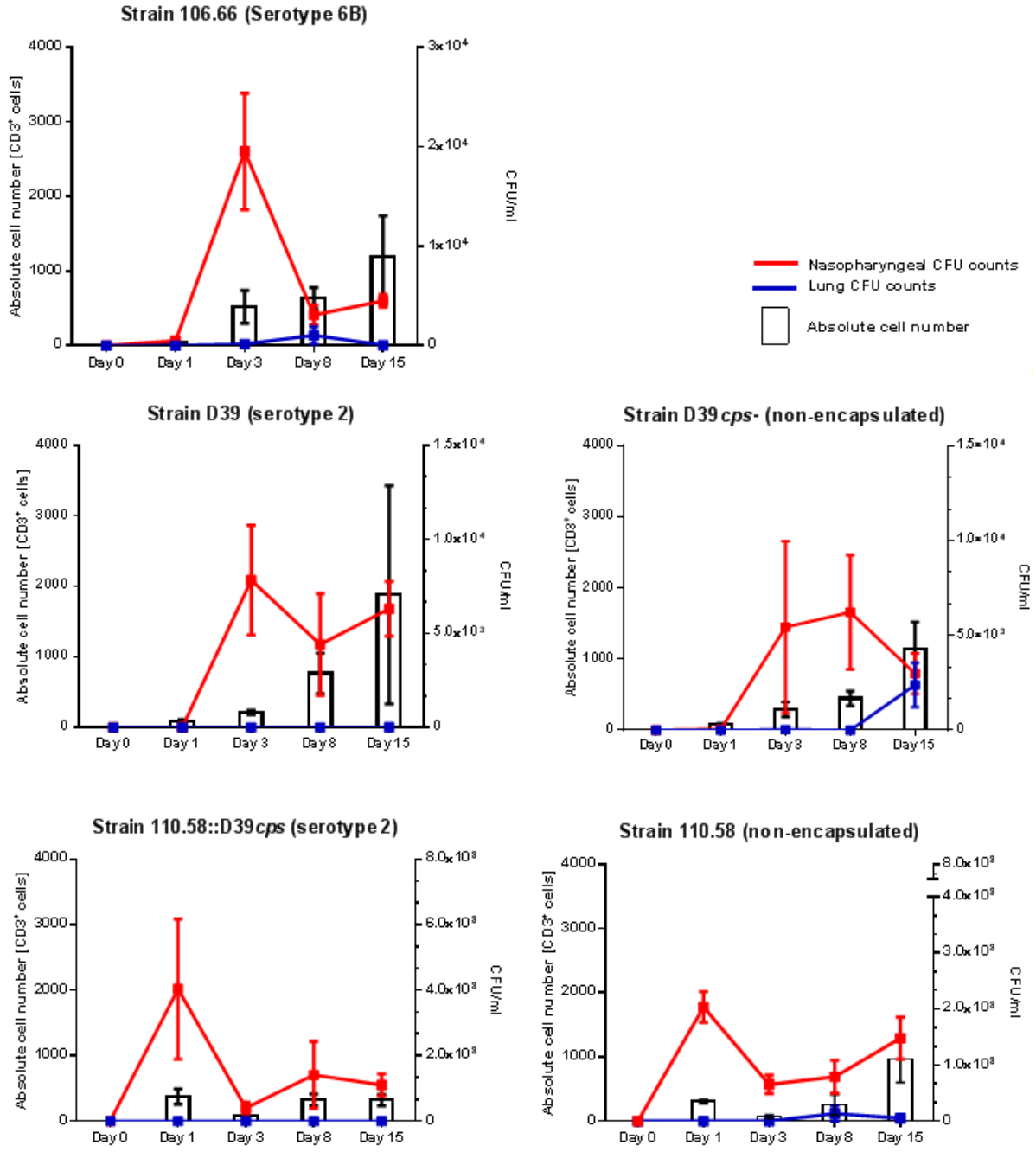
Supplementary Figure S2: *S. pneumoniae* strain D39 successfully infiltrated the lungs in one out of four mice resulting in an increased recruitment of CD45+ cells into the nasal mucosa for this mouse.



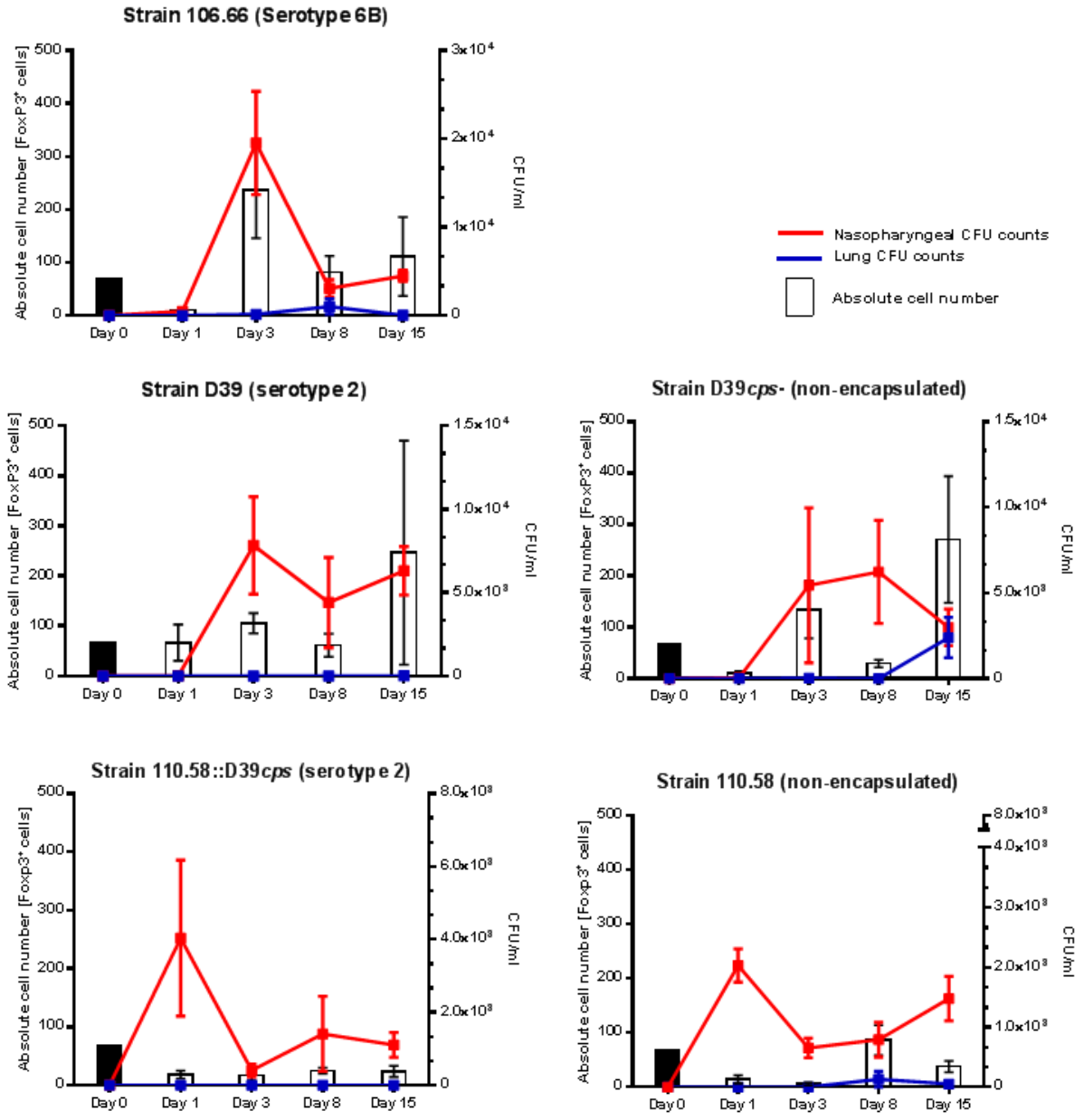
Supplementary Figure S3: Absolute numbers of F4/80-positive cells measured in the nasal mucosa for all different strains tested. Black bar: Reference for absolute cell count on day 0 from different experiments. n = 4. Error bars: Standard error of the mean.



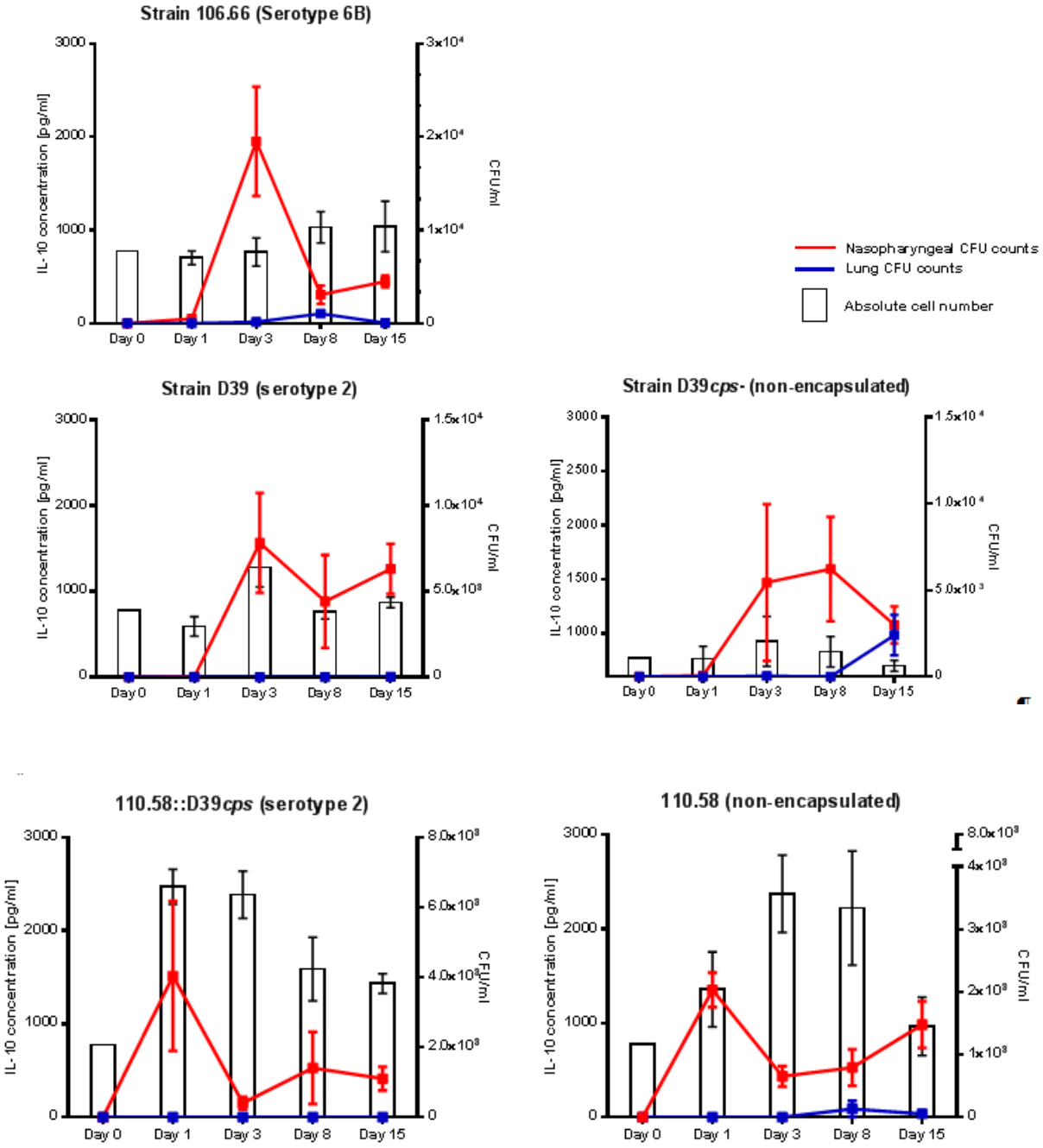
Supplementary Figure S4: Absolute numbers of Gr-1-positive cells measured in the nasal mucosa for all different strains tested. Black bar: Reference for absolute cell count on day 0 from different experiments. n = 4. Error bars: Standard error of the mean.



Supplementary Figure S5: Absolute numbers of CD3-positive cells measured in the nasal mucosa for all different strains tested. Black bar: Reference for absolute cell count on day 0 from different experiments. n = 4. Error bars: Standard error of the mean.



Supplementary Figure S6: Absolute numbers of FoxP3-positive cells measured in the nasal mucosa for all different strains tested. Black bar: Reference for absolute cell count on day 0 from different experiments. n = 4. Error bars: Standard error of the mean.



Supplementary Figure S7: IL-10 concentrations measured in the nasal mucosa for all different strains tested. Red line: CFU counts nasal mucosa. Blue line: CFU counts lungs. Bars: Absolute IL-10 concentration. n = 4. Error bars: Standard error of the mean.

4. Investigating host innate immune responses to Respiratory Syncytial Virus and *Streptococcus pneumoniae:* co-stimulation *in vitro* model of human airway epithelial cells

Investigating host innate immune responses to Respiratory Syncytial Virus and *Streptococcus pneumoniae:* co-stimulation *in vitro* model of human airway epithelial cells

Abstract

The effect of mono- and mixed infections of *Streptococcus pneumoniae* and Respiratory Syncytial Virus (RSV) on the human bronchial epithelial cell line BEAS-2B interleukin-6 (IL-6) and interleukin-8 (CXCL8) release was determined. Mixed infections resulted in more IL-6 and CXCL-8 secretion than mono-infections. Two hours of RSV-priming of the cells also increased IL-6 and CXCL-8 release. The two major pneumococcal virulence factors, capsule and pneumolysin, both had an effect on IL-6 and CXCL-8 secretion: Encapsulated bacteria in combination with RSV resulted in IL-6 secretion by BEAS-2B cells whereas non-encapsulated ones did not induce a clear IL-6 response. Increasing pneumolysin concentrations resulted in increased IL-8 secretion whereas the presence of a capsule played a less significant role for CXCL-8 induction but still, encapsulated bacteria resulted in slightly increased CXCL-8 secretion on RSV-primed cells compared to the non-encapsulated mutant.

Introduction

One of the leading causes of lower respiratory tract infections in infants and children is Respiratory Syncytial Virus (RSV) which causes cold-like symptoms in most healthy adults and children [1, 2]. Factors such as premature birth, immune deficiency, congenital heart disease or chronic lung disease of prematurity increase the risk of suffering more severe infections leading to the need for intensive care and mechanically assisted ventilation or even death [3]. Additionally, the clinical severity can be directly linked to the viral load of an infant. Thus, early antiviral treatment might be an important factor to improve the disease outcome [4]. RSV has also been shown to be an important cause of acute otitis media in older children [5] where the peak seasons for RSV infections are the winter and spring

months [6, 7]. RSV induces only incomplete immunity thus re-infections can occur [8]. According to the World Health Organization (WHO), RSV causes worldwide 64 million infections and 160,000 deaths per year [9].

This non-segmented, negative-strand RNA virus is grouped into the genus *Pneumovirus* [10] of the family *Paramyxoviridae* [11] and was first isolated in 1957 [12]. The first reported case of RSV infection goes back to the year 1931 (Vanderbilt Medical School, Nashville, USA) [13]. 11 proteins are encoded on 15 200 nucleotides. One virion has a diameter of about 200 nm and is composed of a nucleocapsid within a lipid envelope where the lipid bilayer is taken from the host plasma membrane but is completed with viral transmembrane surface glycoproteins of 11-20 nm in size [8].

Upper airway epithelial cells are the main target of RSV infection but there are also cases reported where the virus infected alveolar epithelial cells [13]. Entry of the virus into the cell is the first critical step of infection [8]. In primary airway epithelial cells RSV replication is 100 times greater and cytotoxicity 15 times greater than in the human bronchial epithelial cell line BEAS-2B which might be due to differences in expression of cell surface receptors and the resulting increased viral infection rate [14]. Attachment to the cells occurs mainly via two different surface proteins. One is the G glycoprotein, a glycosylated type II transmembrane protein composed of 289-299 amino acids, depending on the strain, which is used as an accessory protein to increase the efficiency of attachment [15, 16]. The other is the essential F protein which is required for the fusion of the infected cell membranes with uninfected cell membranes or the viral envelope [17] [18]. F protein is also able to activate RhoA, a Ras family protein which results in actin cytoskeleton reorganization [19]. Lung cells show a higher RhoA expression than other cells [20] which results in an increased replication rate for RSV . The third important protein for RSV infection of airway epithelial cells is the M protein which is of major importance for the coordination of assembly of the virus [8, 21].

Flow cytometry of RSV-infected cells has shown that they are more often found in the G_0/G_1-phase than are non-infected controls indicating remodeling of the airway epithelium, beneficial for the virus [22].

During RSV infection lymphocytic infiltrates are observed around small airways as well as cell debris in airway lumens. Neutrophils then attach to epithelial cells infected with RSV and, as a result of activation, start damaging the epithelium [23]. Toll-like receptor 4 (TLR4) on pulmonary cells, stimulated by the major surface glycoproteins G and F, is one of the most important factors for innate immunity against RSV infection. NK cell and monocyte ($CD14^+$cell) responses are stimulated [24]. RSV-induced CXCL-8 production requires interactions between different transcription factors in a cooperative way [25]. IL-6 and CXCL-8 were shown to play an important role in chemotaxis for neutrophils and macrophages and expression of IL-6 at the site of infection is essential for disease progression [24, 26]. Epithelial cells, monocytes and pulmonary epithelial cells increase CXCL-8 secretion after RSV infection [27]. Large amounts of lactate dehydrogenase (LDH) are produced by RSV-infected epithelial cells and thus, LDH can be used as a predictor of disease severity [14, 28].

Genetic predisposition plays a role as it was shown that severe RSV bronchiolitis is linked to a CXCL-8 anomaly (IL8-251A) and children containing such a variation suffer much more often from severe wheezing than children with normal CXCL-8 expression [29, 30]. Additionally, control mechanisms responsible for the regulation of disease pathogenesis and chronicity are determined by the spectrum of cytokines expressed [31]. The following factors are upregulated in response to RSV infections (Table 1):

Cells / Secretions	Factor(s)	Reference
Primary airway epithelial cells	B-cell activating factor (BAFF)	McNamara et al. 2013 [32]
Pulmonary epithelial cells	IL-6 and IL-8	Yokota et al. 2012 [33]
Bronchoalveolar lavage (infants suffering from RSV bronchiolitis)	IL-8, IL-10, MCP-1, MIP-1a/b mRNA	McNamara et al. [34]

Table 1: Factors upregulated during RSV infection.

A Japanese study showed that almost half of the children with bronchopulmonary RSV infection suffered from secondary bacterial infections, most commonly *H. influenzae* and *S. pneumoniae* [35]. Another study found evidence that viral co-infections are associated with more severe invasive pneumococcal disease [7]. In children with higher RSV loads a synergistic interaction between the virus and *S. pneumoniae* was measured which increased the risk of acute otitis media [36]. A major risk for secondary bacterial infections after RSV infections is the upregulation of platelet-activating factor (PAF) receptor and the following enhanced adhesion of pathogenic bacteria, such as *S. pneumoniae* [33]. When the two human epithelial cell lines HEp-2 and A549 were infected with RSV this led to an increased adherence of pneumococcal serotypes 3, 9, 14, 18, 19 and 23, some of the most pathogenic serotypes for infants and young children [37]. The same effect was also shown in another study using serotype 19 with an increase in adherence over time between 24 and 72 hours after viral infection. On the epithelial cell lines A549, BEAS-2B and NHBE, the expression of ICAM-1, CEACAM1 and PAF-r was increased by infection with RSV. Blocking of the receptors ICAM-1 and PAF-r prevented the adhesion of *S. pneumoniae* strain 19 to A549 cells [38].

In our study the effect of two of the main pneumococcal virulence factors, the pore-forming hemolytic toxin pneumolysin [39-42] and the polysaccharide capsule in combination with RSV were analyzed in terms of IL-6 and CXCL-8 induction in BEAS-2B cells.

Material and Methods

The human bronchial epithelial cell line BEAS-2B (ATCC CRL-9609) was used for *in vitro* experiments. As growth medium, Bronchial Epithelial Growth Medium (BEGM) (Lonza, Wokingham, UK) supplemented with the additives listed in Table 2 was used.

Cells were grown in T75 flasks (Sarstedt, Leicester, UK) coated with a mixture of 0.01 mg/ml fibronectin (Sigma-Aldrich), 0.03 mg/ml collagen (Type 1, from human skin, Sigma-Aldrich) and 0.001 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich) dissolved in BEGM for 7 days. Every second day the growth medium was changed.

To perform infection experiments, the cells were seeded in a collagen-coated (fibronectin, collagen and BSA, as above) 96-well plate with 30 000 cells per well. After seeding, the plates were incubated for 2 days at 37°C + 5% CO_2 for 3 days with the medium changed after 2 days. Four different assays of incubation were performed (see Figure 1). The pneumococcal strains or purified virulence factors used in this study are listed in Table 3. The next day, pictures were taken using a 40-fold magnification from all the different incubations, then the supernatant was removed and stored at -80°C in a 96-well plate. CXCL-8 and IL-6 ELISA (R&D systems, Oxford, UK) were performed using a 1:20 dilution of the supernatants.

Results

An IL-6 response higher than the untreated control (5970 pg/ml) was only measured when BEAS-2B cells were stimulated with either encapsulated pneumococcal strain D39 or its mutant PLN-A expressing non-pore forming pneumolysin in combination with RSV. IL-6 response after addition of either RSV (5960 pg/ml), D39 (5830 pg/ml) or PLN-A (3500 pg/ml) as single treatment was lower than the untreated control, but RSV-priming or bacterial priming of the cells or even co-stimulation resulted in IL-6 secretion (Figure 2): For D39, a significantly greater level of IL-6 was measured when the cells were primed with RSV compared to co-stimulation ($p<0.05$) or bacterial priming ($p<0.05$), whereas PLN-A treatment resulted in no significant difference (*p-vaule* ANOVA=*0.7*). When either pneumolysin, the non-pore forming pneumolysin PdB or LPS were used, IL-6 secretion was lower than the untreated control for the mono-treatment as well as in combination with RSV (Supplementary Figure 1).

Priming of BEAS-2B cells with either D39, PLN-A, 25 ng/ml pneumolysin, R36A or PdB resulted in higher CXCL-8 secretion than the untreated control (760 pg/ml). For pneumolysin ($p<0.01$) and PdB ($p<0.05$) the amount of CXCL-8 measured after bacterial priming was significantly greater compared to RSV mono-treatment which resulted in only minimal CXCL-8 induction (23 pg/ml) (Figure 3a). Only mono-treatment with 25 ng/ml

pneumolysin resulted in CXCL-8 secretion, all other bacteria or bacterial products did not induce CXCL-8 higher than the untreated control (Supplementary Figure 2). Co-stimulation of BEAS-2B cells with whole bacteria D39 (734 pg/ml) and PLN-A (588 pg/ml) resulted in CXCL-8 secretion, but the amount of cytokine measured was not significantly increased compared to RSV mono-treatment (*p=0.97* for D39 and *p=0.9* for PLN-A). 730 pg/ml and 580 pg/ml CXCL-8 were measured after RSV-priming and stimulating the cells with D39 or PLN-A, respectively (Figure 3b).

No dose-dependency in terms of IL-6 secretion for pneumolysin was measured. Using 125 ng/ml pneumolysin resulted in IL-6 secretion (1800 pg/ml) without the need of a co-infection using RSV. But no pattern of dose-dependency was observed for RSV priming, as co-stimulation with 5 ng/ml PLY (805 pg/ml) as well as with 125 ng/ml PLY (330 pg/ml) resulted in IL-6 secretion, but not with 25 ng/ml PLY, whereas only priming with 25 ng/ml PLY induced IL-6 (130 pg/ml) (Supplementary Figure 3a). In contrast, CXCL-8 secretion was shown to be dependent on pneumolysin concentration: 5 ng/ml pneumolysin mono-treatment did not result in CXCL-8 secretion higher than the untreated control, whereas after adding 25 ng/ml or 125 ng/ml, 120 pg/ml and 320 pg/ml CXCL-8 were measured. Bacterial priming only resulted in significantly (*p<0.05*) increased CXCL-8 levels compared to RSV mono-treatment after stimulating the BEAS-2B cells with at least 25 ng/ml pneumolysin. A significantly (ANOVA: *p<0.0001*) increased CXCL-8 secretion after RSV-priming compared to bacterial mono-treatment, RSV mono-treatment and bacterial priming was only measured when 125 ng/ml pneumolysin were used (Figure 4). The non-pore-forming pneumolysin mutant protein PdB did not show any concentration-dependency for the concentrations tested (5, 25 and 125 ng), neither in mono- or co-infections nor in RSV- or PdB-primed infections. None of the conditions tested reached a level higher than the uninfected control (Supplementary Figure 3b).

For all the different treatments pictures were also taken. All treatments resulted in necrosis of the BEAS-2B cells, but there were visible differences in the amount of dead cells

(Figure 5). With mono-infections the most cell death occurred with S. pneumoniae strain D39 and with RSV (Figure 5a). Additionally, dose-dependency of purified pneumolysin protein was shown. In contrast, RSV-primed cells (Figure 5b) showed the strongest damage for RSV-treatment, D39 wild-type PLN-A and, to a smaller amount, also for R36A treatment. After adding 125 ng of purified pneumolysin, more necrosis was observed than with either 5 ng or 25 ng protein. Co-infected cells (Figure 5c) produced most necrosis with co-infections of RSV with D39 wild-type strain, R36A and PLN-A. There was no clear dose-depenency of pneumolysin- or PdB-treatment observed. For bacterial-primed cells (Figure 5d) damage of the cell layer was most intensive after priming with D39 wild-type strain, R36A, PLNA and all three different pneumolysin concentrations.

Discussion

IL-6 and CXCL-8 responses of BEAS-2B cells after either mono-infection or co-stimulation with RSV and/or pneumococcus bacteria or bacterial components were compared. Necrosis was viewed by pictures taken of the monolayers after each treatment. For purified LPS, pneumolysin, the non-pore forming pneumolysin PdB as well as for the non-encapsulated pneumococcal strain R36A no differences between mono- or mixed treatments was measured. In contrast, a clear increase in IL-6 secretion was measured after mixed infections using RSV and either the encapsulated *S. pneumoniae* strain D39 (serotype 2) or PLN-A, a pneumolysin-deficient D39-mutant. From previous studies [7, 35, 36] it is known that RSV-infections of epithelial cells also results in more severe bacterial secondary infections. Enhanced adhesion of pathogenic bacteria is the main reason for this increase [33, 37, 38]. Our results for IL-6 secretion confirm these findings as we observed an IL-6 increase in the supernatant of cells primed with RSV for 2 hours and addition of D39 or PLN-A afterwards. Interestingly, this increase was only measured when encapsulated, bacteria were used but not when purified hemolytic or non-hemolytic pneumolysin, LPS or non-encapsulated pneumococcus was added indicating the importance of encapsulation. Co-stimulation of BEAS-2B cells with RSV and pneumococcus or bacterial-primed cells also

showed increased IL-6 secretion compared to mono-infection but for the wild-type D39 strain RSV-primed cells resulted in a higher IL-6 secretion than the other mixed infections which is in accordance to the clinical findings mentioned before [7, 35, 36]. Increasing pneumolysin concentrations did not show an effect on IL-6 secretion and there was also no difference measured between mono- and mixed infections indicating that pneumolysin by itself does not have a major effect on IL-6 induction but that encapsulated bacteria are required to show a clear increase.

The CXCL-8 results of our assays show a similar trend. Again, encapsulated bacteria are required to get an increased response in mixed compared to mono-infected cells. D39 and PLN-A again show a slightly increased CXCL-8 response in mixed infections with RSV compared to mono-infections. But additionally, also an effect of increasing pneumolysin concentrations was measured, which is in accordance with previous findings [43]. Pneumolysin-primed cells resulted in an increased CXCL-8 response when RSV was added compared to RSV mono-infections.

In summary, both IL-6 and CXCL-8 secretion of BEAS-2B cells is increased for mixed infections of RSV and pneumococcus when encapsulated bacteria were used. For CXCL-8 induction pneumolysin plays an important role as was shown for the pharyngeal epithelial cell line Detroit 562 [43]. Our results indicate that priming of BEAS-2B cells with pneumolysin and the associated cell damage favor secondary viral infections but also that priming of bronchial epithelial cells with RSV opens the door for more severe pneumococcal infections.

References

1. Gonzalez PA, Bueno SM, Carreno LJ, Riedel CA, Kalergis AM: **Respiratory syncytial virus infection and immunity**. *Reviews in medical virology* 2012, **22**(4):230-244.
2. Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, Auinger P, Griffin MR, Poehling KA, Erdman D *et al*: **The burden of respiratory syncytial virus infection in young children**. *N Engl J Med* 2009, **360**(6):588-598.
3. Welliver RC: **Review of epidemiology and clinical risk factors for severe respiratory syncytial virus (RSV) infection**. *The Journal of pediatrics* 2003, **143**(5 Suppl):S112-117.
4. El Saleeby CM, Bush AJ, Harrison LM, Aitken JA, Devincenzo JP: **Respiratory syncytial virus load, viral dynamics, and disease severity in previously healthy naturally infected children**. *J Infect Dis* 2011, **204**(7):996-1002.
5. Gomaa MA, Galal O, Mahmoud MS: **Risk of acute otitis media in relation to acute bronchiolitis in children**. *Int J Pediatr Otorhinolaryngol* 2012, **76**(1):49-51.
6. Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA, Singleton RJ, O'Brien KL, Roca A, Wright PF, Bruce N *et al*: **Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis**. *Lancet* 2010, **375**(9725):1545-1555.
7. Techasaensiri B, Techasaensiri C, Mejias A, McCracken GH, Jr., Ramilo O: **Viral coinfections in children with invasive pneumococcal disease**. *Pediatr Infect Dis J* 2010, **29**(6):519-523.
8. Hacking D, Hull J: **Respiratory syncytial virus--viral biology and the host response**. *The Journal of infection* 2002, **45**(1):18-24.
9. **http://www.who.int/vaccine_research/diseases/ari/en/index2.html (consulted: 03/10/2013)**
10. Dudas RA, Karron RA: **Respiratory syncytial virus vaccines**. *Clin Microbiol Rev* 1998, **11**(3):430-439.

11. van Drunen Littel-van den Hurk S, Watkiss ER: **Pathogenesis of respiratory syncytial virus**. *Current opinion in virology* 2012, **2**(3):300-305.

12. Chanock R, Finberg L: **Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). II. Epidemiologic aspects of infection in infants and young children**. *American journal of hygiene* 1957, **66**(3):291-300.

13. Johnson JE, Gonzales RA, Olson SJ, Wright PF, Graham BS: **The histopathology of fatal untreated human respiratory syncytial virus infection**. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2007, **20**(1):108-119.

14. Fonceca AM, Flanagan BF, Trinick R, Smyth RL, McNamara PS: **Primary airway epithelial cultures from children are highly permissive to respiratory syncytial virus infection**. *Thorax* 2012, **67**(1):42-48.

15. Levine S, Klaiber-Franco R, Paradiso PR: **Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus**. *The Journal of general virology* 1987, **68 (Pt 9)**:2521-2524.

16. Cane PA: **Molecular epidemiology of respiratory syncytial virus**. *Reviews in medical virology* 2001, **11**(2):103-116.

17. Teng MN, Collins PL: **Identification of the respiratory syncytial virus proteins required for formation and passage of helper-dependent infectious particles**. *J Virol* 1998, **72**(7):5707-5716.

18. Walsh EE, Brandriss MW, Schlesinger JJ: **Purification and characterization of the respiratory syncytial virus fusion protein**. *The Journal of general virology* 1985, **66 (Pt 3)**:409-415.

19. Pastey MK, Crowe JE, Jr., Graham BS: **RhoA interacts with the fusion glycoprotein of respiratory syncytial virus and facilitates virus-induced syncytium formation**. *J Virol* 1999, **73**(9):7262-7270.

20. Fritz G, Lang P, Just I: **Tissue-specific variations in the expression and regulation of the small GTP-binding protein Rho**. *Biochimica et biophysica acta* 1994, **1222**(3):331-338.

21. Collins PL, Hill MG, Camargo E, Grosfeld H, Chanock RM, Murphy BR: **Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5' proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development**. *Proc Natl Acad Sci U S A* 1995, **92**(25):11563-11567.

22. Wu W, Munday DC, Howell G, Platt G, Barr JN, Hiscox JA: **Characterization of the interaction between human respiratory syncytial virus and the cell cycle in continuous cell culture and primary human airway epithelial cells**. *J Virol* 2011, **85**(19):10300-10309.

23. Aherne W, Bird T, Court SD, Gardner PS, McQuillin J: **Pathological changes in virus infections of the lower respiratory tract in children**. *Journal of clinical pathology* 1970, **23**(1):7-18.

24. Haynes LM, Moore DD, Kurt-Jones EA, Finberg RW, Anderson LJ, Tripp RA: **Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus**. *J Virol* 2001, **75**(22):10730-10737.

25. Mastronarde JG, Monick MM, Mukaida N, Matsushima K, Hunninghake GW: **Activator protein-1 is the preferred transcription factor for cooperative interaction with nuclear factor-kappaB in respiratory syncytial virus-induced interleukin-8 gene expression in airway epithelium**. *J Infect Dis* 1998, **177**(5):1275-1281.

26. Levitz R, Wattier R, Phillips P, Solomon A, Lawler J, Lazar I, Weibel C, Kahn JS: **Induction of IL-6 and CCL5 (RANTES) in human respiratory epithelial (A549) cells by clinical isolates of respiratory syncytial virus is strain specific**. *Virology journal* 2012, **9**:190.

27. Thomas LH, Wickremasinghe MI, Sharland M, Friedland JS: **Synergistic upregulation of interleukin-8 secretion from pulmonary epithelial cells by direct and monocyte-dependent effects of respiratory syncytial virus infection**. *J Virol* 2000, **74**(18):8425-8433.

28. Laham FR, Trott AA, Bennett BL, Kozinetz CA, Jewell AM, Garofalo RP, Piedra PA: **LDH concentration in nasal-wash fluid as a biochemical predictor of bronchiolitis severity**. *Pediatrics* 2010, **125**(2):e225-233.

29. Goetghebuer T, Isles K, Moore C, Thomson A, Kwiatkowski D, Hull J: **Genetic predisposition to wheeze following respiratory syncytial virus bronchiolitis**. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 2004, **34**(5):801-803.

30. Hull J, Thomson A, Kwiatkowski D: **Association of respiratory syncytial virus bronchiolitis with the interleukin 8 gene region in UK families**. *Thorax* 2000, **55**(12):1023-1027.

31. Tripp RA, Oshansky C, Alvarez R: **Cytokines and respiratory syncytial virus infection**. *Proceedings of the American Thoracic Society* 2005, **2**(2):147-149.

32. McNamara PS, Fonceca AM, Howarth D, Correia JB, Slupsky JR, Trinick RE, Al Turaiki W, Smyth RL, Flanagan BF: **Respiratory syncytial virus infection of airway epithelial cells, in vivo and in vitro, supports pulmonary antibody responses by inducing expression of the B cell differentiation factor BAFF**. *Thorax* 2013, **68**(1):76-81.

33. Yokota S, Okabayashi T, Hirakawa S, Tsutsumi H, Himi T, Fujii N: **Clarithromycin suppresses human respiratory syncytial virus infection-induced Streptococcus pneumoniae adhesion and cytokine production in a pulmonary epithelial cell line**. *Mediators Inflamm* 2012, **2012**:528568.

34. McNamara PS, Flanagan BF, Hart CA, Smyth RL: **Production of chemokines in the lungs of infants with severe respiratory syncytial virus bronchiolitis**. *J Infect Dis* 2005, **191**(8):1225-1232.

35. Hishiki H, Ishiwada N, Fukasawa C, Abe K, Hoshino T, Aizawa J, Ishikawa N, Kohno Y: **Incidence of bacterial coinfection with respiratory syncytial virus bronchopulmonary infection in pediatric inpatients**. *Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy* 2011, **17**(1):87-90.

36. Pettigrew MM, Gent JF, Pyles RB, Miller AL, Nokso-Koivisto J, Chonmaitree T: **Viral-bacterial interactions and risk of acute otitis media complicating upper respiratory tract infection**. *J Clin Microbiol* 2011, **49**(11):3750-3755.

37. Hament JM, Aerts PC, Fleer A, Van Dijk H, Harmsen T, Kimpen JL, Wolfs TF: **Enhanced adherence of Streptococcus pneumoniae to human epithelial cells infected with respiratory syncytial virus**. *Pediatr Res* 2004, **55**(6):972-978.

38. Avadhanula V, Rodriguez CA, Devincenzo JP, Wang Y, Webby RJ, Ulett GC, Adderson EE: **Respiratory viruses augment the adhesion of bacterial pathogens to respiratory epithelium in a viral species- and cell type-dependent manner**. *J Virol* 2006, **80**(4):1629-1636.

39. Cole R: **Pneumococcus Hemotoxin**. *J Exp Med* 1914, **20**(4):346-362.

40. Cohen B, Halbert SP, Perkins ME: **Pneumococcal Hemolysin: The Preparation of Concentrates, and Their Action on Red Cells**. *J Bacteriol* 1942, **43**(5):607-627.

41. Morgan PJ, Harrison G, Freestone PP, Crane D, Rowe AJ, Mitchell TJ, Andrew PW, Gilbert RJ: **Structural and functional characterisation of two proteolytic fragments of the bacterial protein toxin, pneumolysin**. *FEBS letters* 1997, **412**(3):563-567.

42. Rossjohn J, Gilbert RJ, Crane D, Morgan PJ, Mitchell TJ, Rowe AJ, Andrew PW, Paton JC, Tweten RK, Parker MW: **The molecular mechanism of pneumolysin, a virulence factor from Streptococcus pneumoniae**. *Journal of molecular biology* 1998, **284**(2):449-461.

43. Dogan S, Zhang Q, Pridmore AC, Mitchell TJ, Finn A, Murdoch C: **Pneumolysin-induced CXCL8 production by nasopharyngeal epithelial cells is dependent on**

calcium flux and MAPK activation via Toll-like receptor 4. *Microbes Infect* 2011, **13**(1):65-75.

Additive	Amount	Supplier
Bovine pituitary extract	50 µg/ml	Invitrogen
Hydrocortisone	0.5 µg/ml	Sigma-Aldrich
Epidermal growth factor	25 ng/ml	Sigma-Aldrich
Epinephrine	0.5 µg/ml	Sigma-Aldrich
Insulin	5 µg/ml	Sigma-Aldrich
Transferrin	10 µg/ml	Sigma-Aldrich
Triiodothyronine	6.5 ng/ml	Sigma-Aldrich
Retinoic acid	0.2 ng/ml	Sigma-Aldrich
Gentamicin	50 mg/ml	Sigma-Aldrich

Table 2: Additives in BEGM medium

Treatment	Concentration
RSV	2.5 Multiplicity of infection (MOI)
S. pneumoniae strain D39	10^5 CFUs
PLN-A (D39 mutant secreting non-pore forming PLY)	10^5 CFUs
Purified pneumolysin (PLY)	5, 25 or 125 ug/ml
Lipopolysaccharide (LPS) (from *Escherichia coli*)	2.5 ug/ml
R36A (D39 non-encapsulated mutant)	10^5 CFUs
PdB (purified non-pore forming PLY)	2, 25, 125 ug/ml

Table 3: Overview of treatments used for infection of BEAS-2B cells

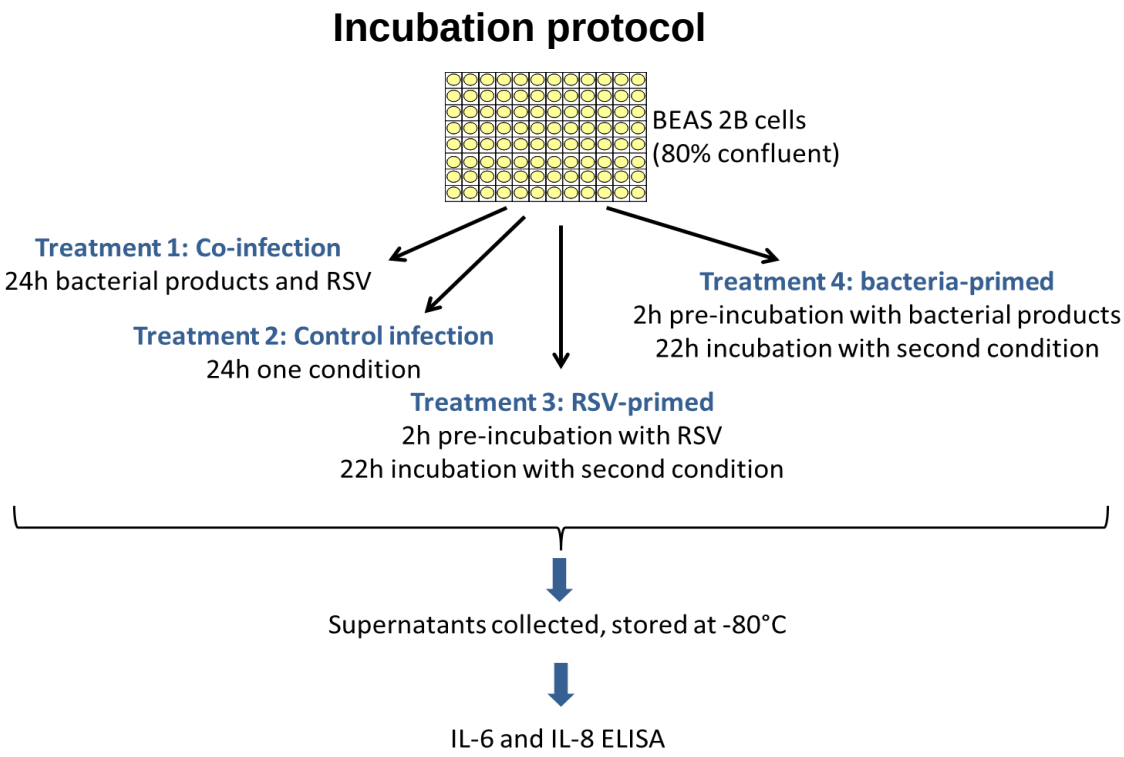


Figure 1: Incubation assays with BEAS 2B cells. Treatment 1: RSV and pneumococci or virulence factors were added at the same time. Treatment 2: Control infections with pneumococcus, pneumococcal virulence factors, or RSV for 24h. Treatment 3: The virus was added first to the cells, incubated for 2h, and then either complete pneumococci or pneumococcal virulence factors were added for another 24h. Treatment 4: First added bacteria or virulence factors for 2h to the cells, then added RSV for 24h.

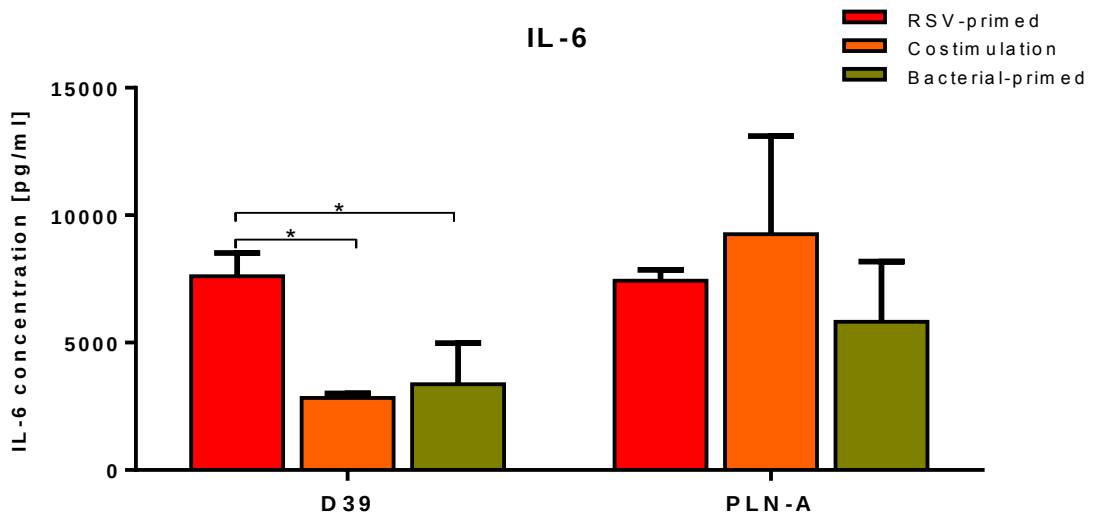


Figure 2: Increased IL6 expression requires encapsulated pneumococci plus RSV infection. D39: *S. pneumoniae* strain D39 (serotype 2), PLN-A: Mutant strain of *S. pneumoniae* strain D39 expressing non-pore forming pneumolysin. Error bars: Standard error of the mean. n = 3. Exceptions: for D39 co-stimulation n = 2. Statistical test: One-way ANOVA with Tukey's test to correct for multiple testing. * = $p<0.05$. ANOVA D39: $p<0.001$

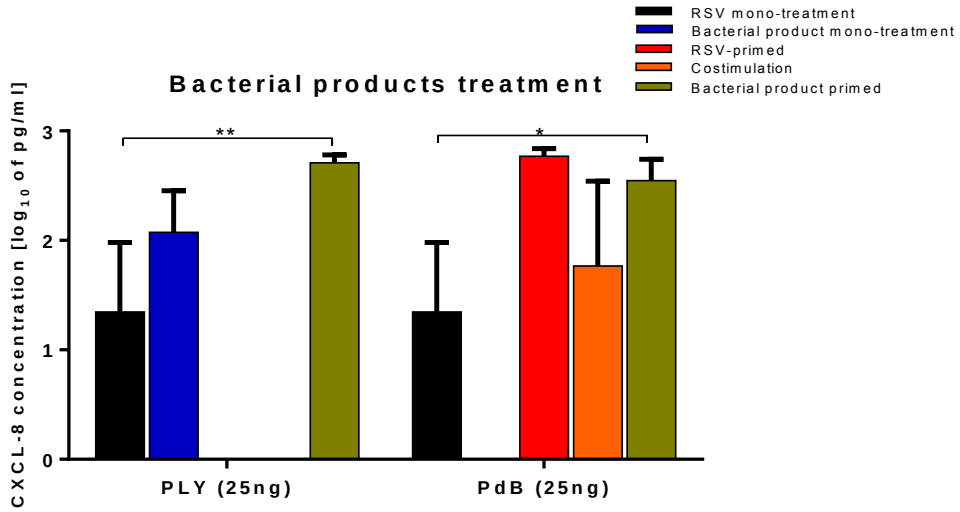


Figure 3a: CXCL-8 secretion of BEAS-2B cells after stimulation with RSV or bacterial products. Priming of BEAS-2B cells with pneumolysin or its non-pore forming mutant PdB resulted in significantly increased CXCL-8 secretion compared to RSV mono-infections. PLY (25ng/ml): 25ng/ml of purified pneumolysin added. PdB (25ng/ml): 25ng/ml of purified non-pore-forming pneumolysin added. Error bars: Standard error of the mean. n = 3. Exceptions: PLN-A and PdB bacterial mono-treatments, PdB RSV-primed n = 2. Statistical test: One-way ANOVA with Tukey's test to correct for multiple testing. * = *p<0.05*; ** = *p<0.01*. ANOVA for PLY (25ng) *p<0.05*; for PdB *p<0.01*

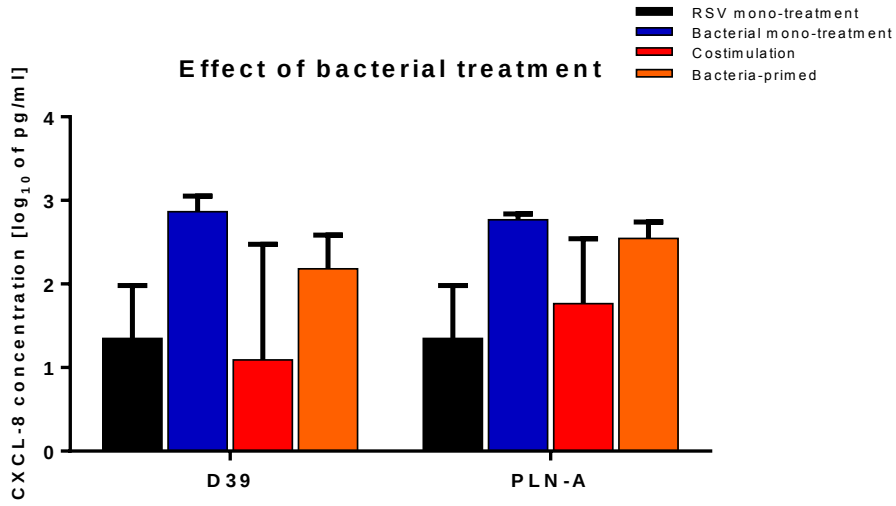


Figure 3b: CXCL-8 secretion of BEAS-2B cells after stimulation with RSV and/or pneumococcus. Mono-treatment with RSV and a mixed treatment using RSV and the encapsulated bacterial strains D39 and PLN-A resulted in CXCL-8 secretion. D39: *S. pneumoniae* strain D39 (serotype 2). PLN-A: Mutant strain of *S. pneumoniae* strain D39 expressing non-pore forming pneumolysin. Error bars: Standard error of the mean. n = 3. CXCL-8 concentration of untreated control was subtracted.

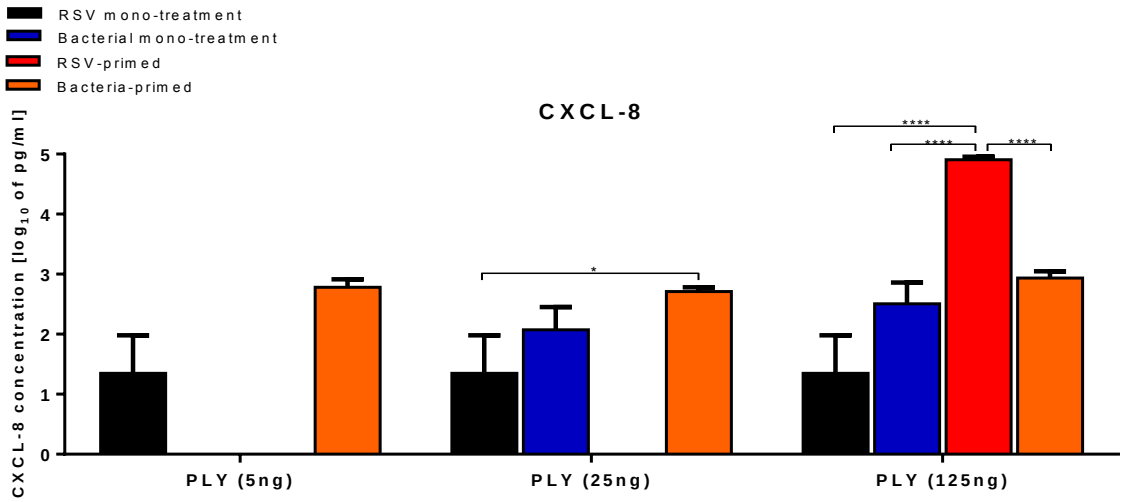


Figure 4: Increasing pneumolysin concentrations result in increased IL-8 secretion. A strong increase in IL-8 expression was also observed after RSV-priming and adding 125 ng of pneumolysin compared to the addition of 5 ng and 25 ng. Error bars: Standard error of the mean. n = 2. ANOVA for PLY (25ng) *p<0.05*; for PLY (125ng) *p<0.0001*

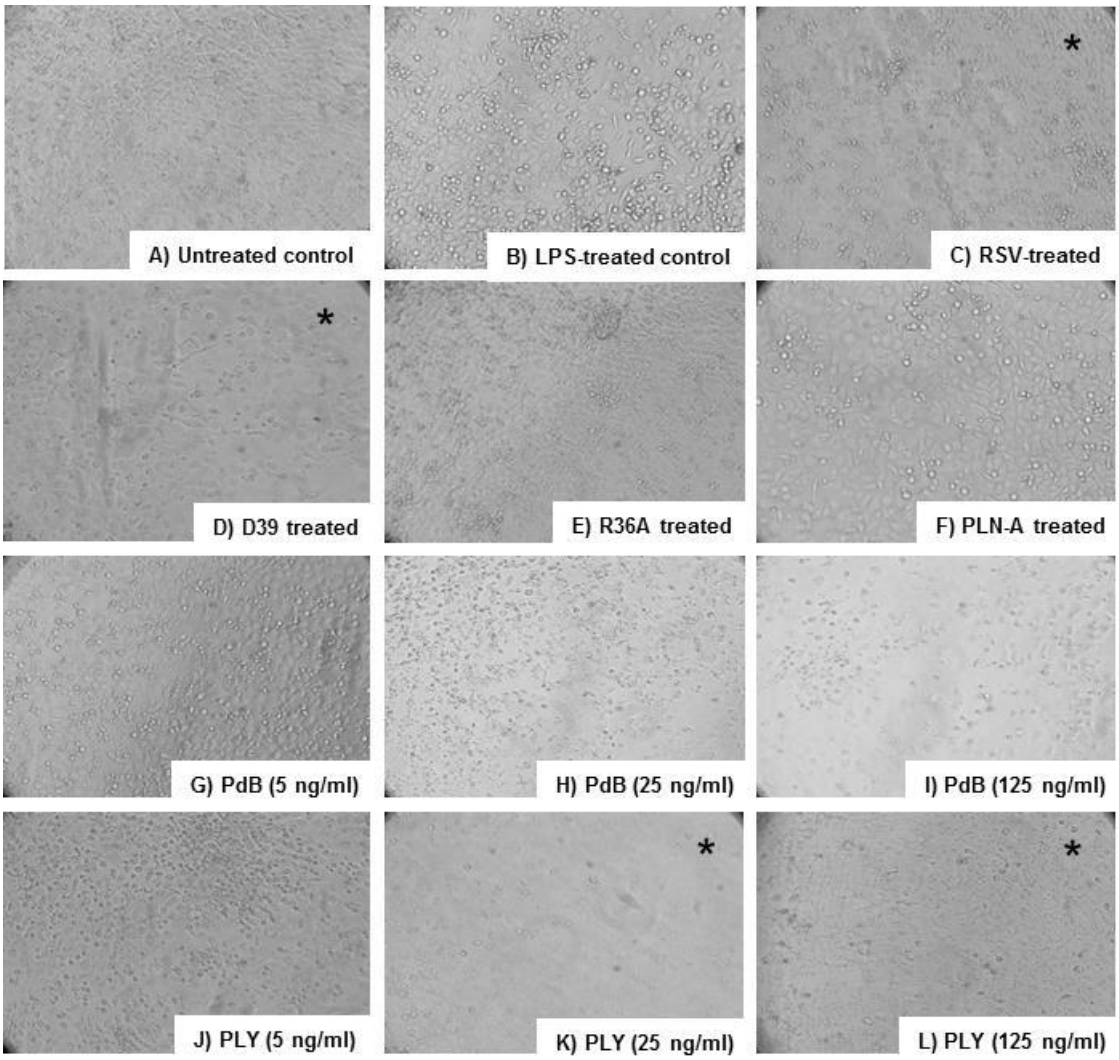


Figure 5a: Mono-infected cells, different treatments. Compared to the untreated control (A), all treatments showed necrosis of BEAS-2B cells. The strongest effects were observed with D39 wild-type (D) and RSV (C). For PLY dose-dependency (J-L) is seen on the pictures; * indicates treatments with strongest effect. LPS: Lipopolysaccharides. RSV: Respiratory syncytial virus. D39: *S. pneumoniae* strain D39 (serotype 2). R36A: non-encapsulated mutant of *S. pneumoniae* strain D39. PLN-A: mutant strain of S. pneumoniae D39 expressing non-pore forming pneumolysin. PdB: non-pore forming pneumolysin. PLY: pneumolysin. Magnification: 40x

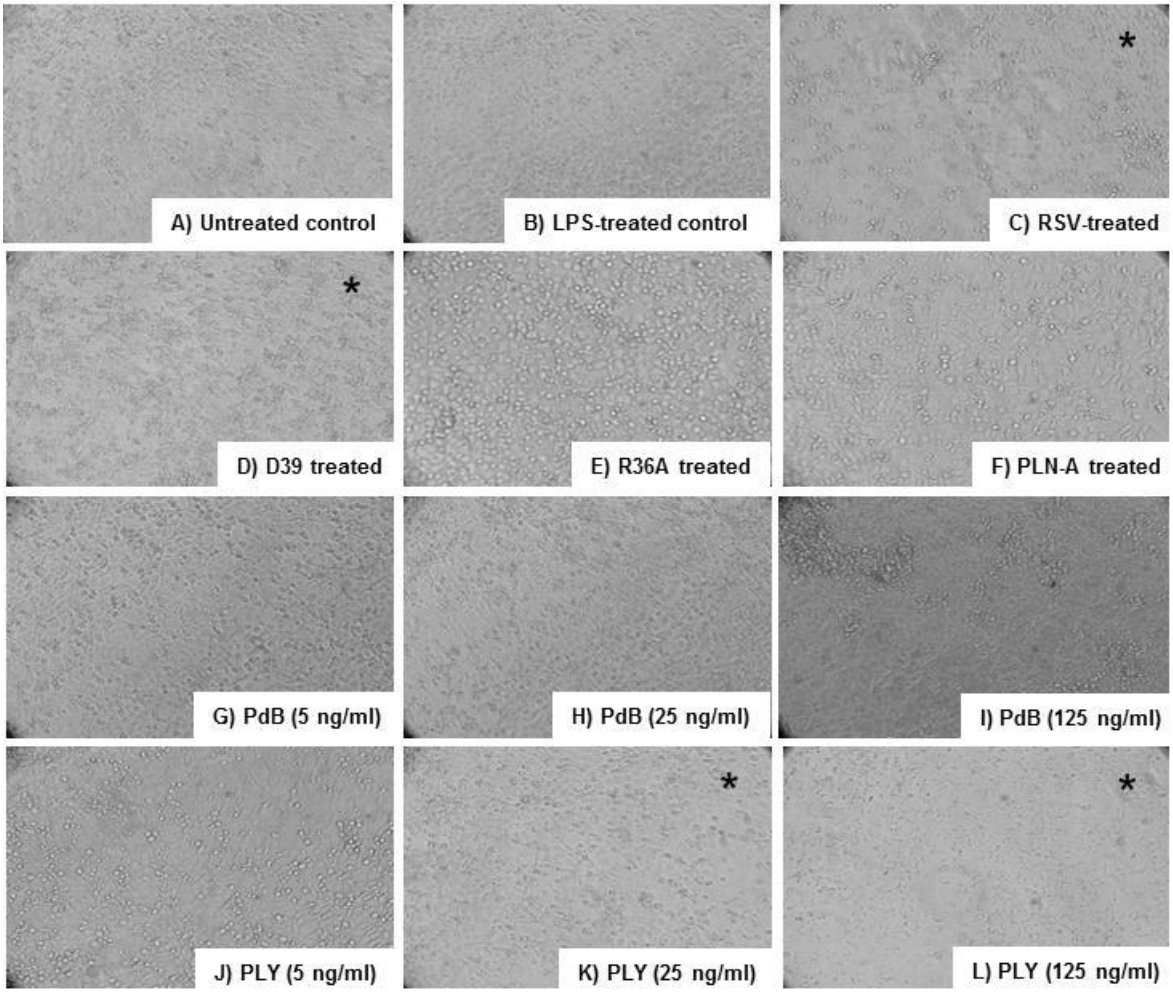


Figure 5b: RSV-primed cells (2h pre-treatment with RSV). Clear signs of necrosis can be observed with all treatments compared to the non-infected control (A). The greatest damage to cell layer is shown for RSV-treatment (C) and D39 wild-type (D). PLN-A treatment (F) and R36A treatment (E) also had a necrotic effect on the cell layer. For pneumolysin treatment, much stronger necrosis is observed after addition of 125 ng (L) than of either 5 ng or 25 ng (J and K). * indicates treatments with strongest effect. LPS: Lipopolysaccharides. RSV: Respiratory syncytial virus. D39: *S. pneumoniae* strain D39 (serotype 2). R36A: non-encapsulated mutant of *S. pneumoniae* strain D39. PLN-A: mutant strain of S. pneumoniae D39 expressing non-pore forming pneumolysin. PdB: non-pore forming pneumolysin. PLY: pneumolysin. Magnification: 40x

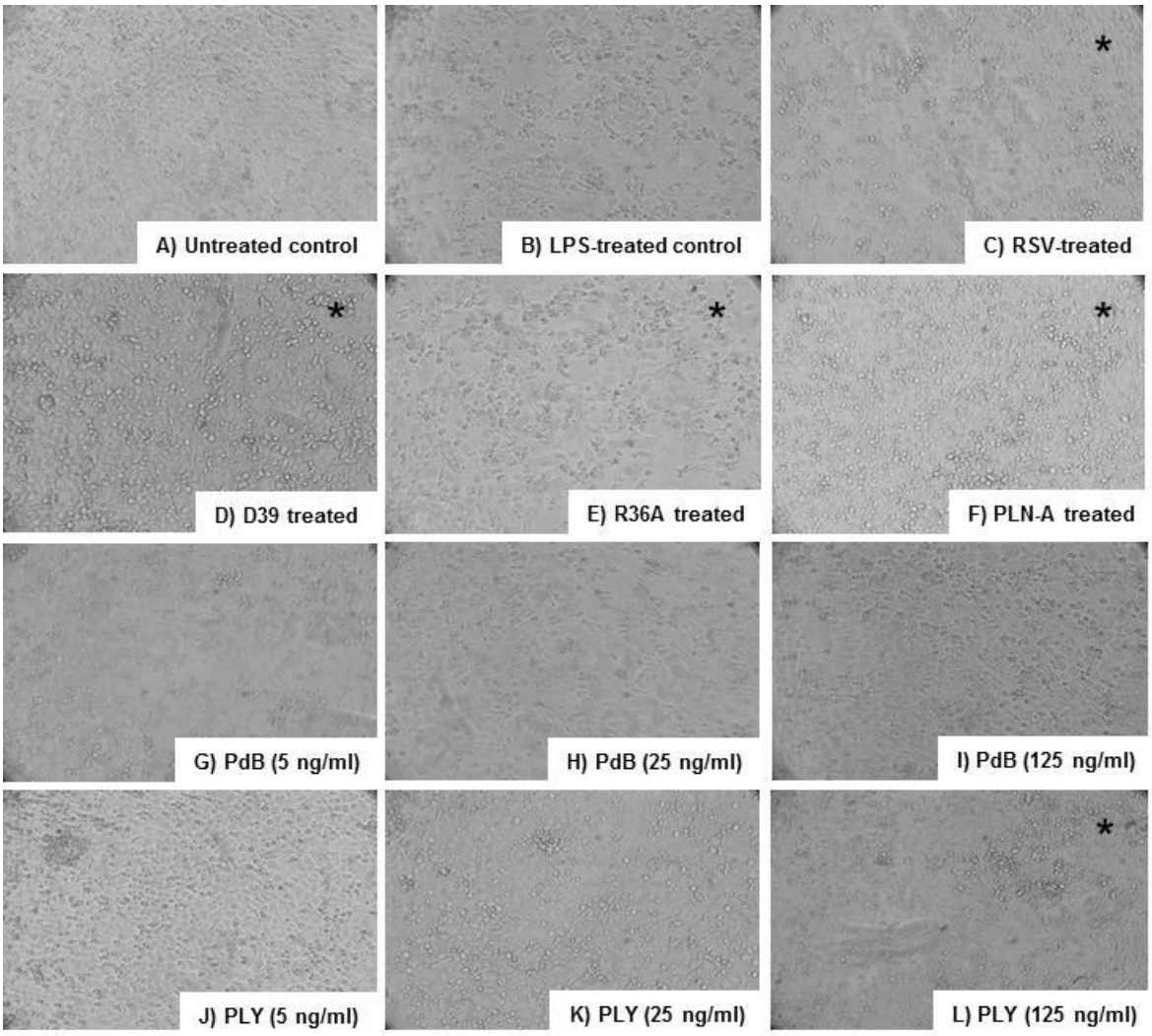


Figure 5c: Co-infected cells. Necrosis is observed for all different conditions tested, but most necrotic cells are shown on cells with co-infections of RSV with D39 wild-type strain, (D), R36A (E) and PLN-A (F). Treating BEAS-2B cells with 125 ng/ml pneumolysin also resulted in increased necrosis. * indicates treatments with strongest effect. LPS: Lipopolysaccharides. RSV: Respiratory syncytial virus. D39: *S. pneumoniae* strain D39 (serotype 2). R36A: non-encapsulated mutant of *S. pneumoniae* strain D39. PLN-A: mutant strain of S. pneumoniae D39 expressing non-pore forming pneumolysin. PdB: non-pore forming pneumolysin. PLY: pneumolysin. Magnification: 40x

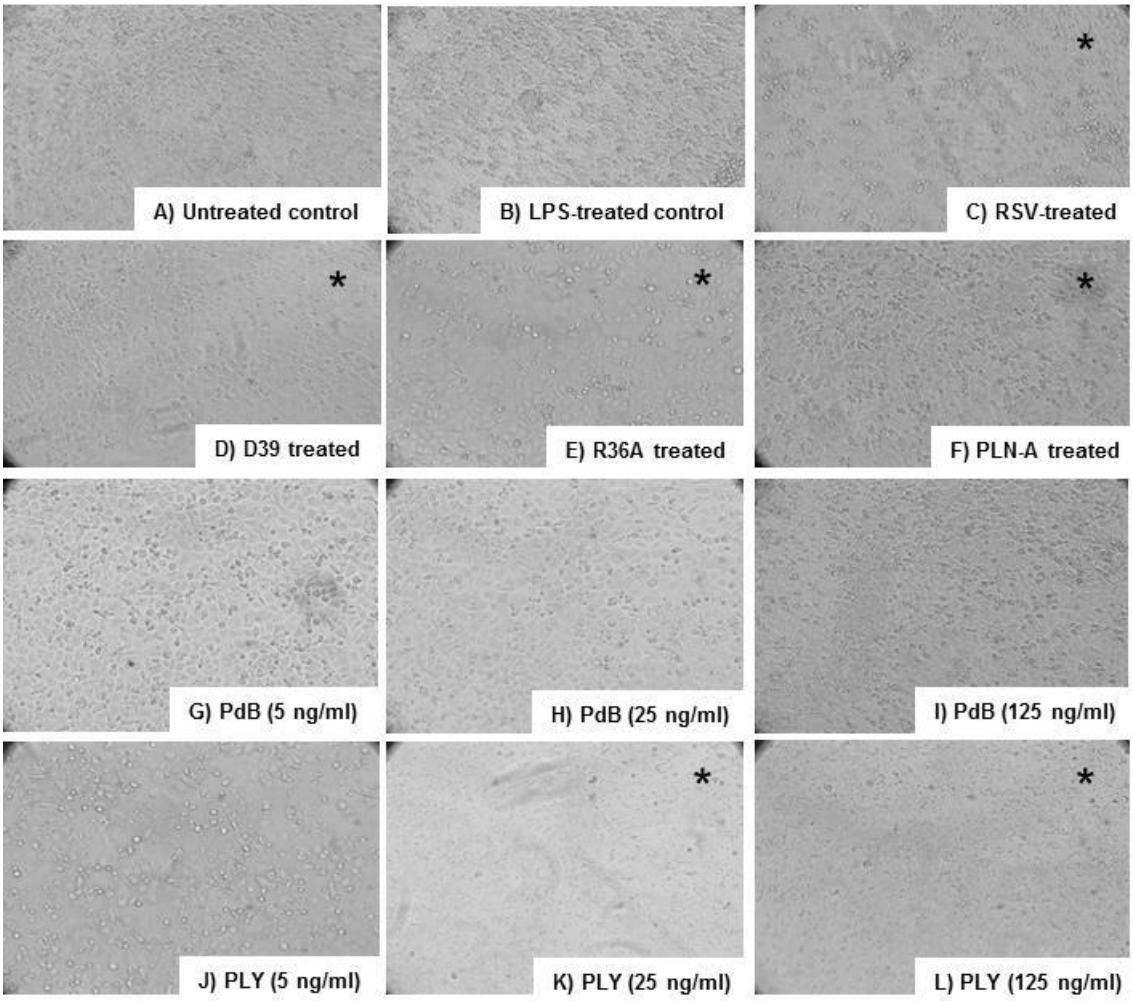
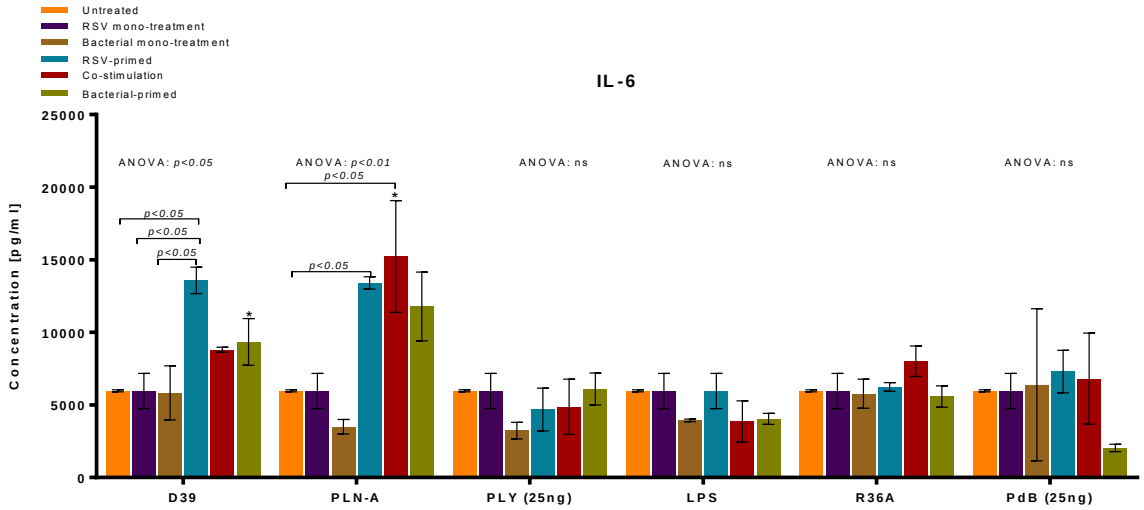


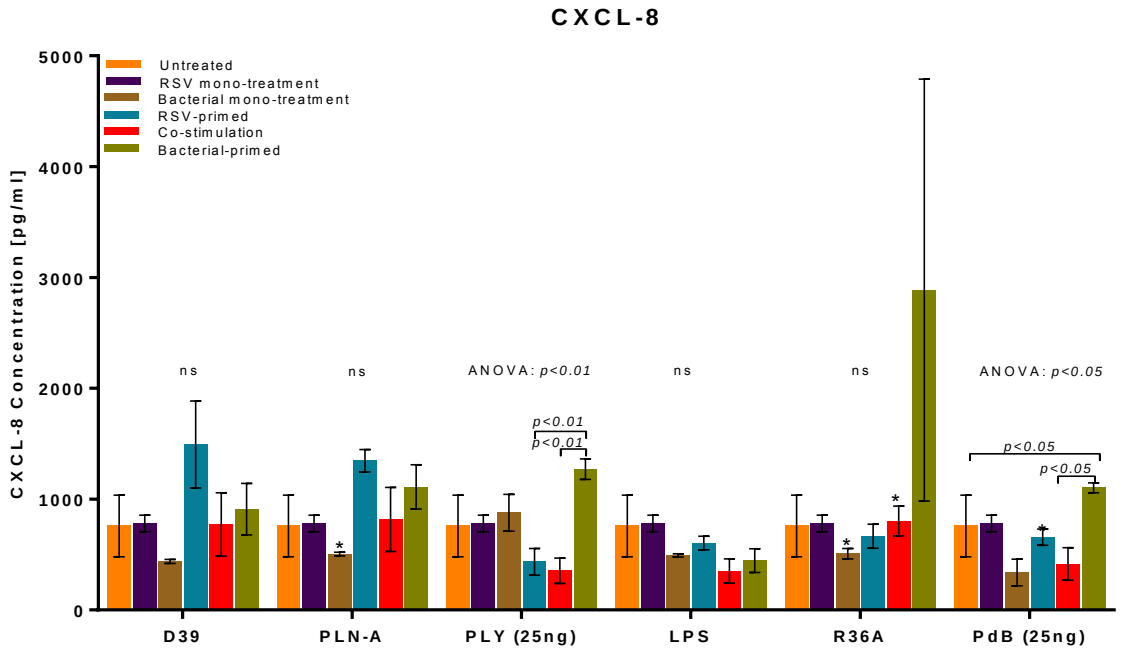
Figure 5d: Bacterial-primed cells (2h of pre-treatment with either bacteria or bacterial products). Necrosis is shown on all pictures compared to untreated control (A), but the strongest effect is observed for D39 wild-type (D), R36A (E), PLN-A (F) and with 25 and 125 ng/ml pneumoylsin (K-L). *indicates treatments with strongest effect. LPS: Lipopolysaccharides. RSV: Respiratory syncytial virus. D39: *S. pneumoniae* strain D39 (serotype 2). R36A: non-encapsulated mutant of *S. pneumoniae* strain D39. PLN-A: mutant strain of S. pneumoniae D39 expressing non-pore forming pneumolysin. PdB: non-pore forming pneumolysin. PLY: pneumolysin. Magnification: 40x

Supplementary Figure 1



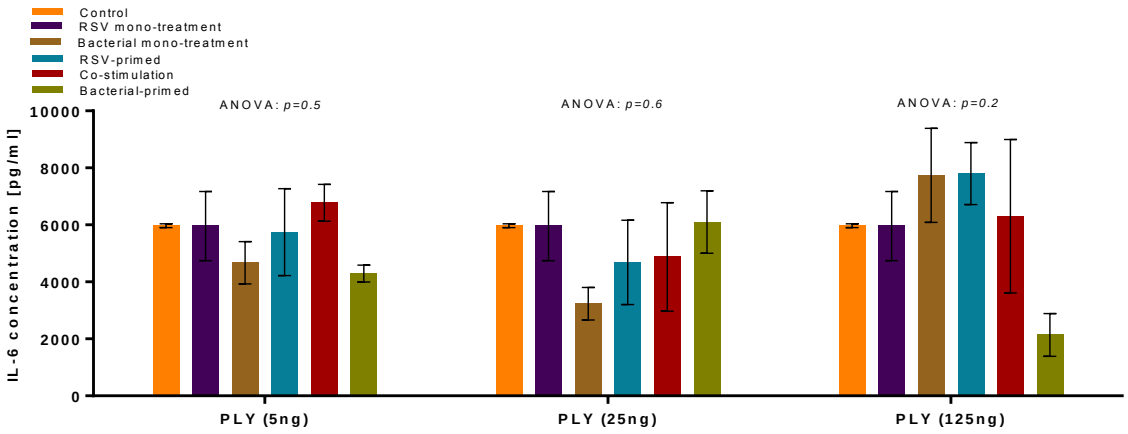
Suplementary Figure 1: Increased IL6 expression requires encapsulated pneumococci plus RSV infection but does not depend on functioning pneumolysin. Addition of purified pneumolysin, LPS, PdB or even non-encapsulated *S. pneumoniae* (R36A) does not result in a IL-6 expression higher than the untreated control (6000 pg/ml). D39: S. pneumoniae strain D39 (serotype 2), PLN-A: Mutant strain of *S. pneumoniae* strain D39 expressing non-pore forming pneumolysin, PLY (25ng): purified pneumolysin LPS: 2.5 µg/ml purified lipopolysaccharides added. R36A: non-encapsulated mutant of D39 (D39*Δcps*). PdB (25ng/ml): 25ng/ml of purified non-pore-forming pneumolysin. Error bars: Standard error of the mean. n = 3. Exceptions: for D39 co-stimulation, LPS bacterial-primed, LPS mono-treatment, R36A mono-treatment, PdB mono-treatment and all untreated controls n = 2. Statistical test: One-way ANOVA with Tukey's test to correct for multiple testing

Supplementary Figure 2

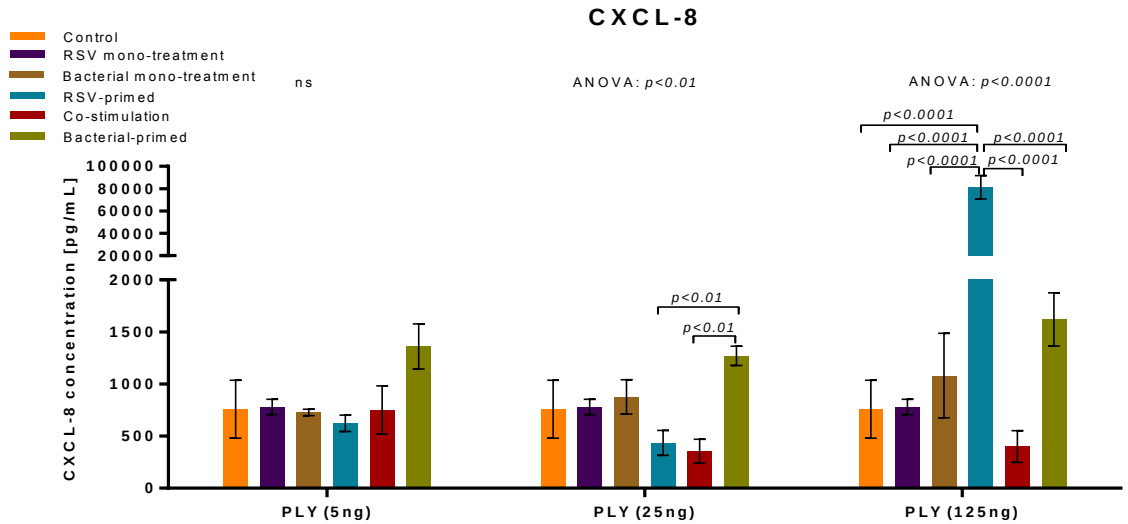


Supplementary Figure 2: CXCL-8 secretion of BEAS-2B cells after stimulation with RSV and/or pneumococcus . Bacterial-priming of BEAS-2B cells results in a trend towards increased IL-8 secretion compared to mono-infections. Mono-infections did not show an increase in IL-8 induction compared to the untreated control, whereas priming with pneumococcus, pneumolysin or the non-pore forming PdB resulted in higher IL-8 concentrations. PLN-A: Mutant strain of *S. pneumoniae* strain D39 not expressing pneumolysin. PLY (25ng/ml): 25ng of purified pneumolysin added. LPS: 2.5 µg/ml purified lipopolysaccharides added. R36A: Mutant strain of S. pneumoniae strain D39 (D39*Δcps*) not expressing serotype 2 capsule. PdB (25ng/ml): 25ng/ml of purified non-pore-forming pneumolysin added. Error bars: Standard error of the mean. n = 3. Exceptions: for all untreated controls, PLN-A, LPS, R36A and PdB bacterial mono-treatments, PdB RSV-primed, R36A co-stimulation and R36A bacterial-priming n = 2. Statistical test: One-way ANOVA with Tukey's test to correct for multiple testing.

Supplementary Figure 3



Supplementary Figure 3a: Increasing pneumolysin concentrations do not have an effect on IL-6 secretion. Adding 5, 25 or 125 ng/µl pneumolysin to BEAS-2B cells did not result in an increased IL-6 secretion compared to the control. Orange: untreated control. Error bars: Standard error of the mean. n =-3. Exceptions: for untreated controls and PLY (125ng) mono-treatment, n =2.



Supplementary Figure 3b: Pneumolysin-priming effect on CXCL-8 secretion is independent of concentration whereas RSV-priming resulted in an increase of chemokine after adding 125 ng of pneumolysin. 2h of priming with either 5 ng, 25 ng or 125 ng of pneumolysin followed by a 24h-incubation with RSV resulted in an equal amount of IL-8 measured in the supernatant for all different concentrations. Co-stimulation also was concentration-independent. A strong increase in IL-8 expression was observed after RSV-priming and adding 125 ng of pneumolysin compared to the addition of 5 ng and 25 ng. Error bars: Standard error of the mean. n = 2

5. Biofilm formation of *Streptococcus pneumoniae* and commensal streptococci

5.1 Manuscript

Differences of initial biofilm formation among pneumococcal serotypes

Eliane Küng, Thierry O. Schaffner, Daniel Wüthrich, Moana Mika, Kathrin Mühlemann, Lucy J. Hathaway and Markus Hilty.

Differences of initial biofilm formation among pneumococcal serotypes

Eliane Küng[1,2], Thierry O. Schaffner [1,2], Daniel Wüthrich[2,3], Moana Mika[1,2] , Kathrin Mühlemann[1†], Lucy J. Hathaway[1,*], Markus Hilty[1, 4,§,*]

[1] Institute for Infectious Diseases, University of Bern, Friedbühlstrasse 51, Postfach 61, 3010 Bern, Switzerland

[2] Graduate School for Cellular and Biomedical Sciences, University of Bern, Theodor Kocher Institute, Freiestrasse 1, 3012 Bern, Switzerland

[3] Department of Biology, Bioinformatics, University of Bern, Baltzerstrasse 6, 3012 Bern, Switzerland

[4] Department of Infectious Diseases, University Hospital, 3010 Bern, Switzerland

[§]Corresponding author: Markus Hilty, PhD - Institute for Infectious Diseases, University of Bern, Friedbühlstrasse 51, Postfach 61, CH-3010, Switzerland - Phone: +41-31-632-4983; Fax +41-31-632-8766. E-mail: Markus.Hilty@ifik.unibe.ch

* Contributed equally

[†] Deceased

Keywords: initial biofilms, serotypes, small colony variants

Short running title: Initial biofilm formation of pneumococcal serotypes

Abstract

Background

More than 90 serotypes of *Streptococcus pneumoniae* have been described and these are determined by the biochemistry of their polysaccharide capsules. Some serotypes such as 6B are associated with colonization whereas others like serotype 7F are more often associated with invasive disease. Capsule-deficient small colony variants (SCV) have been observed for several serotypes and play a role in increasing biofilm biomass and stability. We aimed at investigating initial biofilm formation of 6B and 7F in a quantitative and descriptive (SCV building) manner over time.

Results

No absolute quantitative difference between serotype 6B and 7F in biofilm building was observed. Both formed SCVs in a static biofilm model when grown in a chemically defined medium (CDM) supplemented with either 5 mM galactose or glucose. After 16 h, serotype 7F resulted in more relative SCVs than 6B, but prolonged incubation time led to a gradual increase over time (16 to 64 h) ($p<0.01$) only for serotype 6B. This indicates an expression-regulated mechanism of SCV formation for serotype 6B strains, whereas this process might depend on point mutations for serotype 7F.

Conclusions

An increase in SCV was associated only with the colonizing serotype 6B. This might indicate efficient adaptation to the nutrient-limited environment of the nasopharynx resulting in better biofilm formation and, as a result, in better colonization than a serotype like 7F which is a less efficient colonizer.

Background

The strict human pathogen *Streptococcus pneumoniae* (the pneumococcus) is a Gram-positive diplococcus using the nasopharynx as major ecological reservoir [1]. Currently, 93 different serotypes with differences in capsular structures and antigenicity are known [2]. Pneumococcal biofilms have been isolated from adenoid tissues, mucosal epithelial cells and nasal septa [3-5]. Pneumococci growing in biofilms induce reduced immune responses and have massively increased antibiotic resistance compared to planktonic pneumococci creating a major problem in clinics [5-7].

Differences in biofilm forming capacity have been observed between different pneumococcal serotypes but the relationship between serotype and biofilm building was not clear. [8-14]. Interestingly, growth conditions also have an impact on pneumococcal biofilm formation. Lower temperatures (34°C) and atmospheric oxygen pressure as well as the presence of Fe(III) had a positive effect on biofilm formation, whereas xylitol reduced biofilm growth [14-18]. There is evidence that pneumococci show enhanced biofilm formation in nutrient limited media compared to complex media [15]. LytA-triggered autolysis starting 12-24h after inducing biofilm formation was shown to occur [19, 20].

In a transposon library screen of TIGR4, 69 mutations in the pneumococcal genome were found to affect biofilm growth [21]. Among the genes mainly involved in biofilm formation are the neuraminidase NanA, the serine-rich repeat protein PsrP, the *lic* operon involved in choline uptake, and, for early biofilm formation, the LuxS-controlled quorum sensing system as well as the competence system [13, 16, 20, 22-26]. Natural transformation was increased in biofilm pneumococci [15]. Major differences between planktonic and biofilm growth was also observed in terms of capsular expression. Rough morphology and transparent phenotype are increased [11, 26]. A decrease in *cpsA* and *cps2* expression was measured during biofilm

growth [9, 15]. For serotype 3 biofilms, point mutations in the *cps3DSU* genes were measured, whereas TIGR4 showed insertions in the *cps4E* gene, all resulting in acapsular nonrevertible colony variants. Serotype 19 also generated acapsular non phase-variable variants [11, 21, 27]. Generally, deficiency of capsule resulted in increased biofilm formation [28] and small, acapsular colony variants (SCVs) dominated in the first day of biofilm formation over normal-shaped pneumococci [27, 29].

According to Allegrucci et al. [30] pneumococcal biofilm formation can be divided into three different stages: Initial attachment to the surface occurred during day 1 followed (days 2 and 3) by the aggregation of cellular aggregates, mainly due to the replication of the bacteria attached before. After 6 days of incubation, the biofilms were fully developed, microcolony formation, a maximum cell density and height was measured.

The aim of our study was to investigate the initial attachment of pneumococcus under different growth conditions. As growth medium, CDM supplemented with either glucose or galactose was used to determine the impact of carbon source on biofilm formation. In particular, we aimed to investigate whether there are differences between pneumococcal serotypes 6B and 7F in biofilm formation at two different time points (16h and 64h).

Results

Determination of optimal incubation time for biofilm growth

In order to decide on the ideal time point for experimental biofilm set up, planktonic growth of pneumococcal strains 106.66 (serotype 6B) and 208.41 (serotype 7F) was first performed in CDM glucose and CDM galactose, respectively. We found that the mid-log phase of growth occurred after 3 to 4h in CDM glucose

whereas an incubation time of 16 h was required for CDM galactose (Figure 1A and 1B). The optimal conditions for pneumococcal biofilm growth were therefore chosen with an incubation period of 16 h (CDM galactose) and 4 h (CDM glucose[*]), respectively. In addition we also chose 16 h for CDM glucose in order to evaluate the influence of pneumococcal lysis on our results.

Quantifying CFU of pneumococcal serotypes 6B and 7F at initial biofilm formation

Next, the planktonic and biofilm growth of strains 106.66 (serotype 6B) and 208.41 (serotype 7F) as well as of their isogenic capsule-switch mutants 208.41cps106.66 (serotype 6B) and 106.66cps208.41 (serotype 7F) in CDM glucose after 4h[†] and 16h or in CDM galactose after 16h of incubation was measured (Table 1; Figures 2A and 2B) to investigate whether an increased planktonic growth also results in more biofilm formation. As expected from the growth curves where OD_{450nm} was measured over time, 6B strain resulted in an increased planktonic growth compared to 7F, though this was not significant (*p=0.13*) when counting CFU. The same trend also was observed for the isogenic mutants where 208.41cps106.66 resulted in increased planktonic CFUs compared to 106.66cps208.41 in glucose (*p=0.26*) as well as in galactose (*p<0.05*).

Absolute biofilm CFU counts resulted in no significant differences between serotypes (Wild types: *p=0.25* for CDM glucose, *p=0.42* for CDM galactose; isogenic mutants: *p=0.48* for CDM glucose, *p=0.12* for CDM galactose). Based on our growth curves, pneumococci reach their maximal density in CDM glucose after 5-6 h, and then autolysis begins.

Relative amount of biofilm building

In order to balance out differences in growth behaviour, we next calculated the relative amount of initial biofilm building. For this serotype 7F strains resulted in a

[*] At the time of press, these experiments were not yet analyzed
[†] At the time of press, these experiments were not yet analyzed

significantly increased relative amount of biofilm CFUs from the total of living bacteria counted compared to serotype 6B in CDM glucose (*p<0.01*) as well as in CDM galactose (*p<0.01*). The same finding was true for the isogenic mutants where 208.41cps106.66 showed a significant increase over 106.66cps208.41 in CDM glucose (*p<0.05*) and in CDM galactose (*p<0.0001*) (Figures 2C and 2D), indicating that capsule plays an important role for initial biofilm formation resulting in more CFU counts for serotype 7F after 16h of incubation.

Small colony variant building in biofilms of serotypes 6B and 7F

Wild type serotype 6B and 7F strains both showed SCV formation with a mean of 4.9% (range: 0-27.4) for 106.66 and 8.5% (range: 0-28.6) for 208.41 of total biofilm CFU counts, resulting in a non-significant increase for 208.41 SCVs compared to 106.66 SCVs (*p=0.24*) (Figure 3).

Presence of SCV was confirmed by Fluorescein isothiocyanate-dextran (FITC-Dextran) imaging. To get a better understanding of the stability of SCVs, the bacteria were passaged once in a complex medium (BHI + 5% FCS) before FITC-Dextran imaging was proceeded. SCVs of strain 208.41 remained small (Figure 4A) and a clear difference in bacterial size was observed when compared to the normal phenotype (Figure 4B). SCVs of 106.66 switched to normal size after one passage in a complex medium indicating rapid adaptation to environmental changes (Figure 4C vs Figure 4D).

Initial biofilm building of serotypes 6B and 7F after 64 h

We then were interested in the development of biofilms and SCVs over time and therefore, growth experiments were next performed after 64h of incubation. No significant difference in planktonic CFU counts was measured between serotypes 6B and 7F (*p=0.97*) after 64 h of incubation, and absolute CFU/200 µl of biofilms reached levels comparable to those in 16 h biofilms: $7.6x10^4$ (range: 37-$4.3x10^5$) for

106.66 (*p=0.45*) and $6x10^5$ (range: $2.9x10^3$-$4.7x10^6$) for 208.41 (*p=0.3*), and serotype 7F was non-significantly increased (*p=0.1*) compared to 6B after 64h of incubation (Figure 5A). Strikingly, a significant increase in the relative amount of biofilm CFUs was measured for serotype 6B from 16 h to 64 h of incubation (*p<0.01*), whereas serotype 7F remained stable (*p=0.56*) (Figure 5B). Also, a significant (*p<0.01*), relative increase in SCV formation was measured for serotype 6B from 4.9% (range: 0-27.4) to 21.7% (range: 0-54%) (Figure 5C). Serotype 7F strains showed no pattern in SCV formation over time with 8.5% (range: 0-28.6) at 16h and 11.3% (range: 0-70.8) after 64h of growth and also resulting in a higher variation than data from serotype 6B (Figure 5C).

Discussion

Differences in initial biofilm growth behaviour over time indicate that serotype matters in the process of biofilm formation: To get long-term access to the nasopharynx, it is important to adapt to this nutrient-poor environment. Biofilm formation is one of the most efficient responses to starvation [31]. SCV formation is an important process in biofilm development, as SCVs show enhanced biofilm forming capacity compared to normal colonies, and biofilm biomass increases when there are more SCV [27, 29] . Stable adaptation to distinct host niches has been shown for pneumococci resulting, for example, in different biofilm forming capacities of strains isolated from the human nasopharynx compared to blood isolates [17]. Our results indicate an increase of SCV formation for serotype 6B strains during growth in a nutrient-limited medium, whereas the process of SCV formation in serotype 7F strains might not be dependent on the nutritional environment, but driven by a different mechanism. For example, an increase in point mutations due to growth in a stressful environment might play a role, as in recent studies SCVs of serotypes 3, 8 and 19 were shown to be irreversible due to mutations in their capsule region [27, 29,

32]. We did not observe more biofilm CFU counts after 64h of growth, but this might be due to the limitations of our model. For example, lysis occurs due to the use of a static biofilm model, and when the medium was changed after 16h and 40h, all planktonically growing bacteria were removed and thus, biofilm formation only occurred with the remaining bacteria attaching to the surface. But after 64h of biofilm growth, 6B strains resulted in higher relative and absolute SCV counts compared to 7F strains. This process indicates a faster adaptation capability of serotype 6B strains to a different nutrient environment resulting in an enhanced and organized development of SCVs in a nutrient limited environment.

There are limitations within this study. A static biofilm model was used with an abiotic surface for attachment allowing us to gain general information about the biofilm growth behaviour of pneumococcal serotypes and commensal streptococci. Using a continuous flow bioreactor with living cultures of human cells as published recently [20], would mimic *in vivo* conditions more precisely. Additionally, there were only 4 time points (4, 16, 40 and 64h) analysed which gives a basic picture of biofilm development over time, but no information about its continuity. The exact time point of autolysis induction, the amount of biofilm that is reduced during this process as well as the timing of redevelopment after lysis, is, for example, not evaluated. Future experiments would benefit from confocal microscopy to visualize the biofilms of the different serotypes. Here we only can speculate that there are no full biofilms developed, but that our model only supports adherence and initial biofilm formation. Finally, information about *in vivo* biofilm formation of the pneumococcal serotypes used in this study is missing. Biofilm formation *in vivo* was shown to be highly distinct in shape from *in vitro* biofilms [7] and thus, an important follow-up experiment would be to test our findings *in vivo*.

Conclusions

Our results reveal the difference in initial biofilm formation between two serotypes: After 16 h of incubation, serotype 7F strains showed more relative biofilm CFU counts as well as increased numbers of SCVs compared to 6B. However, only serotype 6B resulted in a gradual increase of SCV numbers and, as a result, in an increase of relative biofilm CFU counts over time. This indicates an expression-regulated mechanism of SCV formation for serotype 6B which allows adaptation to changing nutritional environments. For serotype 7F, which did not show an increase in SCV formation over time, another mechanism as for example the induction of point mutations might be responsible for SCV development

Methods

Bacterial strains and growth conditions

The *S. pneumoniae* strains used for biofilm growth experiments are listed in Table 1.

For biofilm growth, bacteria were plated on Columbia sheep blood agar (CSBA) plates and incubated overnight at 37°C and 5% CO_2, then grown overnight in a waterbath in 5 ml of Chemically Defined medium (CDM) [33] with the following modifications: Copper sulphate solution, manganese sulphate solution and cysteine solution were stored separately from the rest of the medium to prevent precipitations. Copper sulphate and manganese sulphate were stored at -20°C, the cysteine solution at 4°C. For overnight growth, CDM was supplemented with 50 mM of filter-sterilized glucose for strains. The next day, bacteria were subcultured into 5 ml of CDM supplemented with 5 mM of filter-sterilized glucose. Pneumococcal strains were grown to an OD_{600nm} of 0.1 to 0.2 in a water bath, then 10-20% of glycerol was added to the cultures and bacteria were stored at -80°C. After at least 20h at -80°C,

one aliquot was thawed and serial dilutions in phosphate-buffered saline (PBS) were plated out on CSBA plates to count colony forming units (CFUs).

For biofilm formation studies, CDM was supplemented either with 5 mM glucose or 5 mM galactose.

For planktonic growth, bacteria were plated on CSBA plates and incubated overnight at 37°C and 5% CO_2. Then, the strains were grown overnight in 10 ml Lacks medium supplemented with 1.5M glucose as carbon source to an OD_{600nm} of 0.5 [34]. The next day, cultures were centrifuged at 3000 g for 5 min at room temperature (Heraeus Megafuge 16R), and pellet was washed with 10 ml CDM without carbon source. Pellet then was resuspended in 10 ml CDM without carbon source.

For FITC-Dextran imaging after one passage, single colonies of normal size and SCV were taken from CSBA plates used for counting biofilm CFUs, plated out on CSBA plates and incubated overnight at 37°C + 5% CO_2. Then, they were grown overnight in Brain Heart Infusion (BHI) broth supplemented with 5% Fetal Bovine Serum (FBS). The next day, subcultures of overnight growth were performed and liquid cultures grown to an OD_{600nm} of 0.3 to 0.45.

Planktonic growth

96-well microtiter polystyrene plates (Thermo Fisher Scientific, Denmark) were used for planktonic growth. The inside of the plate lid was covered with 3 ml of 0.05% Triton X-100 (Merck, Darmstadt, Germany) in 20% ethanol and air-dried. 192 µl CDM supplemented with either 5.5 mM glucose or 5.5 mM galactose were added per well. 8 µl (dilution = 1:25) of fresh bacterial suspension was added per well. Plate was sealed with parafilm, and OD_{450nm} was measured on an ELISA plate reader (THERMOmax Microplate Reader, Molecular Devices Corporation, California) every 30 minutes for 30 hours using SOFTmax Pro 3.1.2.

Growth of biofilms

96-well microtiter polystyrene plates (Thermo Fisher Scientific, Denmark) were used for biofilm growth. 200 µl of CDM supplemented with either 5 mM glucose or 5 mM galactose and frozen bacterial culture adjusted to $1x10^5$ CFU/200µl was added per well. As extracellular DNA was shown to increase biofilm formation [35, 36], also 2 µl of salmon sperm DNA (Sigma, Zürich, Switzerland) were added per well. The plates then were incubated statically for 16h at 37°C and 5% CO_2 according to previous publications [10, 37]. For 64h of biofilm incubation, the supernatant was removed as published before [9] after 16h and 40h and fresh CDM supplemented with either 5 mM glucose or 5 mM galactose was added.

Colony forming units (CFU) counting of biofilms

After 16 h or 64 h of incubation, the liquid medium and the planktonic cells were removed from the wells using a multichannel pipette. Biofilms then were washed twice with 200 µl PBS and sonicated in the water bath (Branson Water Bath 3210) for 6 seconds. Serial dilutions of biofilms then were plated out on CSBA plates and incubated at 37°C and 5% CO_2. The next day, the bacteria were counted and the CFU/200 µl was calculated.

Colony size differentiation / Fluorescein isothiocyanate-dextran imaging

Serial dilutions of biofilm CFU counts were screened for differences in colony sizes. Small colony variants were discriminated from normal colonies by eye. For clearer discrimination, normal and small colonies were grown in Brain Heart Infusion (see “Bacterial strains and growth conditions”). FITC-Dextran imaging was performed according to previously published protocols (Weinberger et al. 2009 [38], Hathaway et al. [39]). Shortly, bacterial suspensions were centrifuged at 3000 g for 5 minutes at room temperature (Heraeus Megafuge 16R) and pellet was resuspended in 800 µl PBS. 2 µl of FITC-Dextran (10 mg/ml, stored at -20°C, Sigma Aldrich) were mixed with 10 µl of bacterial suspension, 10 µl of mixture placed on a glass slide.

The coverslip then was pressed down firmly. Pictures were taken using a Zeiss Axio Imager.M1 fluorescence microscope with a 100x objective and photographed by a Zeiss AxioCam HRc camera.

Statistical analysis

To compare different groups, One-way ANOVA was used with Tukey's correction for multiple testing. GraphPad Prism 6.3 was used for statistical analysis. For two-group comparisons, Student's t-test was used.

Authors' contributions

Conceived and designed the experiments: MH, LJH, KM, EK. Performed the experiments: MM, EK, TOS. Analysed the data: EK, MH, DW. Wrote the paper: MH, EK.

References

1. Lynch JP, 3rd, Zhanel GG: **Streptococcus pneumoniae: epidemiology, risk factors, and strategies for prevention**. *Semin Respir Crit Care Med* 2009, **30**(2):189-209.

2. Standish AJ, Salim AA, Zhang H, Capon RJ, Morona R: **Chemical inhibition of bacterial protein tyrosine phosphatase suppresses capsule production**. *PLoS One* 2012, **7**(5):e36312.

3. Sanclement JA, Webster P, Thomas J, Ramadan HH: **Bacterial biofilms in surgical specimens of patients with chronic rhinosinusitis**. *Laryngoscope* 2005, **115**(4):578-582.

4. Marks LR, Parameswaran GI, Hakansson AP: **Pneumococcal interactions with epithelial cells are crucial for optimal biofilm formation and colonization in vitro and in vivo**. *Infect Immun* 2012, **80**(8):2744-2760.

5. Garcia-Castillo M, Morosini MI, Valverde A, Almaraz F, Baquero F, Canton R, del Campo R: **Differences in biofilm development and antibiotic susceptibility among Streptococcus pneumoniae isolates from cystic fibrosis samples and blood cultures**. *J Antimicrob Chemother* 2007, **59**(2):301-304.

6. Domenech M, Ramos-Sevillano E, Garcia E, Moscoso M, Yuste J: **Biofilm formation avoids complement immunity and phagocytosis of Streptococcus pneumoniae**. *Infect Immun* 2013, **81**(7):2606-2615.

7. Blanchette-Cain K, Hinojosa CA, Akula Suresh Babu R, Lizcano A, Gonzalez-Juarbe N, Munoz-Almagro C, Sanchez CJ, Bergman MA, Orihuela CJ: **Streptococcus pneumoniae Biofilm Formation Is Strain Dependent,**

Multifactorial, and Associated with Reduced Invasiveness and Immunoreactivity during Colonization. *MBio* 2013, **4**(5).

8. del Prado G, Ruiz V, Naves P, Rodriguez-Cerrato V, Soriano F, del Carmen Ponte M: **Biofilm formation by Streptococcus pneumoniae strains and effects of human serum albumin, ibuprofen, N-acetyl-l-cysteine, amoxicillin, erythromycin, and levofloxacin**. *Diagn Microbiol Infect Dis* 2010, **67**(4):311-318.

9. Camilli R, Pantosti A, Baldassarri L: **Contribution of serotype and genetic background to biofilm formation by Streptococcus pneumoniae**. *Eur J Clin Microbiol Infect Dis* 2011, **30**(1):97-102.

10. Hall-Stoodley L, Nistico L, Sambanthamoorthy K, Dice B, Nguyen D, Mershon WJ, Johnson C, Hu FZ, Stoodley P, Ehrlich GD *et al*: **Characterization of biofilm matrix, degradation by DNase treatment and evidence of capsule downregulation in Streptococcus pneumoniae clinical isolates**. *BMC Microbiol* 2008, **8**:173.

11. McEllistrem MC, Ransford JV, Khan SA: **Characterization of in vitro biofilm-associated pneumococcal phase variants of a clinically relevant serotype 3 clone**. *J Clin Microbiol* 2007, **45**(1):97-101.

12. Tapiainen T, Kujala T, Kaijalainen T, Ikaheimo I, Saukkoriipi A, Renko M, Salo J, Leinonen M, Uhari M: **Biofilm formation by Streptococcus pneumoniae isolates from paediatric patients**. *APMIS* 2010, **118**(4):255-260.

13. Trappetti C, Gualdi L, Di Meola L, Jain P, Korir CC, Edmonds P, Iannelli F, Ricci S, Pozzi G, Oggioni MR: **The impact of the competence quorum**

sensing system on Streptococcus pneumoniae biofilms varies depending on the experimental model. *BMC Microbiol* 2011, **11**:75.

14. Ruiz V, Rodriguez-Cerrato V, Huelves L, Del Prado G, Naves P, Ponte C, Soriano F: **Adherence of Streptococcus pneumoniae to polystyrene plates and epithelial cells and the antiadhesive potential of albumin and xylitol**. *Pediatr Res* 2011, **69**(1):23-27.

15. Marks LR, Reddinger RM, Hakansson AP: **High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in Streptococcus pneumoniae**. *MBio* 2012, **3**(5).

16. Trappetti C, Potter AJ, Paton AW, Oggioni MR, Paton JC: **LuxS mediates iron-dependent biofilm formation, competence, and fratricide in Streptococcus pneumoniae**. *Infect Immun* 2011, **79**(11):4550-4558.

17. Trappetti C, van der Maten E, Amin Z, Potter AJ, Chen AY, van Mourik PM, Lawrence AJ, Paton AW, Paton JC: **Site of isolation determines biofilm formation and virulence phenotypes of Streptococcus pneumoniae serotype 3 clinical isolates**. *Infect Immun* 2013, **81**(2):505-513.

18. Kurola P, Tapiainen T, Sevander J, Kaijalainen T, Leinonen M, Uhari M, Saukkoriipi A: **Effect of xylitol and other carbon sources on Streptococcus pneumoniae biofilm formation and gene expression in vitro**. *APMIS* 2011, **119**(2):135-142.

19. Wei H, Havarstein LS: **Fratricide is essential for efficient gene transfer between pneumococci in biofilms**. *Appl Environ Microbiol* 2012, **78**(16):5897-5905.

20. Vidal JE, Howery KE, Ludewick HP, Nava P, Klugman KP: **Quorum-sensing systems LuxS/autoinducer 2 and Com regulate Streptococcus**

pneumoniae biofilms in a bioreactor with living cultures of human respiratory cells. *Infect Immun* 2013, **81**(4):1341-1353.

21. Munoz-Elias EJ, Marcano J, Camilli A: **Isolation of Streptococcus pneumoniae biofilm mutants and their characterization during nasopharyngeal colonization**. *Infect Immun* 2008, **76**(11):5049-5061.

22. Parker D, Soong G, Planet P, Brower J, Ratner AJ, Prince A: **The NanA neuraminidase of Streptococcus pneumoniae is involved in biofilm formation**. *Infect Immun* 2009, **77**(9):3722-3730.

23. Sanchez CJ, Shivshankar P, Stol K, Trakhtenbroit S, Sullam PM, Sauer K, Hermans PW, Orihuela CJ: **The pneumococcal serine-rich repeat protein is an intra-species bacterial adhesin that promotes bacterial aggregation in vivo and in biofilms**. *PLoS Pathog* 2010, **6**(8):e1001044.

24. Vidal JE, Ludewick HP, Kunkel RM, Zahner D, Klugman KP: **The LuxS-dependent quorum-sensing system regulates early biofilm formation by Streptococcus pneumoniae strain D39**. *Infect Immun* 2011, **79**(10):4050-4060.

25. Oggioni MR, Trappetti C, Kadioglu A, Cassone M, Iannelli F, Ricci S, Andrew PW, Pozzi G: **Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis**. *Mol Microbiol* 2006, **61**(5):1196-1210.

26. Trappetti C, Ogunniyi AD, Oggioni MR, Paton JC: **Extracellular matrix formation enhances the ability of Streptococcus pneumoniae to cause invasive disease**. *PLoS One* 2011, **6**(5):e19844.

27. Allegrucci M, Sauer K: **Formation of Streptococcus pneumoniae non-phase-variable colony variants is due to increased mutation frequency**

present under biofilm growth conditions. *J Bacteriol* 2008, **190**(19):6330-6339.

28. Qin L, Kida Y, Imamura Y, Kuwano K, Watanabe H: **Impaired capsular polysaccharide is relevant to enhanced biofilm formation and lower virulence in Streptococcus pneumoniae**. *Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy* 2013, **19**(2):261-271.

29. Allegrucci M, Sauer K: **Characterization of colony morphology variants isolated from Streptococcus pneumoniae biofilms**. *J Bacteriol* 2007, **189**(5):2030-2038.

30. Allegrucci M, Hu FZ, Shen K, Hayes J, Ehrlich GD, Post JC, Sauer K: **Phenotypic characterization of Streptococcus pneumoniae biofilm development**. *J Bacteriol* 2006, **188**(7):2325-2335.

31. Hathaway LJ, Brugger SD, Morand B, Bangert M, Rotzetter JU, Hauser C, Graber WA, Gore S, Kadioglu A, Muhlemann K: **Capsule type of Streptococcus pneumoniae determines growth phenotype**. *PLoS Pathog* 2012, **8**(3):e1002574.

32. Brueggemann AB, Peto TE, Crook DW, Butler JC, Kristinsson KG, Spratt BG: **Temporal and geographic stability of the serogroup-specific invasive disease potential of Streptococcus pneumoniae in children**. *J Infect Dis* 2004, **190**(7):1203-1211.

33. Weinberger DM, Trzcinski K, Lu YJ, Bogaert D, Brandes A, Galagan J, Anderson PW, Malley R, Lipsitch M: **Pneumococcal capsular polysaccharide structure predicts serotype prevalence**. *PLoS Pathog* 2009, **5**(6):e1000476.

34. Waite RD, Penfold DW, Struthers JK, Dowson CG: **Spontaneous sequence duplications within capsule genes cap8E and tts control phase variation in Streptococcus pneumoniae serotypes 8 and 37**. *Microbiology* 2003, **149**(Pt 2):497-504.

35. Speranza B, Corbo MR, Sinigaglia M: **Effects of nutritional and environmental conditions on Salmonella sp. biofilm formation**. *Journal of food science* 2011, **76**(1):M12-16.

36. van de Rijn I, Kessler RE: **Growth characteristics of group A streptococci in a new chemically defined medium**. *Infect Immun* 1980, **27**(2):444-448.

37. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS: **Extracellular DNA required for bacterial biofilm formation**. *Science* 2002, **295**(5559):1487.

38. Carrolo M, Frias MJ, Pinto FR, Melo-Cristino J, Ramirez M: **Prophage spontaneous activation promotes DNA release enhancing biofilm formation in Streptococcus pneumoniae**. *PLoS One* 2010, **5**(12):e15678.

39. Domenech M, Garcia E, Moscoso M: **In vitro destruction of Streptococcus pneumoniae biofilms with bacterial and phage peptidoglycan hydrolases**. *Antimicrob Agents Chemother* 2011, **55**(9):4144-4148.

Figures

Figure 1 – Planktonic growth of pneumococcal serotypes 6B and 7F in CDM galactose and CDM glucose

22 h of planktonic growth of strains 106.66 (serotype 6B), 208.41 (serotype 7F) in CDM supplemented with A) 5 mM glucose and B) 5 mM galactose.

Error bars: SEM.

Figure 2 – Biofilm growth of pneumococcal strains 106.66 (serotype 6B) and 208. 41 (serotype 7F) and their isogenic capsule-switch mutants

Planktonic and biofilm growth of 106.66 (6B), 208.41cps106.66 (6B),208.41 (7F) and 106.66cps208.41 (7F) in A) CDM glucose and B) CDM galactose after 16h of incubation. Identical experiments are connected with a line. C) and D) show the relative amount of biofilm from total CFU counts in CDM glucose and CDM galactose, respectively.

Statistical tests: Student's t-test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$

Figure 3 – SCV formation of serotypes 6B and 7F

Relative amount of SCVs from total biofilm CFUs in CDM galactose and CDM glucose.

Figure 4 – SCV and normal-sized bacteria of serotypes 6B and 7F after one passage

A) SCV of strain 208.41 (serotype 7F) after passaging once in BHI + 5% FCS and B) normal-sized bacteria of strain 208.41 (serotype 7F) after one passage. C) SCV variant of strain 106.66 (serotype 6B) which reverted to normal size after one passage and D) normal-sized bacteria of strain 106.66 (serotype 6B). Magnification 1000x.

Figure 5 - Development of biofilms and small colony variants of serotypes 6B and 7F over time

A) Planktonic and biofilm growth of 106.66 (6B) and 208.41 (7F) after 64 h of incubation. Identical experiments are connected with a line. B) Relative amount of biofilm CFU counts as percentage of total CFUs for serotypes 6B and 7F after 16 h and 64 h of incubation. C) Relative amount of SCVs from total biofilm CFU counts in CDM galactose after 16 h and 64 h of growth.

Error bars: SEM. Statistical test: Student's t-test. ** *p<0.01*

Tables

Table 1 – Details of pneumococcal strains used in this study.

Table 1

Species	Strain	Sample location	Year of isolation	Country of isolation	Serotype	MLST	Pneumolysin	References
S. pneumoniae	106.66	nasopharynx (otitis media)	1998	CH	6B	2244	yes	[39]
S. pneumoniae	208.41	nasopharynx (otits media)	2001	CH	7F	191	yes	[39]
S. pneumoniae	208.41cps106.66	mutant		CH	6B	191	yes	[39]
S. pneumoniae	106.66cps208.41	mutant		CH	7F	2244	yes	[39]

Figure 1

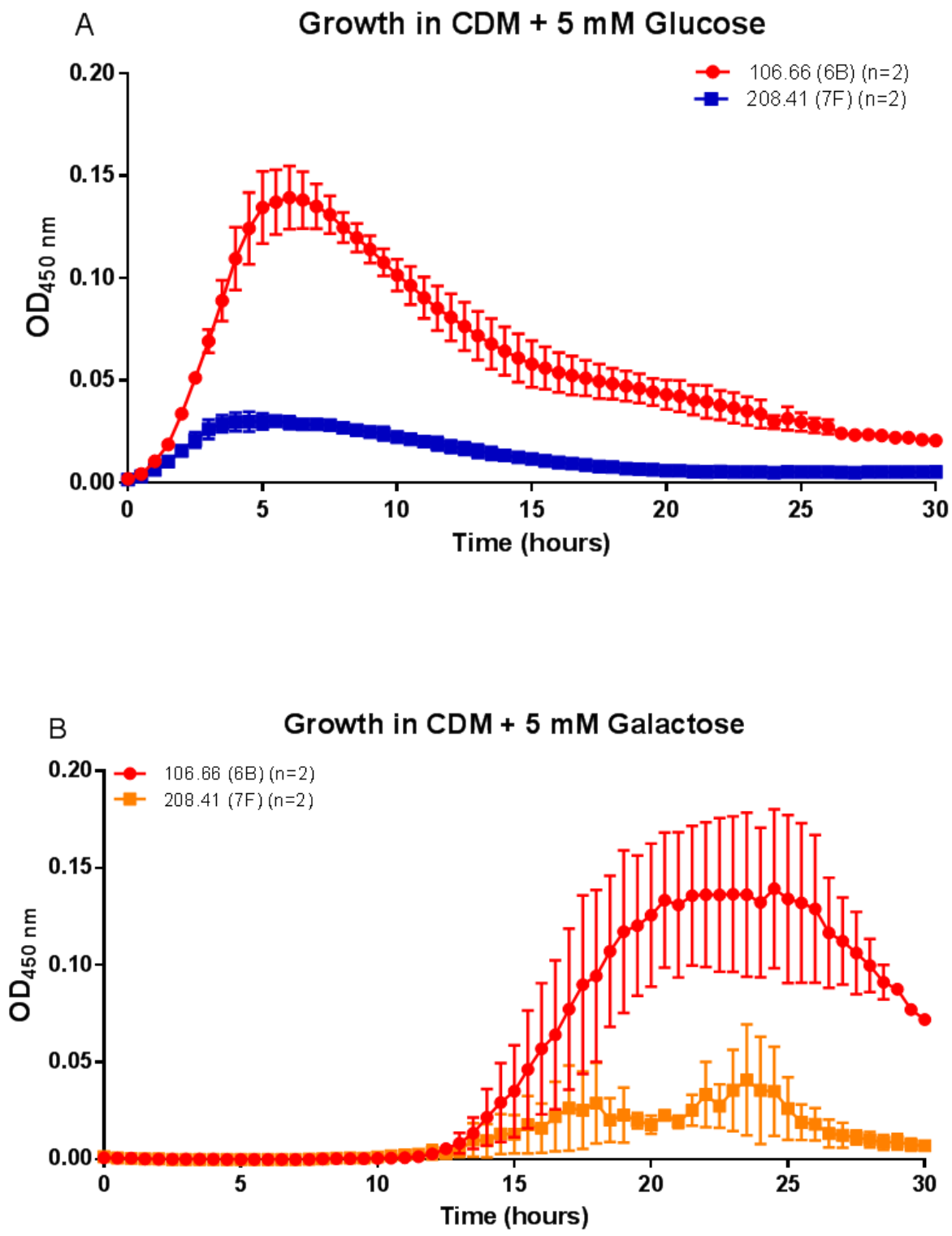
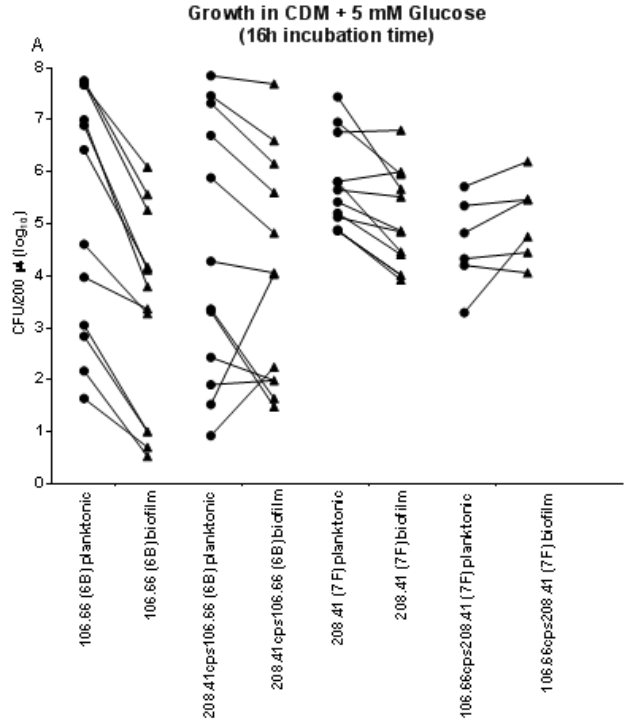
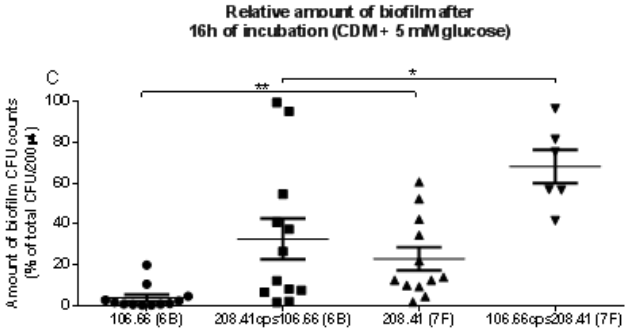
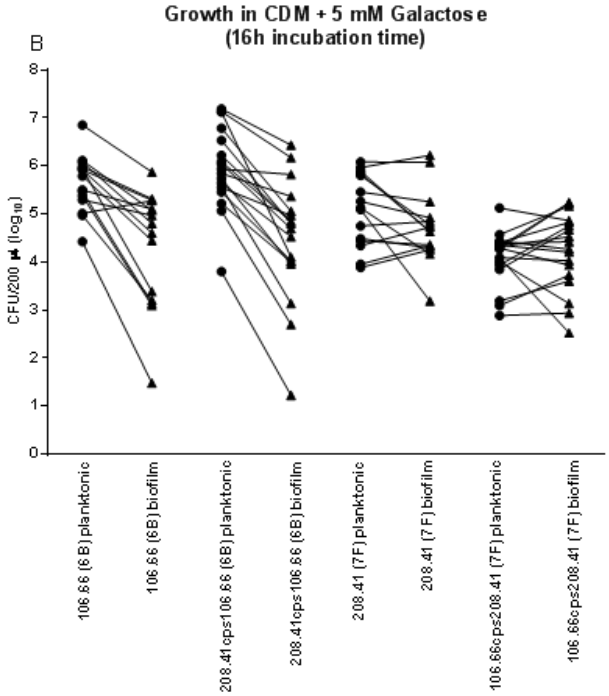

A
Growth in CDM + 5 mM Glucose
106.66 (6B) (n=2)
208.41 (7F) (n=2)
OD450 nm
0.20
0.15
0.10
0.05
0.00
0
5
10
15
20
25
30
Time (hours)
B
Growth in CDM + 5 mM Galactose
106.66 (6B) (n=2)
208.41 (7F) (n=2)
OD450 nm
0.20
0.15
0.10
0.05
0.00
0
5
10
15
20
25
30
Time (hours)

Figure 2


Growth in CDM + 5 mM Glucose
(16h incubation time)
A
CFU/200 µl (log10)
106.66 (6B) planktonic
106.66 (6B) biofilm
208.41cps106.66 (6B) planktonic
208.41cps106.66 (6B) biofilm
208.41 (7F) planktonic
208.41 (7F) biofilm
106.66cps208.41 (7F) planktonic
106.66cps208.41 (7F) biofilm


Relative amount of biofilm after
16h of incubation (CDM+ 5 mM glucose)
C
Amount of biofilm CFU counts
(% of total CFU/200µl)
106.66 (6B)
208.41cps106.66 (6B)
208.41 (7F)
106.66cps208.41 (7F)


Growth in CDM + 5 mM Galactose
(16h incubation time)
B
CFU/200 µl (log10)
106.66 (6B) planktonic
106.66 (6B) biofilm
208.41cps106.66 (6B) planktonic
208.41cps106.66 (6B) biofilm
208.41 (7F) planktonic
208.41 (7F) biofilm
106.66cps208.41 (7F) planktonic
106.66cps208.41 (7F) biofilm

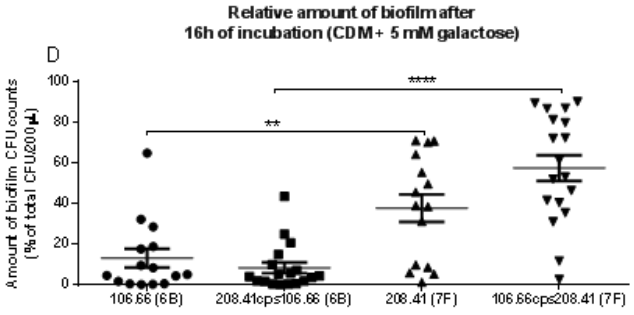

Relative amount of biofilm after
16h of incubation (CDM+ 5 mM galactose)
D
Amount of biofilm CFU counts
(% of total CFU/200µl)
106.66 (6B)
208.41cps106.66 (6B)
208.41 (7F)
106.66cps208.41 (7F)

Figure 3

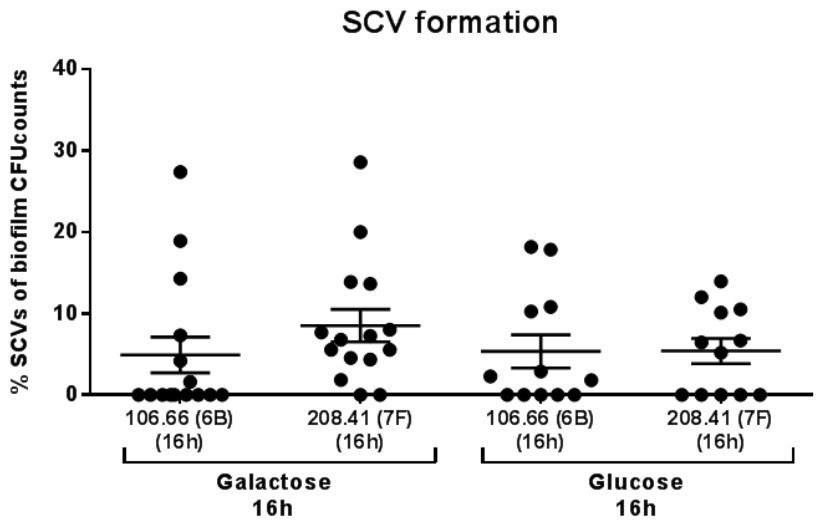

SCV formation
% SCVs of biofilm CFUcounts
40
30
20
10
0
106.66 (6B) (16h)
208.41 (7F) (16h)
106.66 (6B) (16h)
208.41 (7F) (16h)
Galactose 16h
Glucose 16h

Figure 4

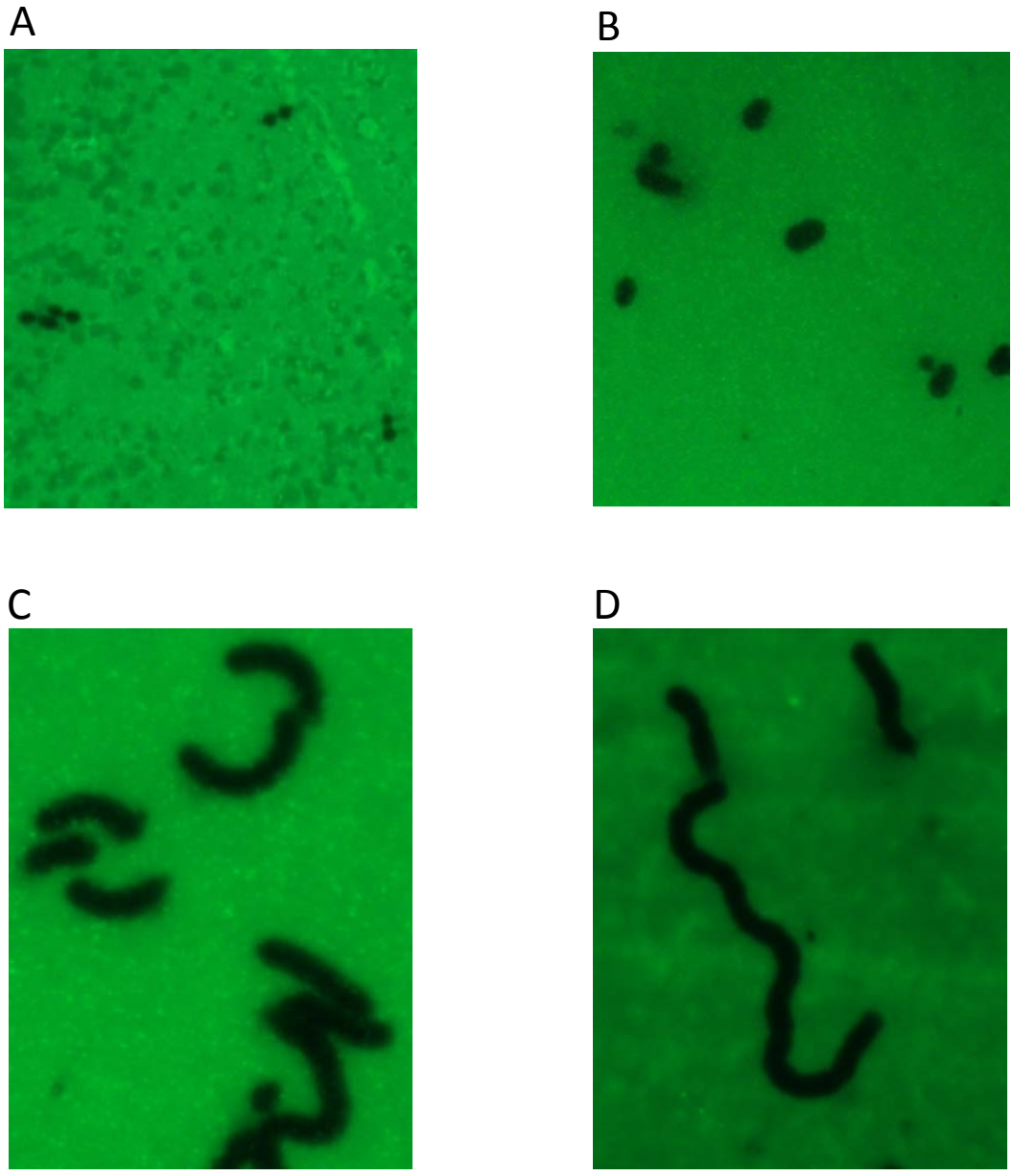
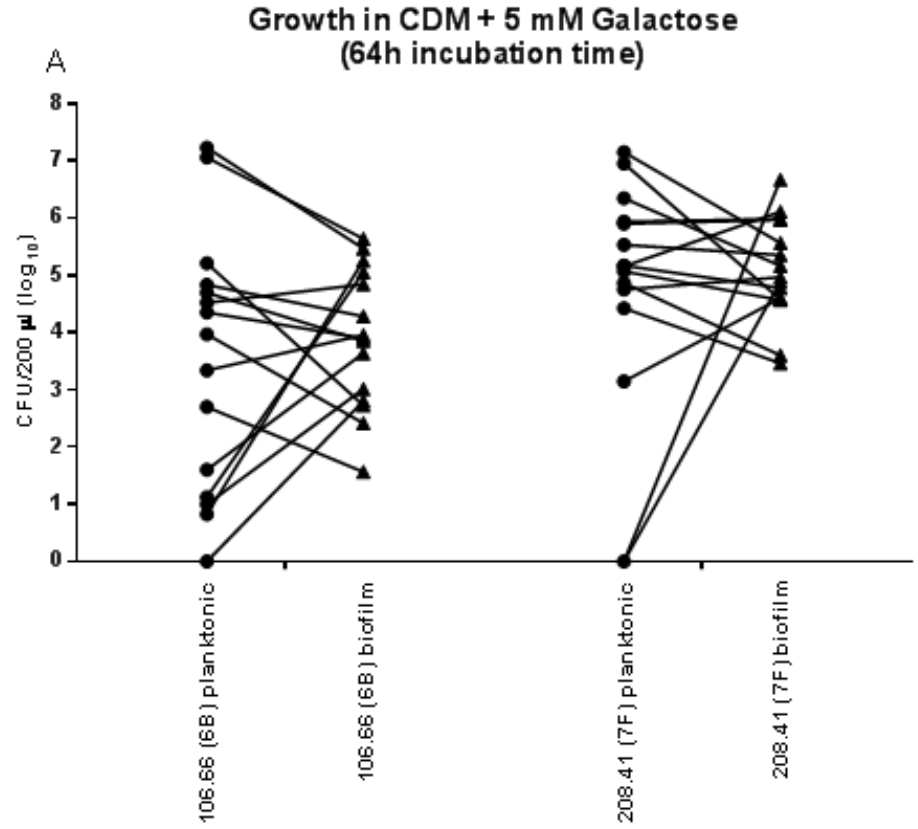
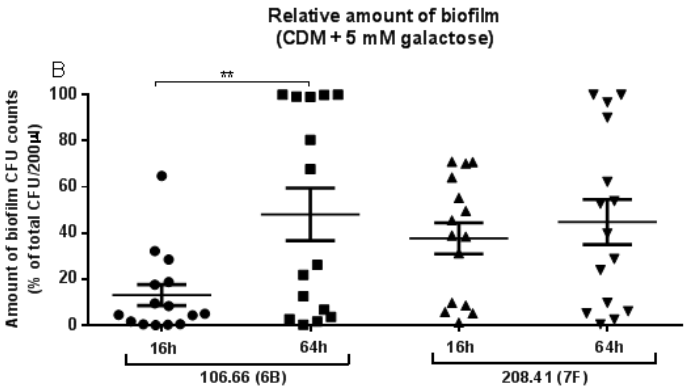
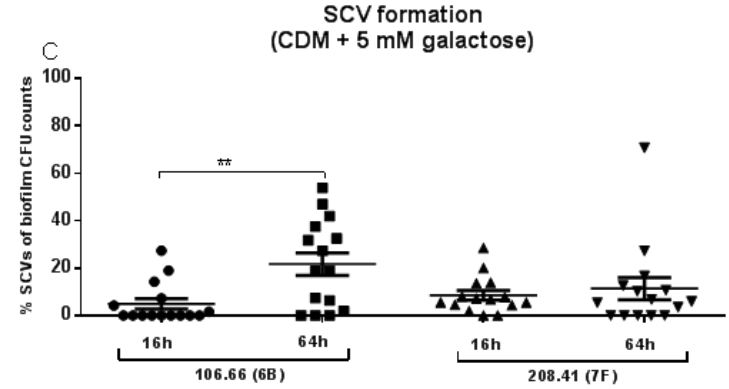


Figure 5


Growth in CDM + 5 mM Galactose
(64h incubation time)
A
CFU/200 µl (log10)
106.66 (6B) planktonic
106.66 (6B) biofilm
208.41 (7F) planktonic
208.41 (7F) biofilm


Relative amount of biofilm
(CDM + 5 mM galactose)
B
Amount of biofilm CFU counts
(% of total CFU/200µl)
**
16h
64h
106.66 (6B)
208.41 (7F)


SCV formation
(CDM + 5 mM galactose)
C
% SCVs of biofilm CFU counts
**
16h
64h
106.66 (6B)
208.41 (7F)

5.2 Interactions in biofilm formation between commensal streptococcal strains and *S. pneumoniae* serotypes

Co-cultivation experiments of commensal streptococcal strains and *S. pneumoniae*

Additional work required: mixed species planktonic growth curves to investigate behaviour over time to compare this data to CFU results

Interactions in biofilm formation between commensal streptococcal strains and *S. pneumoniae* serotypes

Abstract

On the mucosal surfaces of the human nasopharynx biofilms were identified which are generally composed of a high amount of different bacterial species. Between the different species, niche competition occurs where factors like space, nutrient limitation or bacteriocin secretion may play a dominant role.

The commensal streptococci *S. oralis*, *S. mitis* and *S. pseudopneumoniae* with different biofilm forming potential were compared in mono- and mixed species biofilm formation with *S. pneumoniae* serotypes. A decrease of biofilm biomass as well as of biofilm CFU counts resulted from co-cultivation for all species tested. A chemically defined medium (CDM) supplemented with either 5 mM glucose or 5 mM galactose was used resulting in no carbon source-dependent differences in competition. Nutrient limitation only played a significant role for competition of *S. pneumoniae* with *S. oralis*. For the other co-cultivations tested, nutrient limitation is not a major factor. We identified that apart from nutrient limitation and competition for space some other factors as bacteriocins, H_2O_2 production or differences in the structural development of biofilms among the species tested might have a stronger effect on the process of mixed species initial biofilm formation.

Introduction

The human pharynx harbors over 700 different bacterial species [1], including the resident commensal streptococci *S. oralis*, *S. mitis* and *S. pseudopneumoniae* as well as the transient commensal *S. pneumoniae* [2]. Niche competition occurs between commensal bacteria as well as between potential pathogens. Factors as nutrient limitation (carbohydrates, amino acids, micronutrients) [3], secretion of bacteriocins [4, 5], the induction of bacteriophages [6], and modification of the host immune response [7] contribute directly or indirectly to the competition between microorganisms for an anatomical niche. For example, *S. pneumoniae* secrets H_2O_2 which is bactericidal to *S. aureus* and *H. influenzae* [8, 9], and a negative association between carriage of *S. pneumoniae* and *Streptococcus aureus* has been shown [10]. *S. pneumoniae* typically carries the genetic information for bacteriocin production, which inhibits bacteria from the same species as well as *S. mitis* and *S. oralis*; but only a small proportion of clinical strains seems to express bacteriocin activity [11]. The immune-mediated pressure started by *H. influenzae* is able to reduce *S. pneumoniae* density in the nasopharynx [12].

In nature, bacteria are generally organized in biofilms, and also for medical microbiology this growth stage is of main importance [13]. The human nasopharynx is assumed as being nutrient-poor and biofilm formation as the growth type predominating in nutrient-limited environments [3, 14]. Biofilm formation was observed on mucosal surfaces in the nasopharynx as well as in tonsillitis and chronic otitis media [15-18]. Biofilms are generally composed of a high amount of different bacterial species, in the oral cavity up to 50 different species were identified adhering on one location [19]. In oral microbiology it was observed that bacteria producing plaque biofilms do not adhere to teeth randomly but in a highly organized coaggregation profile where differences between early and late colonizing bacteria can be observed suggesting both competitive and symbiontic interactions [20-22].

Three commensal streptococci (*S. pseudopneumoniae*, *S. mitis* and *S. oralis*) generally colonizing the human nasopharynx and with assumed different biofilm building *in*

vitro were used for co-cultivation experiments with *S. pneumoniae* [2, 23]. The primary objective of this study was to develop a static biofilm model to study mixed species growth of *S. pneumoniae* with *S. pseudopneumoniae*, *S. mitis* or *S. oralis*. We further aimed at investigating whether competition occurs between the different bacterial species and whether nutrient limitation is a driving force for this process using chemically defined medium (CDM) supplemented with either glucose or galactose.

Material and methods

Bacterial strains and growth conditions

The pneumococcal strains and commensal streptococci used for mixed-species biofilm growth are listed in Table 1. Bacteria were plated on Columbia Sheep blood agar (CSBA) plates and incubated overnight at 37°C and 5% CO_2, then grown overnight in a water bath in 5 ml of Chemically Defined medium (CDM) [24] with the following modifications: Copper sulphate solution, manganese sulphate solution and cysteine solution were stored separately from the rest of the medium to prevent precipitations. Copper sulphate and manganese sulphate were stored at -20°C, the cysteine solution at 4°C. For overnight growth, CDM was supplemented with 50 mM of filter-sterilized sucrose. The next day, bacteria were subcultured into 5 ml of CDM supplemented with 5 mM of filter-sterilized glucose and grown to an OD_{600} of 0.1 to 0.15 in a water bath. 800 µl aliquots supplemented with 10% glycerol prepared and stored at -80°C.

Biofilm growth

96-well microtiter polystyrene plates (Thermo Fisher Scientific, Denmark) were used for biofilm growth. 200 µl of CDM supplemented with either 5 mM glucose (CDM glucose) or 5 mM galactose (CDM galactose) were added per well. To directly compare mono- and mixed cultural biofilm growth, bacteria were grown on the same day as monocultures as well as in a 1:1 mixture of pneumococcus and a commensal streptococcus. A total amount of 5 µl of frozen stock was used as starting culture per biofilm resulting in only 2.5 µl per species for

mixed culture. But as liquid cultures were only grown to an OD_{600nm} of 0.15 before freezing, differences in starting CFU counts were estimated as non-significant and starting conditions for mono- and mixed species growth approximated as similar. As extracellular DNA was shown to increase biofilm formation [25, 26], also 2 µl of salmon sperm DNA (Sigma, Zürich, Switzerland) with a final concentration of 0.5 ng/ml were added per well. The plates then were incubated statically for 16h at 37°C and 5% CO_2.

Planktonic growth

96-well microtiter polystyrene plates (Thermo Fisher Scientific, Denmark) were used for planktonic growth. The inside of the plate lid was covered with 3 ml of 0.05% Triton X-100 (Merck, Darmstadt, Germany) in 20% ethanol and air-dried. 200 µl CDM supplemented with either 5 mM glucose or 5 mM galactose were added per well. 5 µl of frozen bacterial culture were used for monocultural growth, whereas for mixed species growth 2.5 µl of frozen stock from each species were used (for detailed description, see above). OD_{450nm} was measured on an ELISA plate reader (THERMOmax Microplate Reader, Molecular Devices Corporation, California) every 30 minutes for 22 hours using SOFTmax Pro 3.1.2.

Crystal violet staining of biofilms

After 16h of incubation, the liquid medium with the planktonically growing bacteria was removed from the wells using a multichannel pipette. Biofilms then were washed twice with 200 µl of phosphate buffered saline (PBS), stained with 100 µl of 0.5% crystal violet (CV) (0.5% CV in PBS) for 30 minutes at room temperature. After an additional washing step using 200 µl PBS, plates were air-dried for at least 30 minutes. CV of stained biofilms then was solubilized by adding 200 µl of 95% ethanol and absorbance was measured on an ELISA plate reader (THERMOmax Microplate Reader, Molecular Devices Corporation, California) at 450 nm

Colony forming units (CFU) counting of biofilms

After 16h of incubation, the liquid medium and the planktonic cells were removed from the wells using a multichannel pipette. Biofilms then were washed twice with 200 µl PBS and

sonicated using a sonifier (Branson sonifier 250; duty cycle of 30, output control 2.5, sonicate for 3 seconds). Serial dilutions of biofilms were plated out on pure CSBA plates and on blood plates supplemented with an appropriate antibiotic (see Table 1 for an overview and Table 2 for detailed information to antibiotic resistances of strains used) to distinguish between pneumococci and commensal streptococci. Plates then were incubated at 37°C and CO_2. The next day, the bacteria were counted and the CFU/200 µl were calculated.

CFU counting of planktonically grown bacteria

After 14-16 h of incubation, serial dilutions of the supernatant were plated out on pure CSBA plates and on blood plates supplemented with an appropriate antibiotic (see Table 1 for an overview and Table 2 for detailed information to antibiotic resistances of strains used) to distinguish between pneumococci and commensal streptococci. Plates then were incubated at 37°C and 5% CO_2. The next day, the bacteria were counted and the CFU/200 µl were calculated.

Statistical analysis

GraphPad Prism 6.3 was used for statistical analysis. Student's t-test was used to compare two groups. For mean values, the ratio of data points is added.

Results and Discussion

Biofilm forming potential of S. pneumoniae and commensal streptococci in monoculture

First, CV staining was performed to determine the total adhering biomass (Figures 1A and 1B). *S. oralis* resulted in a non-significantly increased biomass in CDM galactose (*p=0.09*) and CDM glucose (*p=0.14*) compared to all other strains tested, whereas *S. mitis*, *S. pseudopneumoniae* and *S. pneumoniae* showed a similar amount of biofilm (*p=0.7* in CDM glucose, *p=0.3* in CDM galactose).

To get the amount of living bacteria in the biofilm, CFUs were then counted (Figures 1C and 1D). Mean CFU/200 µl of *S. oralis* ($1x10^6$; range: $5.3x10^4$-$3.2x10^6$) were increased compared

to *S. mitis*, *S. pseudopneumoniae* and *S. pneumoniae* in both growth media tested. In CDM glucose, this effect was significant for *S. mitis* ($p<0.05$) and *S. pneumoniae* ($p<0.01$).

Recently, the biofilm forming capacity of *S. oralis*, *S. mitis* and *S. pseudopneumoniae* was studied in C-medium [23]. *S. oralis* and *S. pseudopneumoniae* resulted in better biofilm formation than pneumococci with *S. oralis* resulting in the highest biomass, what is in accordance to our findings. Interestingly, *S. mitis* was shown to be unable to form biofilms in C-medium, whereas our results in CDM (with galactose or glucose) indicate a biofilm forming capacity comparable to *S. pneumoniae*. Individual lineages of *S. mitis* are quite distinct from one another and it was shown for oral streptococci that strains of the same species often differ in their ability to form biofilms [2, 27]. This might explain the different observations in *S. mitis* biofilm forming capacity. Different biofilm capabilities within the same species lineage were also found in our study (data not shown). However, differences in the total adhering biomass were not as apparent as between different species. We therefore pooled biofilm results of the same species for the subsequent experiments.

In conclusion, commensal streptococci and pneumococci included in this study showed different biofilm forming capacity, making them ideal candidate strains to further investigate their different behavior in co-cultivation experiments.

Therefore, we next investigated whether differences in biofilm growth behavior is also observed for mixed species growth (Commensal streptococci with pneumococcus).

Biofilm formation of mixed bacterial species growth (CV staining)

Mixed species biofilms were evaluated by CV staining after 16h in Glucose and Galactose and subsequently compared to the corresponding monocultural growth (Figure 2). Surprisingly, no additive effect in mixed species biofilm growth was measured for any of the combinations tested. When a commensal streptococcus was grown together with *S. pneumoniae*, biofilm formation was always reduced compared to commensal monocultural growth. This finding was significant in the case of *S. oralis* within both, galacotse ($p<0.05$) and glucose ($p<0.05$) but the same trend was also seen for *S. mitis* and *S.*

pseudopneumoniae (Figure 2A and 2B).The same amount of bacteria were added for mono- and mixed cultural growth, but mixed species biofilms resulted in less CFU counts. Space limitation did not occur, as *S. oralis* biofilm biomass reached significantly higher levels than the other species tested. Co-cultures therefore did not result in using all the space provided by our model. Other factors like nutrient limitation or bacteriocin production might influence biofilm growth.

Biofilm formation of mixed bacterial species growth (CFU counting)

To evaluate the amount of living cells of each species in mixed species biofilms, CFUs were then counted. *S. pneumoniae* CFU counts were significantly ($p<0.05$) reduced in mixed cultures compared to monocultural growth. This was true for growth in CDM glucose as well as in CDM galactose and with all commensal streptococci tested (Figure 3A and 3B). In this study, different pneumococcal serotypes (Hungary 19A, strain 106.66 (6B) and strain 110.16 (19F)) showing different planktonic growth behaviour in CDM galactose and glucose were used. Interestingly, CFU counts in mixed species biofilm growth were reduced for all three pneumococci used indicating that the growth phase does not have a major impact on the outcome of competition during initial biofilm formation (data not shown). Using CDM galactose, commensal streptococci resulted in a non-significant decrease of CFU counts in mixed cultures for *S. mitis* ($p=0.18$), whereas the reduction in CFU counts was significant for *S. oralis* ($p<0.05$) and *S. pseudopneumoniae* ($p<0.05$). The same trend was observed with CDM glucose, where the reduction of CFU counts in mixed cultures was significant for *S. pseudopneumoniae* ($p<0.05$) and *S. oralis* ($p<0.0001$), whereas the reduction was not significant for *S. mitis* ($p=0.7$) (Figures 3C and 3D).

Planktonic growth of mixed bacterial species (CFU counting)

We were interested in investigating if nutrient limitation might play a dominant role in CFU reduction during mixed species growth. For this reason, planktonic growth of mixed cultures was performed. If nutrient limitation would occur, planktonic growth also might show

reduced amounts of CFU counts in mixed compared to monocultural growth, whereas other biofilm-specific factors like the more compact packing of bacteria and thus the closer interactions would have no influence.

For planktonic growth in CDM galactose CFU counts of pneumococci remained on a slightly decreased level when monocultural growth was compared to planktonic growth in a mixture with *S. pseudopneumoniae* (*p=0.3*) and with *S. mitis* (*p=0.4*) whereas a non-significant decrease was measured with *S. oralis* (*p=0.06*) (Figure 4A). In CDM glucose mixed cultural growth resulted in a non-significant (*p=0.5*) increase of pneumococcal CFU counts compared to *S. pneumoniae* monocultures (Figure 4B). Commensal streptococcal CFU counts in mixed cultures were non-significantly increased for *S. pseudopneumoniae* (*p=0.25*), remained stable *S. mitis* (*p=0.51*) and were non-significantly reduced for *S. oralis* (*p=0.25*) when growth was performed in CDM galactose (Figure 4C). In CDM glucose CFU counts of monocultures and mixed cultures showed no significant difference for *S. pseudopneumoniae* (*p=0.7*), *S. mitis* (*p=0.7*) and *S. oralis* (*p=0.42*) (Figure 4D).

During planktonic growth, nutrient limitation is supposed to be the major factor influencing competition in mixed cultural growth. This was true for mixed cultural growth of pneumococcus with *S. oralis*: planktonic growth resulted in a decrease of CFU counts for both species and thus, nutrient limitation is a major factor of competition between these two species. The opposite effect occurred for mixed cultural growth of *S. pneumoniae* with *S. pseudopneumoniae* as increased levels of the commensal streptococcus were measured. For co-cultural growth of *S. pneumoniae* with *S. mitis*, CFU counts remained stable compared to monocultures. This indicates that nutrient limitation is not a driving force in competition among these species.

There are clear limitations of our study: Competition occurs during initial biofilm formation in mixed species growth, but no information is available on which components play a dominant role. No conclusion about the importance of H_2O_2 or bacteriocins can be drawn. Additionally, no microscopic analysis are yet available to compare structures of mono- or

mixed species biofilms and to gain additional informations about how differences in the structural composition might influence initial biofilm formation.

In summary, our results show that nutrient limitation is a major factor influencing growth of *S. pneumoniae* in mix with *S. oralis*, which was identified to be a good biofilm builder. But interestingly, competition also occurs in initial biofilm growth in mixed cultures of pneumococcus with *S. pseudopneumoniae* or *S. mitis* where nutrient limitation is not a major factor. Competition for space also does not occur as *S. oralis* monocultures resulted in more biofilm biomass than all other species tested indicating that co-cultures did not succeed in filling in all the space provided by our model with biofilm biomass. Thus, despite the competition for space and nutrients, other factors as bacteriocins [4, 5], H_2O_2 [8] or differences in the structural composition might play a more important role in the development of co-cultured initial biofilms.

References

1. Kadioglu, A., et al., *The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease.* Nat Rev Microbiol, 2008. **6**(4): p. 288-301.
2. Kilian, M., et al., *Evolution of Streptococcus pneumoniae and its close commensal relatives.* PLoS One, 2008. **3**(7): p. e2683.
3. Hathaway, L.J., et al., *Capsule type of Streptococcus pneumoniae determines growth phenotype.* PLoS Pathog, 2012. **8**(3): p. e1002574.
4. Dawid, S., M.E. Sebert, and J.N. Weiser, *Bacteriocin activity of Streptococcus pneumoniae is controlled by the serine protease HtrA via posttranscriptional regulation.* J Bacteriol, 2009. **191**(5): p. 1509-18.
5. Kochan, T.J. and S. Dawid, *The HtrA protease of Streptococcus pneumoniae controls density-dependent stimulation of the bacteriocin blp locus via disruption of pheromone secretion.* J Bacteriol, 2013. **195**(7): p. 1561-72.
6. Selva, L., et al., *Killing niche competitors by remote-control bacteriophage induction.* Proc Natl Acad Sci U S A, 2009. **106**(4): p. 1234-8.
7. Lysenko, E.S., et al., *The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces.* PLoS Pathog, 2005. **1**(1): p. e1.
8. Regev-Yochay, G., et al., *In vitro bactericidal activity of Streptococcus pneumoniae and bactericidal susceptibility of Staphylococcus aureus strains isolated from cocolonized versus noncocolonized children.* J Clin Microbiol, 2008. **46**(2): p. 747-9.
9. Pericone, C.D., et al., *Inhibitory and bactericidal effects of hydrogen peroxide production by Streptococcus pneumoniae on other inhabitants of the upper respiratory tract.* Infect Immun, 2000. **68**(7): p. 3990-7.
10. Regev-Yochay, G., et al., *Association between carriage of Streptococcus pneumoniae and Staphylococcus aureus in Children.* JAMA, 2004. **292**(6): p. 716-20.
11. Lux, T., et al., *Diversity of bacteriocins and activity spectrum in Streptococcus pneumoniae.* J Bacteriol, 2007. **189**(21): p. 7741-51.

12. Margolis, E., *Hydrogen peroxide-mediated interference competition by Streptococcus pneumoniae has no significant effect on Staphylococcus aureus nasal colonization of neonatal rats.* J Bacteriol, 2009. **191**(2): p. 571-5.

13. Oggioni, M.R., et al., *Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis.* Mol Microbiol, 2006. **61**(5): p. 1196-210.

14. Speranza, B., M.R. Corbo, and M. Sinigaglia, *Effects of nutritional and environmental conditions on Salmonella sp. biofilm formation.* J Food Sci, 2011. **76**(1): p. M12-6.

15. Lizcano, A., et al., *Early biofilm formation on microtiter plates is not correlated with the invasive disease potential of Streptococcus pneumoniae.* Microb Pathog, 2010. **48**(3-4): p. 124-30.

16. Marks, L.R., G.I. Parameswaran, and A.P. Hakansson, *Pneumococcal interactions with epithelial cells are crucial for optimal biofilm formation and colonization in vitro and in vivo.* Infect Immun, 2012. **80**(8): p. 2744-60.

17. Sanclement, J.A., et al., *Bacterial biofilms in surgical specimens of patients with chronic rhinosinusitis.* Laryngoscope, 2005. **115**(4): p. 578-82.

18. Garcia-Castillo, M., et al., *Differences in biofilm development and antibiotic susceptibility among Streptococcus pneumoniae isolates from cystic fibrosis samples and blood cultures.* J Antimicrob Chemother, 2007. **59**(2): p. 301-4.

19. Aas, J.A., et al., *Defining the normal bacterial flora of the oral cavity.* J Clin Microbiol, 2005. **43**(11): p. 5721-32.

20. Kolenbrander, P.E., et al., *Oral multispecies biofilm development and the key role of cell-cell distance.* Nat Rev Microbiol, 2010. **8**(7): p. 471-80.

21. Kolenbrander, P.E., et al., *Bacterial interactions and successions during plaque development.* Periodontol 2000, 2006. **42**: p. 47-79.

22. Hojo, K., et al., *Bacterial interactions in dental biofilm development.* J Dent Res, 2009. **88**(11): p. 982-90.

23. Moscoso, M., E. Garcia, and R. Lopez, *Biofilm formation by Streptococcus pneumoniae: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion.* J Bacteriol, 2006. **188**(22): p. 7785-95.

24. van de Rijn, I. and R.E. Kessler, *Growth characteristics of group A streptococci in a new chemically defined medium.* Infect Immun, 1980. **27**(2): p. 444-8.

25. Whitchurch, C.B., et al., *Extracellular DNA required for bacterial biofilm formation.* Science, 2002. **295**(5559): p. 1487.

26. Carrolo, M., et al., *Prophage spontaneous activation promotes DNA release enhancing biofilm formation in Streptococcus pneumoniae.* PLoS One, 2010. **5**(12): p. e15678.

27. Loo, C.Y., D.A. Corliss, and N. Ganeshkumar, *Streptococcus gordonii biofilm formation: identification of genes that code for biofilm phenotypes.* J Bacteriol, 2000. **182**(5): p. 1374-82.

Figure legends

Figure 1 - Biofilm growth of commensal streptococci and pneumococcus in monoculture

CV staining of monocultural biofilms grown in A) CDM galactose or B) CDM glucose and incubated for 16h of *S. oralis*, *S. pseudopneumoniae*, *S. mitis* and *S. pneumoniae*. Three different pneumococcal serotypes were used: 19A, 6B and 19F.

CFU counts of 16h initial biofilms grown in CDM galactose C) or CDM glucose D) of *S. oralis*, *S. pseudopneumoniae* and *S. mitis*. For *S. pneumoniae*, serotypes 19A, 6B and 19F were used.

Statistical test: One-way ANOVA with Tukey's test to correct for multiple comparisons

**p<0.05*; ***p<0.01*. Error bars: SEM

Figure 2 – Comparison of mono- and mixed cultural biofilm biomass of commensal streptococci and *S. pneumoniae* by CV staining

CV staining of mono- and mixed cultural biofilm growth incubated for 16 h in A) CDM galactose and B) CDM glucose. Three different pneumococcal serotypes were used: 19A, 6B and 19F.

Statistical test: Student's t-test to compare monocultural with mixed cultural growth. **p<0.05*. Error bars: SEM.

Figure 3 – CFU counts of mono- and mixed cultural biofilms of commensal streptococci and *S. pneumoniae*

S. pneumoniae (serotypes 6B, 19A and 19F) CFU counts in biofilm monocultural growth and in mixed cultures with *S. pseudopneumoniae*, *S. mitis* and *S. oralis* in A) CDM galactose and B) CDM glucose. C) CFU counts of commensal streptococci in mono- and mixed cultural growth with *S. pneumoniae* in biofilm growth using CDM galactose and D) CDM glucose. Statistical test: Student's t-test to compare monocultural with mixed cultural growth. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Error bars: SEM

Figure 4 – CFU counts of mono- and mixed cultural planktonic growth of commensal streptococci and *S. pneumoniae*

S. pneumoniae (serotypes 19A and 19F) CFU counts in planktonic monocultural growth and in mixed cultures with *S. pseudopneumoniae*, *S. mitis* and *S. oralis* in A) CDM galactose and B) CDM glucose. C) CFU counts of commensal streptococci in mono- and mixed cultural growth with *S. pneumoniae* in biofilm growth using CDM galactose and D) CDM glucose. Statistical test: Student's t-test to compare monocultural with mixed cultural growth. *$p<0.05$. Error bars: SEM

Species	Strain	Sample location	Year of isolation	Country of isolation	Serotype	MLST	Pneumolysin	References	Remarks
S. pseudopneumoniae	411	nasopharynx	early 1990s	UK			yes	This study	Used for discrimination experiments (CFU counts in co-culturing)
S. pseudopneumoniae	412	nasopharynx	early 1990s	UK			yes	This study	
S. pseudopneumoniae	413	nasopharynx	early 1990s	UK			yes	This study	
S. mitis	414	blood	early 1990s	UK			yes	This study	Used for discrimination experiments (CFU counts in co-culturing)
S. mitis	415	blood	early 1990s	UK			no	This study	Used for discrimination experiments (CFU counts in co-culturing)
S. mitis	416	blood	early 1990s	UK			no	This study	
S. oralis	417	blood	early 1990s	UK			no	This study	Used for discrimination experiments (CFU counts in co-culturing)
S. oralis	418	blood	early 1990s	UK			no	This study	Used for discrimination experiments (CFU counts in co-culturing)
S. oralis	419	blood	early 1990s	UK			no	This study	Used for discrimination experiments (CFU counts in co-culturing)
S. pneumoniae	110.16	nasopharynx (otitis media)	1999	CH	19F		yes		tetracycline for discrimination
S. pneumoniae	Hungary 19A-6	lab strain		Internat. Reference	19A	268	yes		tetracycline for discrimination
S. pneumoniae	106.66	nasopharynx (otitis media)	1998	CH	6B	2244	Yes	[3]	tetracycline for discrimination

Table 1: Detailed information to strains used in this study.

number	Comment	erythromycin	clindamycin	oxacillin	levofloxacin	chloramphenicol	tetracycline	sulpha-trimethoprim	vancomycin	clindamycin Etest	penicillin MIC Etest
411	*S. pseudopneumoiae* UK	39	30	30	32	31	40.5	25.5	27	0.094	0.023
412	*S. pseudopneumoiae* UK	16	31	30	30	30	16.5	26	29	0.125	0.023
413	*S. pseudopneumoiae* UK	40	32	20	30	35	16	25	26	0.064	0.047
414	*S. mitis* UK	46	38	30	23	36.5	23	25.5	28	0.023	0.012
415	*S. mitis* UK	20	27	26.5	18	26	30	20	22.5	0.19	0.023
416	*S. mitis* UK	20	30	27	21	29	13	0	21	0.125	0.023
417	*S. oralis* UK	37	30	11.5	26	30	38.5	29	25	0.19	0.19
418	*S. oralis* UK	39	34	14	22	32	39	30	27.5	0.094	0.125
419	*S. oralis* UK	19	30	0	22	27.5	35	27	23	0.19	6
1.69	*S. pneumoiae* Hungary 19A-6	0	0	0	32	20.5	14	0	31	256+	1
110.16	*S. pneumoiae* (19F)	0	10	18	26	25	10	22.5	26.5	2	0.064

Table 2: Overview antibiotic resistances of commensal and pneumococcal strains used in this study. No color: susceptible. Yellow: intermediate. Red: resistant. N.B. No antibiogram was performed for strain 106.66 (serotype 6B), but only resistance to tetracycline was tested.

Figure 1

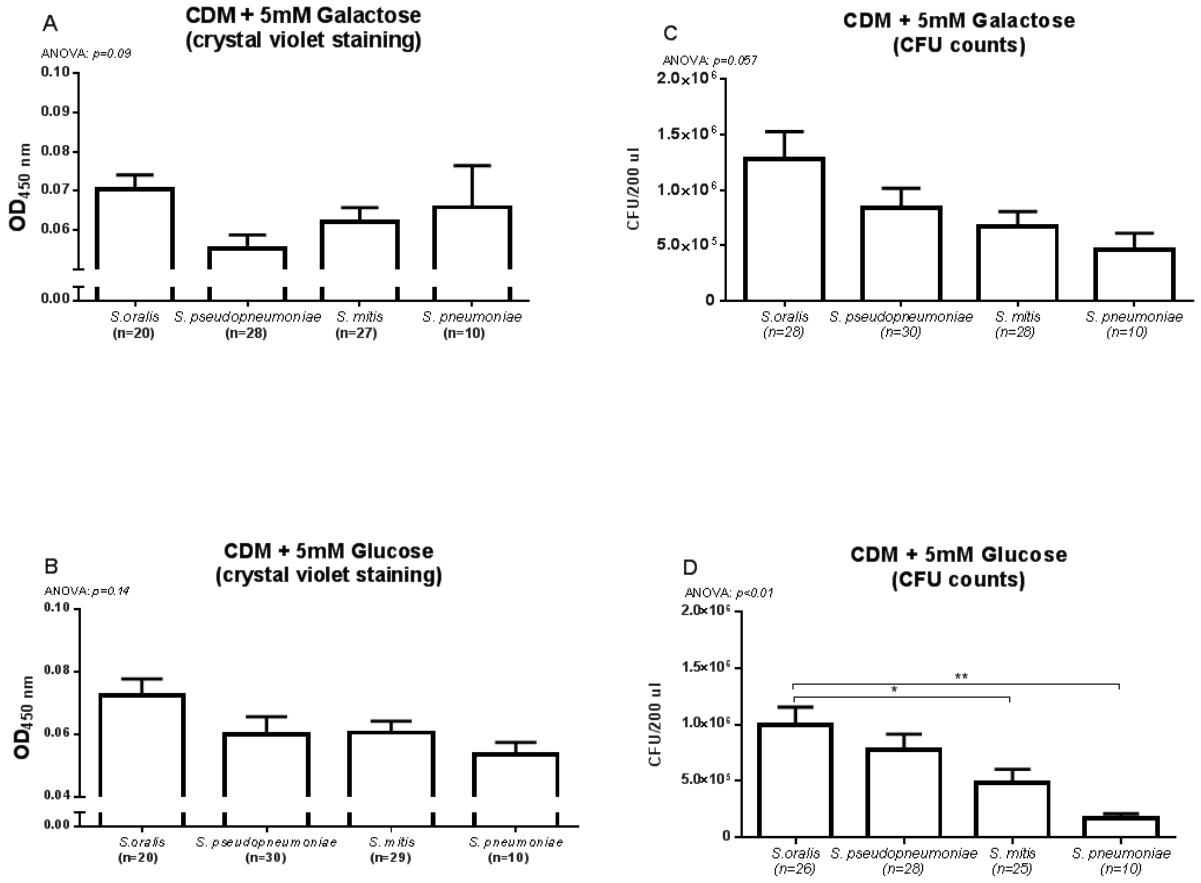

A
CDM + 5mM Galactose
(crystal violet staining)
ANOVA: p=0.09
OD450 nm
0.10
0.09
0.08
0.07
0.06
0.00
S.oralis (n=20)
S. pseudopneumoniae (n=28)
S. mitis (n=27)
S. pneumoniae (n=10)
C
CDM + 5mM Galactose
(CFU counts)
ANOVA: p=0.057
CFU/200 ul
2.0x10^6
1.5x10^6
1.0x10^6
5.0x10^5
0
S.oralis (n=28)
S. pseudopneumoniae (n=30)
S. mitis (n=28)
S. pneumoniae (n=10)
B
CDM + 5mM Glucose
(crystal violet staining)
ANOVA: p=0.14
OD450 nm
0.10
0.08
0.06
0.04
0.00
S.oralis (n=20)
S. pseudopneumoniae (n=30)
S. mitis (n=29)
S. pneumoniae (n=10)
D
CDM + 5mM Glucose
(CFU counts)
ANOVA: p<0.01
CFU/200 ul
2.0x10^6
1.5x10^6
1.0x10^6
5.0x10^5
0
**
*
S.oralis (n=26)
S. pseudopneumoniae (n=28)
S. mitis (n=25)
S. pneumoniae (n=10)

Figure 2

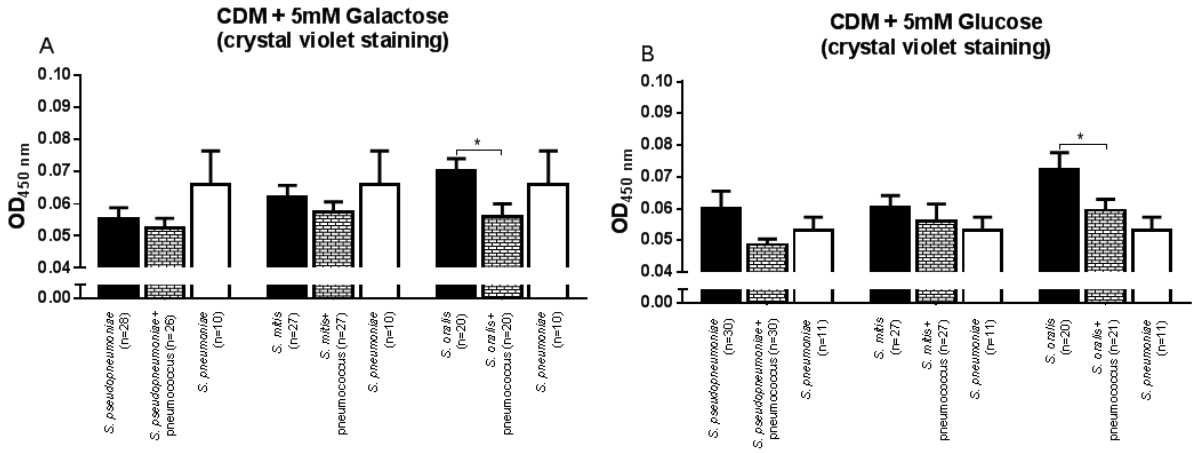

A
CDM + 5mM Galactose
(crystal violet staining)
OD450 nm
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.00
*
S. pseudopneumoniae (n=28)
S. pseudopneumoniae + pneumococcus (n=28)
S. pneumoniae (n=10)
S. mitis (n=27)
S. mitis+ pneumococcus (n=27)
S. pneumoniae (n=10)
S. oralis (n=20)
S. oralis+ pneumococcus (n=20)
S. pneumoniae (n=10)
B
CDM + 5mM Glucose
(crystal violet staining)
OD450 nm
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.00
*
S. pseudopneumoniae (n=30)
S. pseudopneumoniae + pneumococcus (n=30)
S. pneumoniae (n=11)
S. mitis (n=27)
S. mitis+ pneumococcus (n=27)
S. pneumoniae (n=11)
S. oralis (n=20)
S. oralis+ pneumococcus (n=21)
S. pneumoniae (n=11)

Figure 3

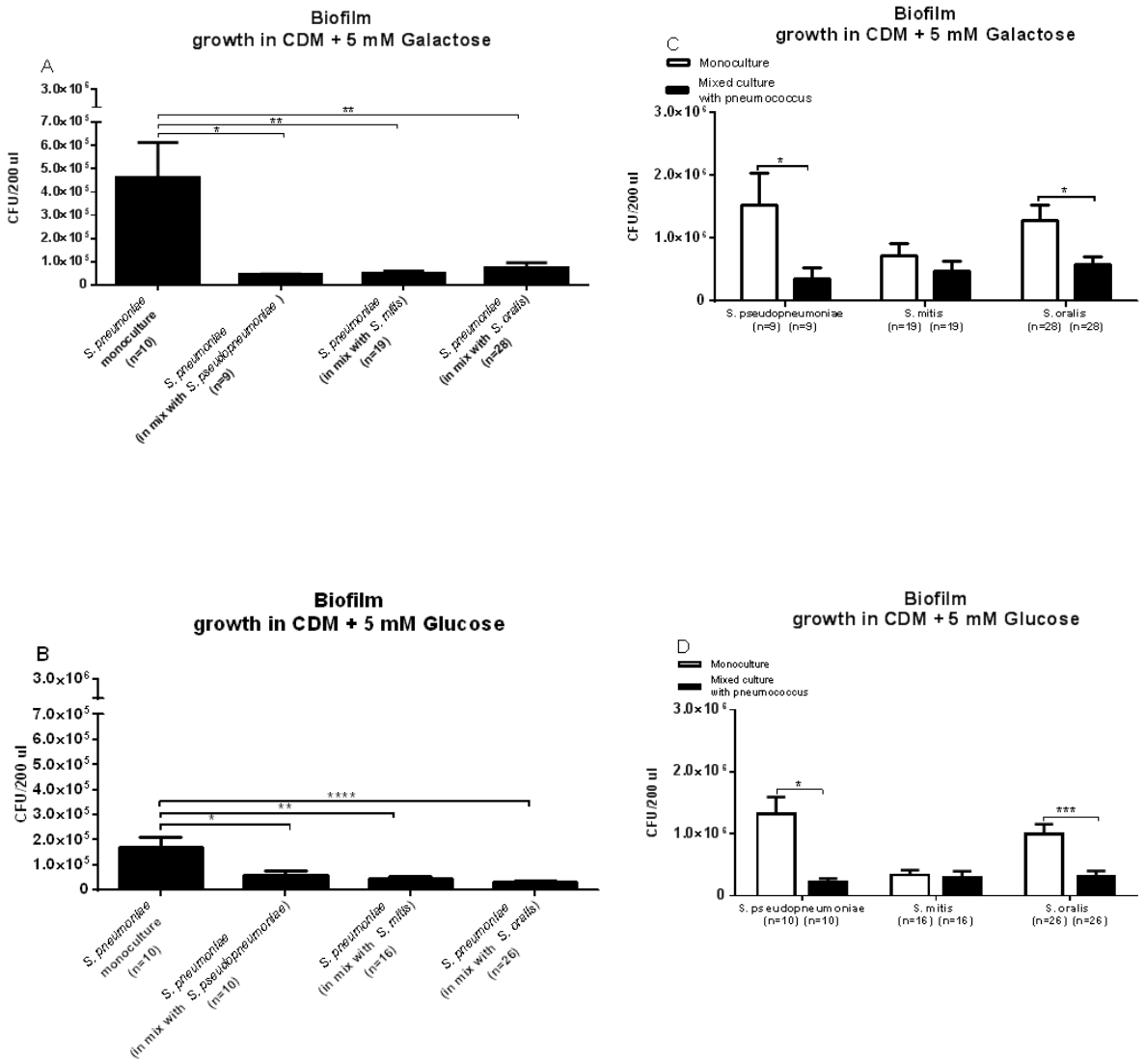
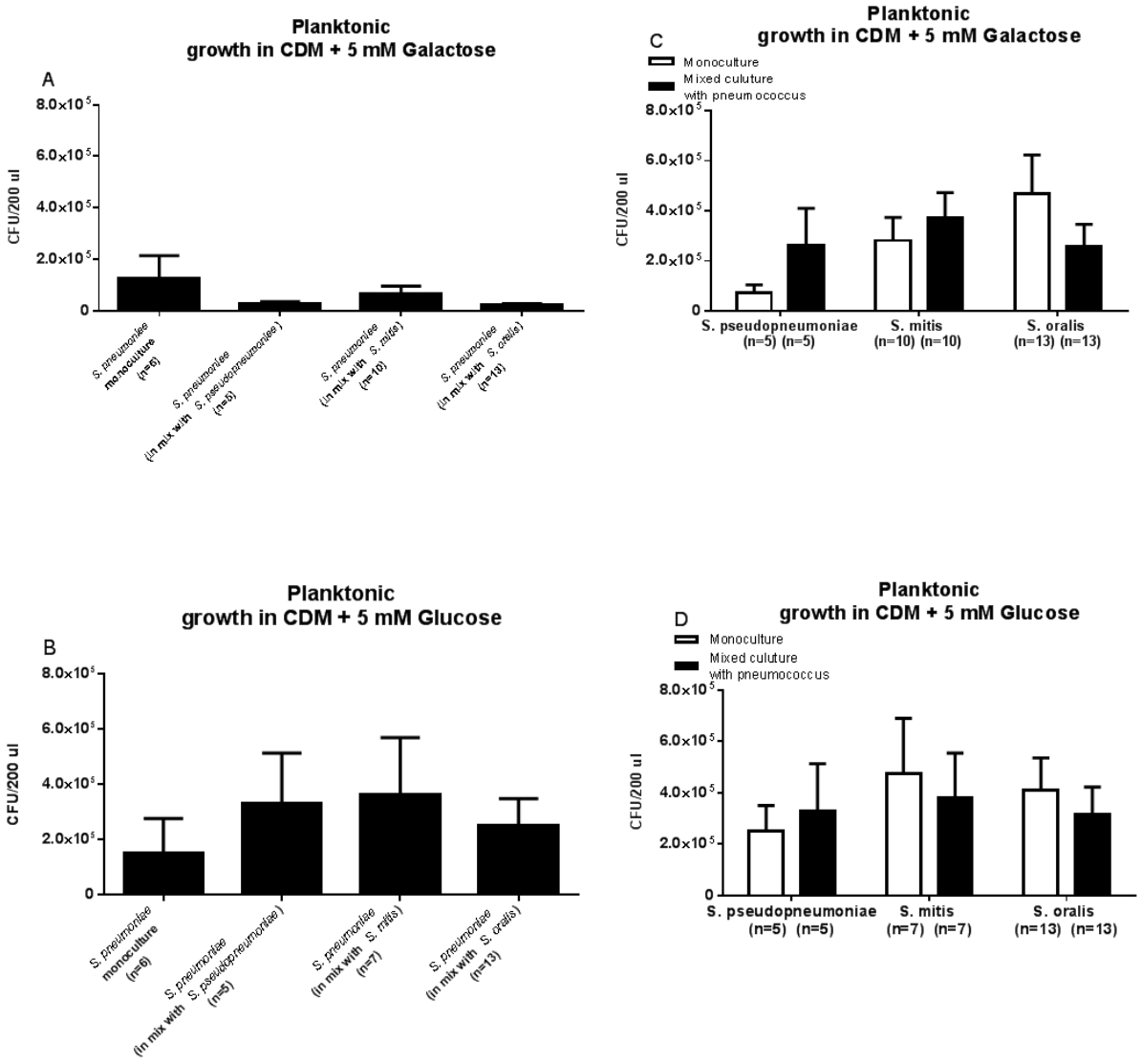

A
Biofilm
growth in CDM + 5 mM Galactose
CFU/200 ul
3.0x10^6
7.0x10^5
6.0x10^5
5.0x10^5
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
0
*
**
**
S. pneumoniae monoculture (n=10)
S. pneumoniae (in mix with S. pseudopneumoniae) (n=9)
S. pneumoniae (in mix with S. mitis) (n=19)
S. pneumoniae (in mix with S. oralis) (n=28)
C
Biofilm
growth in CDM + 5 mM Galactose
Monoculture
Mixed culture with pneumococcus
CFU/200 ul
3.0x10^6
2.0x10^6
1.0x10^6
0
*
*
S. pseudopneumoniae (n=9) (n=9)
S. mitis (n=19) (n=19)
S. oralis (n=28) (n=28)
B
Biofilm
growth in CDM + 5 mM Glucose
CFU/200 ul
3.0x10^6
7.0x10^5
6.0x10^5
5.0x10^5
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
0
****
**
*
S. pneumoniae monoculture (n=10)
S. pneumoniae (in mix with S. pseudopneumoniae) (n=10)
S. pneumoniae (in mix with S. mitis) (n=16)
S. pneumoniae (in mix with S. oralis) (n=26)
D
Biofilm
growth in CDM + 5 mM Glucose
Monoculture
Mixed culture with pneumococcus
CFU/200 ul
3.0x10^6
2.0x10^6
1.0x10^6
0
*
***
S. pseudopneumoniae (n=10) (n=10)
S. mitis (n=16) (n=16)
S. oralis (n=26) (n=26)

Figure 4


Planktonic
growth in CDM + 5 mM Galactose
A
CFU/200 ul
8.0x10^5
6.0x10^5
4.0x10^5
2.0x10^5
0
S. pneumoniae monoculture (n=6)
S. pneumoniae (in mix with S. pseudopneumoniae) (n=5)
S. pneumoniae (in mix with S. mitis) (n=10)
S. pneumoniae (in mix with S. oralis) (n=13)
Planktonic
growth in CDM + 5 mM Galactose
C
Monoculture
Mixed culuture with pneumococcus
CFU/200 ul
S. pseudopneumoniae (n=5) (n=5)
S. mitis (n=10) (n=10)
S. oralis (n=13) (n=13)
Planktonic
growth in CDM + 5 mM Glucose
B
CFU/200 ul
S. pneumoniae monoculture (n=6)
S. pneumoniae (in mix with S. pseudopneumoniae) (n=5)
S. pneumoniae (in mix with S. mitis) (n=7)
S. pneumoniae (in mix with S. oralis) (n=13)
Planktonic
growth in CDM + 5 mM Glucose
D
Monoculture
Mixed culuture with pneumococcus
CFU/200 ul
S. pseudopneumoniae (n=5) (n=5)
S. mitis (n=7) (n=7)
S. oralis (n=13) (n=13)

6. Discussion

The human nasopharynx, the major ecological reservoir of *S. pneumoniae,* is considered to be a nutrient-poor environment and in this environment pneumococcal biofilms have been isolated from mucosal epithelial cells [154, 180]. The niche is colonized by up to 700 different bacterial species competing for nutrients (e.g. carbohydrates) and space and influencing one another's growth by the secretion of bacteriocins or the induction of bacteriophages [173, 174, 180, 181]. Additionally, the upper airway epithelial cells are also the main target of RSV infection, which increases the adherence potential of *S. pneumoniae* resulting in more severe infections and modifies the host's immune response [182-185]. Pneumococcal colonization of the nasopharynx generally occurs asymptomatically and transiently, with recruitment of FoxP3+ regulatory T-cells and induction of IL-10 secretion resulting in a reduction of immunological reactivity [5, 108, 186-188]. However, *S. pneumoniae* also has a serotype-associated potential to cause invasive diseases such as pneumonia, sepsis or meningitis [189-191]. As soon as the lungs are invaded, a massive infiltration of neutrophils and monocytes/macrophages is measured. T-cells also play an important role in early pneumococcal immunity [105, 108, 192].

The polysaccharide capsule of *S. pneumoniae* plays an important role in colonization and growth behaviour, but also has major impacts on invasiveness of pneumococcal strains and on the host's immune responses [74, 180, 189, 190]. Capsule was the main focus of our studies. We first investigated the role of capsule and pneumolysin, the other main pneumococcal virulence factor [88], in CXCL8 and IL-6 induction *in vitro* and *in vivo* and studied the difference in lung infiltration between encapsulated and non-encapsulated isogenic pneumococcal strains as well as the resulting immune responses by measuring various cell types of innate immune response. Additionally, we were also interested in the impact of serotype on initial biofilm formation and structure in a static biofilm model *in vitro*, where a serotype 6B and a 7F strain were compared for the amount of biofilm formation as well as in its composition with a main focus on SCV formation. As competition between

pneumococci and commensal streptococci plays an important role in nasopharyngeal colonization, initial biofilm formation of monoculture and mixed cultures of *S. pneumoniae* with *S. oralis*, *S. mitis* and *S. pseudopneumoniae* was analyzed and the impact of nutrient limitation investigated by mixed culture planktonic growth. Lastly, the impact of RSV as mono-treatment or together with encapsulated and non-encapsulated *S. pneumoniae* or pneumolysin on CXCL8 and IL-6 secretion from bronchial epithelial cells was studied.

Effect of pneumococcal capsule and pneumolysin on CXCL8 and IL-6 secretion

The release of CXCL8 and IL-6 from the human pharyngeal cell line Detroit 562 was shown to be suppressed by the presence of capsule. This finding was true for *S. pneumoniae* strain D39 and its isogenic non-encapsulated mutant D39*cps*- as well as for the naturally non-encapsulated clinical strain 110.58 and its encapsulated isogenic mutant 110.58::D39*cps*. The human bronchial epithelial cell line iHBEC resulted in much less CXCL8 and IL-6 release, but the levels also were affected by the presence of a capsule. In nasopharyngeal carriage in a mouse model the increase in CXCL8 after capsule deletion was only observed when pneumolysin also was deleted indicating that the effect of capsule on suppression of CXCL8 release was hidden by pneumolysin which is known to have a major effect on CXCL8 induction [177].

Thus far, the effect of pneumococcal capsule on CXCL8 response has mainly been tested using laboratory strains (D39, TIGR4) [193, 194]. However, it would be interesting to investigate whether different serotypes result in different levels of CXCL8 secretion using isogenic capsule switch mutants which we already have available in our laboratory.

Nasopharyngeal carriage, lung infiltration and innate immune response

In a nasopharyngeal carriage mouse model, only non-encapsulated strains were able to infiltrate the lungs. Encapsulated bacteria are responsible for the initiation of innate immunity leading to the infiltration of neutrophils and macrophages to enter the nasopharynx resulting in efficient opsonophagocytosis of the bacteria due to the polysaccharide capsule. CXCL8 chemotactic activity is important for immune cell infiltration in the nasopharynx, and restriction of the initial step of CXCL8 release might increase bacterial survival and result in longer term survival in the nasopharynx without host mediated clearance. Nasopharyngeal carriage was also observed to be associated with the stimulation of FoxP3+ regulatory T-cells and an increase in IL-10 secretion involved in immune homeostatic processes [186, 188], resulting in a moderate immune response and a prolonged carriage state of pneumococci in the upper respiratory tract. But as soon as lung infiltration occurs, a strong inflammatory response is initiated clearing not only the bacteria from the lungs but also from the nasopharynx.

As in vivo studies of immune responses to pneumococcal strains are complex and influenced by a huge amount of different factors, responses to encapsulated and non-encapsulated strains could be studied on cell lines of macrophages or neutrophils *in vitro* to get a more detailed picture of the response generated by one cell type. Additionally, to get a deeper understanding of the differences in the infiltrating potential between different serotypes and to study differences in immune responses against encapsulated and nonencapsulated pneumococcal strains, clinical isolates which naturally cause infections and their encapsulated or nonencapsulated isogenic mutants should be tested *in vivo*. There are several clinical isolates and their isogenic mutants available in our lab.

RSV and pneumococcus: impact on CXCL8 and IL-6 secretion of bronchial epithelial cells

IL-6 secretion of human bronchial epithelial cells *in vitro* is increased for mixed treatment with RSV and pneumococcus compared to mono-stimulation with either pathogen when encapsulated bacteria are used. This trend was not observed for non-encapsulated pneumococci or free pneumolysin which did not have an effect on IL-6 secretion. The CXCL8 response also was increased for encapsulated bacteria in combination with RSV compared to non-encapsulated pneumococci, but additionally, also an effect of increasing pneumolysin concentrations was measured, which is in accordance with previous findings [177]. In summary, priming of epithelial cells with RSV opens the door for more severe pneumococcal infections, and damaging the cells with pneumolysin also favors secondary viral infections.

Recently it was shown that epithelial cell lines and primary epithelial cells from bronchial brushings from children differ strongly in their response to RSV infections [178, 179]. Thus, our cytokine responses should be confirmed using primary epithelial cells from healthy children. Additionally, primary epithelial cells from cystic fibrosis (CF) patients also will be tested. Evidence from epidemiological studies and from animal models suggests that prior or ongoing viral infections of the respiratory tract may predispose individuals to the development of invasive pneumococcal disease and this is particularly true in CF patients in whom bacterial and viral co-infections are particularly common. Thus, a comparison of bacterial-viral interactions between primary cells from healthy children and from CF patients would be of interest. On other human cell lines such as macrophages and neutrophils interactions between virus and bacteria also can be examined. Additionally, cytokine results also might be studied *in vivo* using an infection mouse model.

Influence of serotype on biofilm formation

Serotypes 6B as well as 7F both were able to form SCVs during biofilm growth in CDM supplemented with 5 mM glucose as well as in CDM supplemented with 5 mM galactose after 16h of incubation in a static *in vitro* biofilm model. The growth medium did not have an effect on SCV formation. We then also were interested in the development of biofilms and SCVs over time and analyzed growth after 64h of incubation. The biofilm composition was shown to be different between the serotypes: Serotype 6B resulted in a gradual increase in SCV formation and also in an increase of relative biofilm CFU counts between 16 and 64 h, whereas levels remained stable for serotype 7F. Our findings indicate an expression-regulated mechanism of SCV formation for serotype 6B allowing the adaptation to changing nutritional environments. Serotype 7F strains show a different mechanism which might be associated with the development of point mutations. As adaptation to a nutrient-poor environment and stable biofilm formation is important to get long-term access to the nasopharynx, this process also is interesting in terms of different colonizing and invading potential of different pneumococcal serotypes [158, 180, 195].

The changes in biofilm composition in terms of SCV and normal colonies over time might be of interest, and timepoints in between 4 and 65h could be analyzed. Biofilm formation on a biotic surface, e.g. on fixed pharyngeal epithelial cells, or the use of a continuous flow bioreactor would give additional information about biofilm development in an environment more closely related to nasopharyngeal conditions.

Planktonic and biofilm growth of mixed species cultures

The commensal streptococci *S. oralis*, *S. mitis* and *S. pseudopneumoniae* all were able to form initial biofilms in a static *in vitro* model using CDM supplemented with 5 mM glucose or 5 mM galactose as growth medium, with *S. oralis* resulting in an increased amount of biofilm CFU counts compared to *S. mitis*, *S. pseudopneumoniae* and *S.*

pneumoniae. In mixed species biofilms of *S. pneumoniae* with *S. oralis*, *S. mitis* or *S. pseudopneumoniae*, *S. pneumoniae* as well as the commensal streptococci all showed a reduction of CFU counts compared to mono-species biofilm growth. Nutrient limitation tested by mixed species planktonic growth was shown to only play a significant role for growth of *S. pneumoniae* with *S. oralis*, but not for growth with either of the other two commensal streptococci. Initial biofilm formation in mixed species cultures of *S. pneumoniae* with *S. mitis* or *S. pseudopneumoniae* also resulted in a decrease of CFU counts for both species indicating that bacteriocins [173, 174], the production of hydrogen peroxide [175] or other bactericidal factors might play a more significant role in biofilm formation where bacteria are closer and interactions might be stronger than in planktonic growth. Additionally, differences in the biofilm structure between commensal streptococci and pneumococcus might also result in competition for adherence space.

Thus far, only CFU counts of planktonically grown bacteria in mixed cultural growth after 15h of incubation were counted, but no information about the growth behaviour over time is available. The next step would be to perform growth curves measuring OD_{450} over 22h to investigate the impact of carbon source on mixed cultural planktonic growth over time and thus, to gain information about when the maximal OD_{450} is reached and to study the impact of pneumococcal autolysis.

For further experiments, the advantage of growth in CDM could be used: as all components of the medium are known and thus, differences in the composition after growing either pneumococci or commensal streptococcal species biofilms could be analyzed to determine differences in the amount of the different components metabolized. This might give a closer insight into metabolic differences between pneumococcus and commensal streptococci. Additionally, also the supernatant of mixed species biofilm cultures could be analyzed to study the secretion of bactericidal substances as bacteriocins or hydrogen peroxide. In a follow-up experiment to study competition in a model more closely related to

nasopharyngeal conditions where a constant flow of nutrients and other components is influencing the micro-ecosystem, a flow model as published previously [176] could be used.

7. References

1. Hahn H., K.S.H.E., Schultz Th.F., Suerbaum S., *Medizinische Mikrobiologie und Infektiologie (Eds.).* 2009. **XXIV**: p. 213-218.
2. Auld, A.G., *Remarks on the Morphology and Chemical Products of the Diplococcus Pneumoniae, and some Results of Vaccination.* Br Med J, 1897. **1**(1891): p. 775-7.
3. Im, H., et al., *The crystal structure of alanine racemase from Streptococcus pneumoniae, a target for structure-based drug design.* BMC Microbiol, 2011. **11**: p. 116.
4. Hava, D.L., J. LeMieux, and A. Camilli, *From nose to lung: the regulation behind Streptococcus pneumoniae virulence factors.* Mol Microbiol, 2003. **50**(4): p. 1103-10.
5. Lynch, J.P., 3rd and G.G. Zhanel, *Streptococcus pneumoniae: epidemiology, risk factors, and strategies for prevention.* Semin Respir Crit Care Med, 2009. **30**(2): p. 189-209.
6. Hardie, J.M. and R.A. Whiley, *Classification and overview of the genera Streptococcus and Enterococcus.* Soc Appl Bacteriol Symp Ser, 1997. **26**: p. 1S-11S.
7. Tettelin, H., et al., *Complete genome sequence of a virulent isolate of Streptococcus pneumoniae.* Science, 2001. **293**(5529): p. 498-506.
8. Skov Sorensen, U.B., et al., *Ultrastructural localization of capsules, cell wall polysaccharide, cell wall proteins, and F antigen in pneumococci.* Infect Immun, 1988. **56**(8): p. 1890-6.
9. Standish, A.J., et al., *Chemical inhibition of bacterial protein tyrosine phosphatase suppresses capsule production.* PLoS One, 2012. **7**(5): p. e36312.
10. Bentley, S.D., et al., *Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes.* PLoS Genet, 2006. **2**(3): p. e31.
11. Kadioglu, A., et al., *The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease.* Nat Rev Microbiol, 2008. **6**(4): p. 288-301.
12. Griffith, F., *The Significance of Pneumococcal Types.* J Hyg (Lond), 1928. **27**(2): p. 113-59.
13. Avery, O.T., C.M. Macleod, and M. McCarty, *Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types : Induction of Transformation by a Desoxyribonucleic Acid Fraction Isolated from Pneumococcus Type Iii.* J Exp Med, 1944. **79**(2): p. 137-58.
14. Havarstein, L.S., R. Hakenbeck, and P. Gaustad, *Natural competence in the genus Streptococcus: evidence that streptococci can change pherotype by interspecies recombinational exchanges.* J Bacteriol, 1997. **179**(21): p. 6589-94.
15. Lancefield, R.C., *A Serological Differentiation of Human and Other Groups of Hemolytic Streptococci.* J Exp Med, 1933. **57**(4): p. 571-95.
16. Kawamura, Y., et al., *Determination of 16S rRNA sequences of Streptococcus mitis and Streptococcus gordonii and phylogenetic relationships among members of the genus Streptococcus.* Int J Syst Bacteriol, 1995. **45**(2): p. 406-8.
17. Arbique, J.C., et al., *Accuracy of phenotypic and genotypic testing for identification of Streptococcus pneumoniae and description of Streptococcus pseudopneumoniae sp. nov.* J Clin Microbiol, 2004. **42**(10): p. 4686-96.
18. Clavel, T., C. Charrier, and D. Haller, *Streptococcus danieliae sp. nov., a novel bacterium isolated from the caecum of a mouse.* Arch Microbiol, 2013. **195**(1): p. 43-9.
19. Facklam, R., *What happened to the streptococci: overview of taxonomic and nomenclature changes.* Clin Microbiol Rev, 2002. **15**(4): p. 613-30.
20. Henriques-Normark, B., et al., *The rise and fall of bacterial clones: Streptococcus pneumoniae.* Nat Rev Microbiol, 2008. **6**(11): p. 827-37.
21. Lee, A.W., H. Tettelin, and S. Chancey, *Genomic Analyses of Clonal Isolates Provide Clues to the Evolution of Streptococcus pneumoniae.* Front Microbiol, 2011. **2**: p. 63.
22. Martin, B., et al., *Independent evolution of competence regulatory cascades in streptococci?* Trends Microbiol, 2006. **14**(8): p. 339-45.

23. Hakenbeck, R., *Transformation in Streptococcus pneumoniae: mosaic genes and the regulation of competence.* Res Microbiol, 2000. **151**(6): p. 453-6.
24. Johnsborg, O. and L.S. Havarstein, *Regulation of natural genetic transformation and acquisition of transforming DNA in Streptococcus pneumoniae.* FEMS Microbiol Rev, 2009. **33**(3): p. 627-42.
25. Kilian, M., et al., *Evolution of Streptococcus pneumoniae and its close commensal relatives.* PLoS One, 2008. **3**(7): p. e2683.
26. Salyers A.a., W.D.D., *Bacterial pathogenesis: A molecular approach*. 2nd ed. 2002, ASM Press Washington.
27. Coates, H., et al., *The role of chronic infection in children with otitis media with effusion: evidence for intracellular persistence of bacteria.* Otolaryngol Head Neck Surg, 2008. **138**(6): p. 778-81.
28. Dagan, R. and K.L. O'Brien, *Modeling the association between pneumococcal carriage and child-care center attendance.* Clin Infect Dis, 2005. **40**(9): p. 1223-6.
29. Millar, E.V., et al., *Indirect effect of 7-valent pneumococcal conjugate vaccine on pneumococcal colonization among unvaccinated household members.* Clin Infect Dis, 2008. **47**(8): p. 989-96.
30. Hammitt, L.L., et al., *Indirect effect of conjugate vaccine on adult carriage of Streptococcus pneumoniae: an explanation of trends in invasive pneumococcal disease.* J Infect Dis, 2006. **193**(11): p. 1487-94.
31. Jourdain, S., et al., *Differences in nasopharyngeal bacterial carriage in preschool children from different socio-economic origins.* Clin Microbiol Infect, 2011. **17**(6): p. 907-14.
32. Pereiro, I., et al., *Risk factors for invasive disease among children in Spain.* J Infect, 2004. **48**(4): p. 320-9.
33. Picard, C., et al., *Primary immunodeficiencies associated with pneumococcal disease.* Curr Opin Allergy Clin Immunol, 2003. **3**(6): p. 451-9.
34. Halasa, N.B., et al., *Incidence of invasive pneumococcal disease among individuals with sickle cell disease before and after the introduction of the pneumococcal conjugate vaccine.* Clin Infect Dis, 2007. **44**(11): p. 1428-33.
35. Castagnola, E. and F. Fioredda, *Prevention of life-threatening infections due to encapsulated bacteria in children with hyposplenia or asplenia: a brief review of current recommendations for practical purposes.* Eur J Haematol, 2003. **71**(5): p. 319-26.
36. Meisel, R., et al., *Increased risk for invasive pneumococcal diseases in children with acute lymphoblastic leukaemia.* Br J Haematol, 2007. **137**(5): p. 457-60.
37. Alanee, S.R., et al., *Association of serotypes of Streptococcus pneumoniae with disease severity and outcome in adults: an international study.* Clin Infect Dis, 2007. **45**(1): p. 46-51.
38. Kalin, M., et al., *Prospective study of prognostic factors in community-acquired bacteremic pneumococcal disease in 5 countries.* J Infect Dis, 2000. **182**(3): p. 840-7.
39. Watanakunakorn, C., et al., *Pneumococcal bacteremia in three community teaching hospitals from 1980 to 1989.* Chest, 1993. **103**(4): p. 1152-6.
40. Farinas-Alvarez, C., et al., *Prognostic factors for pneumococcal bacteremia in a university hospital.* Eur J Clin Microbiol Infect Dis, 2000. **19**(10): p. 733-41.
41. Whitney, C.G. and P.J. Nuorti. *http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5911a1.htm*. 2010 07/11/2013].
42. Williams, C. and R. Masterton, *Pneumococcal immunisation in the 21st century.* J Infect, 2008. **56**(1): p. 13-9.
43. *Pneumokokken fact sheet*. 2013, Bundesamt für Gesundheit (BAG), Eidgenössische Kommission für Impffragen: Bern.
44. Brueggemann, A.B., et al., *Vaccine escape recombinants emerge after pneumococcal vaccination in the United States.* PLoS Pathog, 2007. **3**(11): p. e168.

45. Weintraub, A., *Immunology of bacterial polysaccharide antigens.* Carbohydr Res, 2003. **338**(23): p. 2539-47.
46. Miyaji, E.N., et al., *Serotype-independent pneumococcal vaccines.* Cell Mol Life Sci, 2013. **70**(18): p. 3303-26.
47. Hilty, M., et al., *Nasopharyngeal microbiota in infants with acute otitis media.* J Infect Dis, 2012. **205**(7): p. 1048-55.
48. Tagg, J.R. and K.P. Dierksen, *Bacterial replacement therapy: adapting 'germ warfare' to infection prevention.* Trends Biotechnol, 2003. **21**(5): p. 217-23.
49. Margolis, E., A. Yates, and B.R. Levin, *The ecology of nasal colonization of Streptococcus pneumoniae, Haemophilus influenzae and Staphylococcus aureus: the role of competition and interactions with host's immune response.* BMC Microbiol, 2010. **10**: p. 59.
50. Shelburne, S.A., 3rd, et al., *A direct link between carbohydrate utilization and virulence in the major human pathogen group A Streptococcus.* Proc Natl Acad Sci U S A, 2008. **105**(5): p. 1698-703.
51. Hendriksen, W.T., et al., *CodY of Streptococcus pneumoniae: link between nutritional gene regulation and colonization.* J Bacteriol, 2008. **190**(2): p. 590-601.
52. Bernhardt, J., et al., *Bacillus subtilis during feast and famine: visualization of the overall regulation of protein synthesis during glucose starvation by proteome analysis.* Genome Res, 2003. **13**(2): p. 224-37.
53. Dawid, S., A.M. Roche, and J.N. Weiser, *The blp bacteriocins of Streptococcus pneumoniae mediate intraspecies competition both in vitro and in vivo.* Infect Immun, 2007. **75**(1): p. 443-51.
54. Mackowiak, P.A., *The normal microbial flora.* N Engl J Med, 1982. **307**(2): p. 83-93.
55. Moscoso, M., E. Garcia, and R. Lopez, *Biofilm formation by Streptococcus pneumoniae: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion.* J Bacteriol, 2006. **188**(22): p. 7785-95.
56. Tonnaer, E.L., et al., *Advances in understanding the pathogenesis of pneumococcal otitis media.* Pediatr Infect Dis J, 2006. **25**(6): p. 546-52.
57. Exley, R.M., et al., *Neisseria meningitidis lactate permease is required for nasopharyngeal colonization.* Infect Immun, 2005. **73**(9): p. 5762-6.
58. Trappetti, C., et al., *Sialic acid: a preventable signal for pneumococcal biofilm formation, colonization, and invasion of the host.* J Infect Dis, 2009. **199**(10): p. 1497-505.
59. Roos, K., E.G. Hakansson, and S. Holm, *Effect of recolonisation with "interfering" alpha streptococci on recurrences of acute and secretory otitis media in children: randomised placebo controlled trial.* BMJ, 2001. **322**(7280): p. 210-2.
60. Murphy, T.F., L.O. Bakaletz, and P.R. Smeesters, *Microbial interactions in the respiratory tract.* Pediatr Infect Dis J, 2009. **28**(10 Suppl): p. S121-6.
61. Laufer, A.S., et al., *Microbial communities of the upper respiratory tract and otitis media in children.* MBio, 2011. **2**(1): p. e00245-10.
62. Charlson, E.S., et al., *Disordered microbial communities in the upper respiratory tract of cigarette smokers.* PLoS One, 2010. **5**(12): p. e15216.
63. Hammerschmidt, S., *Adherence molecules of pathogenic pneumococci.* Curr Opin Microbiol, 2006. **9**(1): p. 12-20.
64. Zhang, J.R., et al., *The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells.* Cell, 2000. **102**(6): p. 827-37.
65. Ogunniyi, A.D., P. Giammarinaro, and J.C. Paton, *The genes encoding virulence-associated proteins and the capsule of Streptococcus pneumoniae are upregulated and differentially expressed in vivo.* Microbiology, 2002. **148**(Pt 7): p. 2045-53.
66. Hammerschmidt, S., et al., *Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells.* Infect Immun, 2005. **73**(8): p. 4653-67.

67. Rajam, G., et al., *A functional epitope of the pneumococcal surface adhesin A activates nasopharyngeal cells and increases bacterial internalization.* Microb Pathog, 2008. **44**(3): p. 186-96.
68. Bogaert, D., R. De Groot, and P.W. Hermans, *Streptococcus pneumoniae colonisation: the key to pneumococcal disease.* Lancet Infect Dis, 2004. **4**(3): p. 144-54.
69. Ramos-Sevillano, E., et al., *Nasopharyngeal colonization and invasive disease are enhanced by the cell wall hydrolases LytB and LytC of Streptococcus pneumoniae.* PLoS One, 2011. **6**(8): p. e23626.
70. Wartha, F., et al., *Capsule and D-alanylated lipoteichoic acids protect Streptococcus pneumoniae against neutrophil extracellular traps.* Cell Microbiol, 2007. **9**(5): p. 1162-71.
71. Mitchell, T.J., *The pathogenesis of streptococcal infections: from tooth decay to meningitis.* Nat Rev Microbiol, 2003. **1**(3): p. 219-30.
72. Levitz, R., et al., *Induction of IL-6 and CCL5 (RANTES) in human respiratory epithelial (A549) cells by clinical isolates of respiratory syncytial virus is strain specific.* Virol J, 2012. **9**: p. 190.
73. Abeyta, M., G.G. Hardy, and J. Yother, *Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of Streptococcus pneumoniae.* Infect Immun, 2003. **71**(1): p. 218-25.
74. Weiser, J.N., *The pneumococcus: why a commensal misbehaves.* J Mol Med (Berl), 2010. **88**(2): p. 97-102.
75. Crum, N.F., et al., *An outbreak of conjunctivitis due to a novel unencapsulated Streptococcus pneumoniae among military trainees.* Clin Infect Dis, 2004. **39**(8): p. 1148-54.
76. Ertugrul, N., et al., *BOX-polymerase chain reaction-based DNA analysis of nonserotypeable Streptococcus pneumoniae implicated in outbreaks of conjunctivitis.* J Infect Dis, 1997. **176**(5): p. 1401-5.
77. Sorensen, U.B., et al., *Covalent linkage between the capsular polysaccharide and the cell wall peptidoglycan of Streptococcus pneumoniae revealed by immunochemical methods.* Microb Pathog, 1990. **8**(5): p. 325-34.
78. Kamerling, J., *Pneumococcal Polysaccharides: A Chemical View. Streptococcus pneumoniae. Larchmont: Mary Ann Liebert. pp. 81-114*. 2000.
79. Nelson, A.L., et al., *Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance.* Infect Immun, 2007. **75**(1): p. 83-90.
80. Hyams, C., et al., *The Streptococcus pneumoniae capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms.* Infect Immun, 2010. **78**(2): p. 704-15.
81. Magee, A.D. and J. Yother, *Requirement for capsule in colonization by Streptococcus pneumoniae.* Infect Immun, 2001. **69**(6): p. 3755-61.
82. Hathaway, L.J., P. Battig, and K. Muhlemann, *In vitro expression of the first capsule gene of Streptococcus pneumoniae, cpsA, is associated with serotype-specific colonization prevalence and invasiveness.* Microbiology, 2007. **153**(Pt 8): p. 2465-71.
83. Morona, J.K., et al., *Tyrosine phosphorylation of CpsD negatively regulates capsular polysaccharide biosynthesis in streptococcus pneumoniae.* Mol Microbiol, 2000. **35**(6): p. 1431-42.
84. Yother, J., *Capsules of Streptococcus pneumoniae and Other Bacteria: Paradigms for Polysaccharide Biosynthesis and Regulation.* Annu Rev Microbiol, 2010.
85. Aanensen, D.M., et al., *Predicted functions and linkage specificities of the products of the Streptococcus pneumoniae capsular biosynthetic loci.* J Bacteriol, 2007. **189**(21): p. 7856-76.
86. Weiser, J.N., et al., *Changes in availability of oxygen accentuate differences in capsular polysaccharide expression by phenotypic variants and clinical isolates of Streptococcus pneumoniae.* Infect Immun, 2001. **69**(9): p. 5430-9.
87. Morona, J.K., et al., *The effect that mutations in the conserved capsular polysaccharide biosynthesis genes cpsA, cpsB, and cpsD have on virulence of Streptococcus pneumoniae.* J Infect Dis, 2004. **189**(10): p. 1905-13.

88. Marriott, H.M., T.J. Mitchell, and D.H. Dockrell, *Pneumolysin: a double-edged sword during the host-pathogen interaction.* Curr Mol Med, 2008. **8**(6): p. 497-509.
89. Mitchell, A.M. and T.J. Mitchell, *Streptococcus pneumoniae: virulence factors and variation.* Clin Microbiol Infect, 2010. **16**(5): p. 411-8.
90. Rubins, J.B. and E.N. Janoff, *Pneumolysin: a multifunctional pneumococcal virulence factor.* J Lab Clin Med, 1998. **131**(1): p. 21-7.
91. Hirst, R.A., et al., *The role of pneumolysin in pneumococcal pneumonia and meningitis.* Clin Exp Immunol, 2004. **138**(2): p. 195-201.
92. Kadioglu, A., et al., *Upper and lower respiratory tract infection by Streptococcus pneumoniae is affected by pneumolysin deficiency and differences in capsule type.* Infect Immun, 2002. **70**(6): p. 2886-90.
93. Berry, A.M., et al., *Reduced virulence of a defined pneumolysin-negative mutant of Streptococcus pneumoniae.* Infect Immun, 1989. **57**(7): p. 2037-42.
94. Abbas A, L., AH and Pillai, S, *Cellular and Molecular Immunology.* 2007. **6th Edition ed. Elsevier, Philadelphia**.
95. Kadioglu, A. and P.W. Andrew, *The innate immune response to pneumococcal lung infection: the untold story.* Trends Immunol, 2004. **25**(3): p. 143-9.
96. Obaro, S. and R. Adegbola, *The pneumococcus: carriage, disease and conjugate vaccines.* J Med Microbiol, 2002. **51**(2): p. 98-104.
97. Simell, B., et al., *The fundamental link between pneumococcal carriage and disease.* Expert Rev Vaccines, 2012. **11**(7): p. 841-55.
98. Yoshimura, A., et al., *Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2.* J Immunol, 1999. **163**(1): p. 1-5.
99. Koppe, U., N. Suttorp, and B. Opitz, *Recognition of Streptococcus pneumoniae by the innate immune system.* Cell Microbiol, 2012. **14**(4): p. 460-6.
100. Malley, R., et al., *Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection.* Proc Natl Acad Sci U S A, 2003. **100**(4): p. 1966-71.
101. Dockrell, D.H., M.K. Whyte, and T.J. Mitchell, *Pneumococcal pneumonia: mechanisms of infection and resolution.* Chest, 2012. **142**(2): p. 482-91.
102. Paterson, G.K. and T.J. Mitchell, *Innate immunity and the pneumococcus.* Microbiology, 2006. **152**(Pt 2): p. 285-93.
103. Calbo, E. and J. Garau, *Of mice and men: innate immunity in pneumococcal pneumonia.* Int J Antimicrob Agents, 2010. **35**(2): p. 107-13.
104. Underhill, D.M. and A. Ozinsky, *Phagocytosis of microbes: complexity in action.* Annu Rev Immunol, 2002. **20**: p. 825-52.
105. Haslett, C., *Granulocyte apoptosis and its role in the resolution and control of lung inflammation.* Am J Respir Crit Care Med, 1999. **160**(5 Pt 2): p. S5-11.
106. Kolling, U.K., et al., *Leucocyte response and anti-inflammatory cytokines in community acquired pneumonia.* Thorax, 2001. **56**(2): p. 121-5.
107. Biffl, W.L., et al., *Interleukin-6 delays neutrophil apoptosis via a mechanism involving platelet-activating factor.* J Trauma, 1996. **40**(4): p. 575-8; discussion 578-9.
108. Neill, D.R., et al., *T regulatory cells control susceptibility to invasive pneumococcal pneumonia in mice.* PLoS Pathog, 2012. **8**(4): p. e1002660.
109. Shen, S., et al., *Treg cell numbers and function in patients with antibiotic-refractory or antibiotic-responsive Lyme arthritis.* Arthritis Rheum, 2010. **62**(7): p. 2127-37.
110. Bogdan, C., Y. Vodovotz, and C. Nathan, *Macrophage deactivation by interleukin 10.* J Exp Med, 1991. **174**(6): p. 1549-55.
111. Dallaire, F., et al., *Microbiological and inflammatory factors associated with the development of pneumococcal pneumonia.* J Infect Dis, 2001. **184**(3): p. 292-300.

112. Hament, J.M., et al., *Respiratory viral infection predisposing for bacterial disease: a concise review.* FEMS Immunol Med Microbiol, 1999. **26**(3-4): p. 189-95.
113. Schwarzmann, S.W., et al., *Bacterial pneumonia during the Hong Kong influenza epidemic of 1968-1969.* Arch Intern Med, 1971. **127**(6): p. 1037-41.
114. Avadhanula, V., et al., *Respiratory viruses augment the adhesion of bacterial pathogens to respiratory epithelium in a viral species- and cell type-dependent manner.* J Virol, 2006. **80**(4): p. 1629-36.
115. van der Flier, M., et al., *Adherence of Streptococcus pneumoniae to immobilized fibronectin.* Infect Immun, 1995. **63**(11): p. 4317-22.
116. Tan, T.T., et al., *The respiratory pathogen Moraxella catarrhalis adheres to epithelial cells by interacting with fibronectin through ubiquitous surface proteins A1 and A2.* J Infect Dis, 2005. **192**(6): p. 1029-38.
117. Jiang, Z., et al., *Fimbria-mediated enhanced attachment of nontypeable Haemophilus influenzae to respiratory syncytial virus-infected respiratory epithelial cells.* Infect Immun, 1999. **67**(1): p. 187-92.
118. Bosch, A.A., et al., *Viral and bacterial interactions in the upper respiratory tract.* PLoS Pathog, 2013. **9**(1): p. e1003057.
119. Kane, M., et al., *Successful transmission of a retrovirus depends on the commensal microbiota.* Science, 2011. **334**(6053): p. 245-9.
120. Dudas, R.A. and R.A. Karron, *Respiratory syncytial virus vaccines.* Clin Microbiol Rev, 1998. **11**(3): p. 430-9.
121. Gonzalez, P.A., et al., *Understanding respiratory syncytial virus infection to improve treatment and immunity.* Curr Mol Med, 2012.
122. Hall, C.B., et al., *The burden of respiratory syncytial virus infection in young children.* N Engl J Med, 2009. **360**(6): p. 588-98.
123. WHO. http://www.who.int/vaccine_research/diseases/ari/en/index2.html. 03/10/2013].
124. Villenave, R., M.D. Shields, and U.F. Power, *Respiratory syncytial virus interaction with human airway epithelium.* Trends Microbiol, 2013. **21**(5): p. 238-44.
125. Raza, M.W., et al., *Effect of respiratory syncytial virus infection on binding of Neisseria meningitidis and Haemophilus influenzae type b to a human epithelial cell line (HEp-2).* Epidemiol Infect, 1993. **110**(2): p. 339-47.
126. Saadi, A.T., et al., *Factors enhancing adherence of toxigenic Staphylococcus aureus to epithelial cells and their possible role in sudden infant death syndrome.* Epidemiol Infect, 1993. **110**(3): p. 507-17.
127. Franke-Ullmann, G., et al., *Alteration of pulmonary macrophage function by respiratory syncytial virus infection in vitro.* J Immunol, 1995. **154**(1): p. 268-80.
128. Raza, M.W., et al., *Bactericidal activity of a monocytic cell line (THP-1) against common respiratory tract bacterial pathogens is depressed after infection with respiratory syncytial virus.* J Med Microbiol, 2000. **49**(3): p. 227-33.
129. Avadhanula, V., et al., *Nontypeable Haemophilus influenzae and Streptococcus pneumoniae bind respiratory syncytial virus glycoprotein.* J Med Microbiol, 2007. **56**(Pt 9): p. 1133-7.
130. Vlastarakos, P.V., et al., *Biofilms in ear, nose, and throat infections: how important are they?* Laryngoscope, 2007. **117**(4): p. 668-73.
131. Hall-Stoodley, L. and P. Stoodley, *Evolving concepts in biofilm infections.* Cell Microbiol, 2009. **11**(7): p. 1034-43.
132. Moscoso, M., E. Garcia, and R. Lopez, *Pneumococcal biofilms.* Int Microbiol, 2009. **12**(2): p. 77-85.
133. Kievit, T., *Biofilms*. 2011, University of Manitoba: Winnipeg, MB, Canada. p.547-558.
134. Stewart, P.S. and M.J. Franklin, *Physiological heterogeneity in biofilms.* Nat Rev Microbiol, 2008. **6**(3): p. 199-210.

135. Speranza, B., M.R. Corbo, and M. Sinigaglia, *Effects of nutritional and environmental conditions on Salmonella sp. biofilm formation.* J Food Sci, 2011. **76**(1): p. M12-6.
136. Webb, J.S., M. Givskov, and S. Kjelleberg, *Bacterial biofilms: prokaryotic adventures in multicellularity.* Curr Opin Microbiol, 2003. **6**(6): p. 578-85.
137. Costerton, J.W., *Introduction to biofilm.* Int J Antimicrob Agents, 1999. **11**(3-4): p. 217-21; discussion 237-9.
138. Costerton, J.W., P.S. Stewart, and E.P. Greenberg, *Bacterial biofilms: a common cause of persistent infections.* Science, 1999. **284**(5418): p. 1318-22.
139. Zhang, K., et al., *Effects of quorum sensing on cell viability in Streptococcus mutans biofilm formation.* Biochem Biophys Res Commun, 2009. **379**(4): p. 933-8.
140. Sanchez, C.J., et al., *Streptococcus pneumoniae in Biofilms Are Unable to Cause Invasive Disease Due to Altered Virulence Determinant Production.* PLoS One, 2011. **6**(12): p. e28738.
141. Beckford-Ball, J. *http://woundsinternational.files.wordpress.com/2011/02/schematic-representation-of-polymicrobial-biofilm-formation.jpg*. 18.02.2011 16.11.2013].
142. Xiao, J. and H. Koo, *Structural organization and dynamics of exopolysaccharide matrix and microcolonies formation by Streptococcus mutans in biofilms.* J Appl Microbiol, 2010. **108**(6): p. 2103-13.
143. Whitchurch, C.B., et al., *Extracellular DNA required for bacterial biofilm formation.* Science, 2002. **295**(5559): p. 1487.
144. Hall-Stoodley, L. and P. Stoodley, *Developmental regulation of microbial biofilms.* Curr Opin Biotechnol, 2002. **13**(3): p. 228-33.
145. Steinberger, R.E. and P.A. Holden, *Extracellular DNA in single- and multiple-species unsaturated biofilms.* Appl Environ Microbiol, 2005. **71**(9): p. 5404-10.
146. Vilain, S., et al., *DNA as an adhesin: Bacillus cereus requires extracellular DNA to form biofilms.* Appl Environ Microbiol, 2009. **75**(9): p. 2861-8.
147. Mulcahy, H., L. Charron-Mazenod, and S. Lewenza, *Extracellular DNA chelates cations and induces antibiotic resistance in Pseudomonas aeruginosa biofilms.* PLoS Pathog, 2008. **4**(11): p. e1000213.
148. Lewenza, S., *Extracellular DNA-induced antimicrobial peptide resistance mechanisms in Pseudomonas aeruginosa.* Front Microbiol, 2013. **4**: p. 21.
149. Allesen-Holm, M., et al., *A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms.* Mol Microbiol, 2006. **59**(4): p. 1114-28.
150. Shak, S., et al., *Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum.* Proc Natl Acad Sci U S A, 1990. **87**(23): p. 9188-92.
151. Carrolo, M., et al., *Prophage spontaneous activation promotes DNA release enhancing biofilm formation in Streptococcus pneumoniae.* PLoS One, 2010. **5**(12): p. e15678.
152. Oggioni, M.R., et al., *Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis.* Mol Microbiol, 2006. **61**(5): p. 1196-210.
153. Sanclement, J.A., et al., *Bacterial biofilms in surgical specimens of patients with chronic rhinosinusitis.* Laryngoscope, 2005. **115**(4): p. 578-82.
154. Marks, L.R., G.I. Parameswaran, and A.P. Hakansson, *Pneumococcal interactions with epithelial cells are crucial for optimal biofilm formation and colonization in vitro and in vivo.* Infect Immun, 2012. **80**(8): p. 2744-60.
155. Shak, J.R., et al., *Novel role for the Streptococcus pneumoniae toxin pneumolysin in the assembly of biofilms.* MBio, 2013. **4**(5): p. e00655-13.
156. Tapiainen, T., et al., *Biofilm formation by Streptococcus pneumoniae isolates from paediatric patients.* APMIS, 2010. **118**(4): p. 255-60.
157. Hall-Stoodley, L., et al., *Characterization of biofilm matrix, degradation by DNase treatment and evidence of capsule downregulation in Streptococcus pneumoniae clinical isolates.* BMC Microbiol, 2008. **8**: p. 173.

158. del Prado, G., et al., *Biofilm formation by Streptococcus pneumoniae strains and effects of human serum albumin, ibuprofen, N-acetyl-l-cysteine, amoxicillin, erythromycin, and levofloxacin.* Diagn Microbiol Infect Dis, 2010. **67**(4): p. 311-8.
159. Allegrucci, M. and K. Sauer, *Characterization of colony morphology variants isolated from Streptococcus pneumoniae biofilms.* J Bacteriol, 2007. **189**(5): p. 2030-8.
160. Waite, R.D., et al., *Spontaneous sequence duplications within capsule genes cap8E and tts control phase variation in Streptococcus pneumoniae serotypes 8 and 37.* Microbiology, 2003. **149**(Pt 2): p. 497-504.
161. Allegrucci, M. and K. Sauer, *Formation of Streptococcus pneumoniae non-phase-variable colony variants is due to increased mutation frequency present under biofilm growth conditions.* J Bacteriol, 2008. **190**(19): p. 6330-9.
162. Crowley, R.C., et al., *Differential protein expression in Streptococcus uberis under planktonic and biofilm growth conditions.* Appl Environ Microbiol, 2011. **77**(1): p. 382-4.
163. Wen, Z.T., H.V. Baker, and R.A. Burne, *Influence of BrpA on critical virulence attributes of Streptococcus mutans.* J Bacteriol, 2006. **188**(8): p. 2983-92.
164. Inagaki, S., et al., *Effects of recombinase A deficiency on biofilm formation by Streptococcus mutans.* Oral Microbiol Immunol, 2009. **24**(2): p. 104-8.
165. Li, Y.H., et al., *A quorum-sensing signaling system essential for genetic competence in Streptococcus mutans is involved in biofilm formation.* J Bacteriol, 2002. **184**(10): p. 2699-708.
166. Qin, L., et al., *Impaired capsular polysaccharide is relevant to enhanced biofilm formation and lower virulence in Streptococcus pneumoniae.* J Infect Chemother, 2013. **19**(2): p. 261-71.
167. Mercier, C., et al., *Positive role of peptidoglycan breaks in lactococcal biofilm formation.* Mol Microbiol, 2002. **46**(1): p. 235-43.
168. Blanchette-Cain, K., et al., *Streptococcus pneumoniae Biofilm Formation Is Strain Dependent, Multifactorial, and Associated with Reduced Invasiveness and Immunoreactivity during Colonization.* MBio, 2013. **4**(5).
169. Vidal, J.E., et al., *Quorum-sensing systems LuxS/autoinducer 2 and Com regulate Streptococcus pneumoniae biofilms in a bioreactor with living cultures of human respiratory cells.* Infect Immun, 2013. **81**(4): p. 1341-53.
170. Sanderson, A.R., J.G. Leid, and D. Hunsaker, *Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis.* Laryngoscope, 2006. **116**(7): p. 1121-6.
171. Drago, L., et al., *Biofilm formation by bacteria isolated from upper respiratory tract before and after adenotonsillectomy.* APMIS, 2012. **120**(5): p. 410-6.
172. Chole, R.A. and B.T. Faddis, *Anatomical evidence of microbial biofilms in tonsillar tissues: a possible mechanism to explain chronicity.* Arch Otolaryngol Head Neck Surg, 2003. **129**(6): p. 634-6.
173. Dawid, S., M.E. Sebert, and J.N. Weiser, *Bacteriocin activity of Streptococcus pneumoniae is controlled by the serine protease HtrA via posttranscriptional regulation.* J Bacteriol, 2009. **191**(5): p. 1509-18.
174. Kochan, T.J. and S. Dawid, *The HtrA protease of Streptococcus pneumoniae controls density-dependent stimulation of the bacteriocin blp locus via disruption of pheromone secretion.* J Bacteriol, 2013. **195**(7): p. 1561-72.
175. Regev-Yochay, G., et al., *In vitro bactericidal activity of Streptococcus pneumoniae and bactericidal susceptibility of Staphylococcus aureus strains isolated from cocolonized versus noncocolonized children.* J Clin Microbiol, 2008. **46**(2): p. 747-9.
176. Narisawa, N., et al., *Competence-dependent endogenous DNA rearrangement and uptake of extracellular DNA give a natural variant of Streptococcus mutans without biofilm formation.* J Bacteriol, 2011. **193**(19): p. 5147-54.

177. Dogan, S., et al., *Pneumolysin-induced CXCL8 production by nasopharyngeal epithelial cells is dependent on calcium flux and MAPK activation via Toll-like receptor 4.* Microbes Infect, 2011. **13**(1): p. 65-75.
178. Fonceca, A.M., et al., *Primary airway epithelial cultures from children are highly permissive to respiratory syncytial virus infection.* Thorax, 2012. **67**(1): p. 42-8.
179. McNamara, P.S., et al., *Respiratory syncytial virus infection of airway epithelial cells, in vivo and in vitro, supports pulmonary antibody responses by inducing expression of the B cell differentiation factor BAFF.* Thorax, 2013. **68**(1): p. 76-81.
180. Hathaway, L.J., et al., *Capsule type of Streptococcus pneumoniae determines growth phenotype.* PLoS Pathog, 2012. **8**(3): p. e1002574.
181. Selva, L., et al., *Killing niche competitors by remote-control bacteriophage induction.* Proc Natl Acad Sci U S A, 2009. **106**(4): p. 1234-8.
182. Hishiki, H., et al., *Incidence of bacterial coinfection with respiratory syncytial virus bronchopulmonary infection in pediatric inpatients.* J Infect Chemother, 2011. **17**(1): p. 87-90.
183. Techasaensiri, B., et al., *Viral coinfections in children with invasive pneumococcal disease.* Pediatr Infect Dis J, 2010. **29**(6): p. 519-23.
184. Hament, J.M., et al., *Enhanced adherence of Streptococcus pneumoniae to human epithelial cells infected with respiratory syncytial virus.* Pediatr Res, 2004. **55**(6): p. 972-8.
185. Yokota, S., et al., *Clarithromycin suppresses human respiratory syncytial virus infection-induced Streptococcus pneumoniae adhesion and cytokine production in a pulmonary epithelial cell line.* Mediators Inflamm, 2012. **2012**: p. 528568.
186. Verhagen, L.M., et al., *Bacterial respiratory pathogens in children with inherited immune and airway disorders: nasopharyngeal carriage and disease risk.* Pediatr Infect Dis J, 2013. **32**(4): p. 399-404.
187. Obert, C., et al., *Identification of a Candidate Streptococcus pneumoniae core genome and regions of diversity correlated with invasive pneumococcal disease.* Infect Immun, 2006. **74**(8): p. 4766-77.
188. Zhang, Q., et al., *Characterisation of regulatory T cells in nasal associated lymphoid tissue in children: relationships with pneumococcal colonization.* PLoS Pathog, 2011. **7**(8): p. e1002175.
189. Brueggemann, A.B., et al., *Temporal and geographic stability of the serogroup-specific invasive disease potential of Streptococcus pneumoniae in children.* J Infect Dis, 2004. **190**(7): p. 1203-11.
190. Kronenberg, A., et al., *Distribution and invasiveness of Streptococcus pneumoniae serotypes in Switzerland, a country with low antibiotic selection pressure, from 2001 to 2004.* J Clin Microbiol, 2006. **44**(6): p. 2032-8.
191. Weinberger, D.M., et al., *Pneumococcal capsular polysaccharide structure predicts serotype prevalence.* PLoS Pathog, 2009. **5**(6): p. e1000476.
192. Kadioglu, A., et al., *Host cellular immune response to pneumococcal lung infection in mice.* Infect Immun, 2000. **68**(2): p. 492-501.
193. Bootsma, H.J., M. Egmont-Petersen, and P.W. Hermans, *Analysis of the in vitro transcriptional response of human pharyngeal epithelial cells to adherent Streptococcus pneumoniae: evidence for a distinct response to encapsulated strains.* Infect Immun, 2007. **75**(11): p. 5489-99.
194. Marriott, H.M., et al., *Interleukin-1beta regulates CXCL8 release and influences disease outcome in response to Streptococcus pneumoniae, defining intercellular cooperation between pulmonary epithelial cells and macrophages.* Infect Immun, 2012. **80**(3): p. 1140-9.
195. Trappetti, C., et al., *Site of isolation determines biofilm formation and virulence phenotypes of Streptococcus pneumoniae serotype 3 clinical isolates.* Infect Immun, 2013. **81**(2): p. 505-13.

196. Lacks, S., *Integration efficiency and genetic recombination in pneumococcal transformation.* Genetics, 1966. **53**(1): p. 207-35.
197. Adams, M.H. and A.S. Roe, *A Partially Defined Medium for Cultivation of Pneumococcus.* J Bacteriol, 1945. **49**(4): p. 401-9.
198. van de Rijn, I. and R.E. Kessler, *Growth characteristics of group A streptococci in a new chemically defined medium.* Infect Immun, 1980. **27**(2): p. 444-8.

8. Acknowledgements

First, I would like to thank Prof. Kathrin Mühlemann for giving me the opportunity to join her lab and for all her support. Her death on the 1st of November was a terrible loss, scientifically, but also personally.

Special thanks go to Dr. Markus Hilty and Dr. Lucy J. Hathaway for all their great support after Kathrin's death. I appreciate very much all their help, their contributions of time and all the fruitful discussions about my projects. They were always available and willing to help, to correct and improve reports and also to give guidance.

I am deeply grateful to Prof. Dr. Aras Kadioglu from the University of Liverpool for giving me the opportunity to join his lab for 6 months and to learn immunological *in vivo* and *in vitro* techniques. Special thanks also go to Dr. Daniel Neill, Laura Bricio Moreno, Dr. Marie Yang and Suzanna Gore for their support and advice for all *in vivo* experiments.

Many thanks go to Dr. Angela Fonceca for introducing me into the RSV *in vitro* project, to Dr. William Coward for the collaboration on the CXCL8 project and to Daniel Wüthrich for all his help on whole genome sequencing.

I thank my lab mates from Bern, Moana Mika, Hansjürg Engel, Thierry Schaffner, Corinne Ruppen and Salome Seiffert, and to Reham Yahya, Hesham Malak, Mathieu Bangert, Laura Jacques and Stavros Panagiotou from Liverpool for the technical and moral support they gave me all the time. Many thanks also to Suzanne Aebi, Marianne Küffer and Rolf Troller for their great technical assistance.

I am grateful to Prof. Dr. Andrew Hemphill, my mentor and to my co-referee Prof. Vincent Perreten for their support during my Ph.D.

Many thanks also go to my family, especially to my parents and my sisters, Gabriela, Adaja and Sulamith, for all their support and patience.

And finally, I would like to thank Joel for all his encouragement, support, patience and love.

9. Curriculum vitae

Contact information

Last name	Küng
First name	Eliane
Date of birth	30.03.1988
Home address	Weid 82A, 3622 Homberg b. Thun (CH)
	Email: kueelia@gmail.com
Working address	Institute for Infectious Diseases
	Friedbühlstrasse 51, 3010 Bern (CH)
	Email: eliane.kueng@ifik.unibe.ch

Education

2010-2013	PhD in Microbiology / Infectiology, University of Bern, Switzerland, Supervisors: Prof. Dr. Dr. K. Mühlemann (deceased 01/11/2012), Dr. M. Hilty, Dr. L. J. Hathaway, Institute for Infectious Diseases
2009-2010	Master in Molecular life sciences (special qualification: Microbiology/ Immunology) University of Bern Supervisor: Prof. Dr. J. Frey, Institute of Veterinary Bacteriology,
2006-2009	Bachelor in Cell biology, University of Bern

Attendance at meetings

May 2013	Poster presentation on “Pneumococcal biofilm formation: Influence of growth medium and serotype” at the “11th European Meeting on the Molecular Biology of the Pneumococcus” in Madrid, Spain
September 2012	Attendance at the “The Northwest Microbiology Group Annual Meeting” in Liverpool, United Kingdom
June 2011	Attendance at the “10th European Meeting on the Molecular Biology of the Pneumococcus” in Amsterdam, The Netherlands
March 2010	Poster presentation on “Identification and characterization of a novel RTX-like leukotoxin in *Avibacterium paragallinarum*” at the Swiss Molecular Microbiology Meeting in Thun, Switzerland

Publications

Küng, E and J. Frey (2013). “**AvxA, a composite serine-protease-RTX toxin of Avibacterium paragallinarum**.” Vet Microbiol 163(3-4): 290-298.

Eliane Küng, William R. Coward, Daniel R. Neill, Hesham Malak, Kathrin Mühlemann, Aras Kadioglu, Markus Hilty and Lucy J. Hathaway (2013). **The pneumococcal polysaccharide capsule and pneumolysin differentially affect CXCL8 and IL-6 release from cells of the respiratory trac**t (submitted)

Patents

Frey, Joachim and Küng, Eliane (2013) ***Avibacterium paragallinarum*** **RTX toxin**. WIPO/OMPI international patent publication number 2013/034687 A1, filed March 2013. Patent pending

Languages

German	mother tongue
English	fluent, spoken and written
French	Diplôme d'études en langue française (DELF) A1 to A6
Spanish	2 years of courses at Gymnasium, basic knowledge

10. Appendix

Chemically defined medium (CDM, modified from [198])

Substance	Provider	Product No.
Iron (II) sulfate, heptahydrate	Merck	3965
Iron (III) nitrate, nonahydrate	Merck	3883
Potassium phosphate dibasic, anhydrous	Fluka	17835
Potassium phosphate monobasic, anhydrous	Merck	4873
Magnesiumsulfate, anhydrous	Sigma	M7506
Manganous sulfate, monohydrate(min.98%)	Merck	5941
L-Alanine (200x)	Sigma	A7627
L-Arginine (200x)	Sigma	A5006
L-Aspartic acid (200x)	Sigma	A9256
L-Phenylalanine (200x) warm up to dissolve	Merck	7256
L-Proline 200x)	Sigma	P0380
L-Serine (200x)	Sigma	S4500
L-Glutamic acid, monosodium salt, monohydrate	Sigma	G2834
L-Glutamine (200x)	Merck	289
Glycine (200x)	Merck	4201
L-Threonine (200x)	Merck	8411
L-Trypotophan (200x)	Sigma	8941
L-Tyrosine (200x)	Merck	8371
L-Valine (200x)	Merck	8495
L-Histidine monohydrochloride, monohydrate	Sigma	H8125
L-Isoleucine (200x)	Merck	5362
L-Leucine (200x)	Sigma	L8912
L-Lysine monochloride (200x)	Sigma	L5626
L-Methionine (200x)	Merck	5707
Folic acid (min. 98%)	Sigma	F8758
Biotin (min. 99%)	Sigma	B4501
Niacinamide	Sigma	N3376
D-Calcium pantothenate	Sigma	P2250
Para-aminobenzoic acid	Sigma	A9878
Pyridoxal hydrochloride	Sigma	P9130
Pyridoxine	Sigma	P9755
Pyridoxamine, dihydrochloride	Sigma	P9380
Thiamine hydrochloride	Sigma	T4625
Cyanocobalamin	Sigma	V2876
(-)-Riboflavine	Sigma	R4500
Guanine hydrochloride	Sigma	51030
Diadenine sulfate salt, dihydrate	Sigma	A9126
Uracil	Sigma	U0750
Calcium chloride, anhydrous	Merck	2083
Sodium acetate, anhydrous	Merck	6268
Sodium phosphate dibasic, anhydrous	Merck	6586
Sodium phosphate monobasic, monohydrate	Merck	6346
Choline chloride	Sigma	C7527
Sodium bicarbonate (min. 99%)	Merck	6329
Cystein	Sigma	C7477
β-NAD	Sigma	N1511
Copper (II)sulfate	Sigma	C7631
Manganese sulfate	Merck	5941

Table A1: List of chemicals required for CDM production

Substance	Concentration	for 1 l		Remarks	Aliquots / storage
Iron (II) sulfate, heptahydrate	10mg/ml RWT1	0.5	ml	always prepare freshly	
Iron (III) nitrate, nonahydrate	10mg/ml RWT1	0.1	ml	always prepare freshly	
Potassium phosphate dibasic, anhydrous		0.2	g	always weigh in freshly (in SB)	
Potassium phosphate monobasic, anhydrous		1	g	always weigh in freshly (in SB)	
Magnesiumsulfate, anhydrous	11.966 g/70 ml RWT1	2	ml	dissolve in ice bath	sterile filtered, 12 ml aliq./-15°C - -25°C
Manganous sulfate, monohydrate(min.98%)	200 mg/40 ml RWT1	1	ml		sterile filtered, 6 ml aliq./-15°C - -25°C
L-Alanine (200x)	2 g/100 ml RWT1	5	ml		autoclaved, 2°C - 8 °C
L-Arginine (200x)	2 g/100 ml RWT1	5	ml		autoclaved, 2°C - 8 °C
L-Aspartic acid (200x)	2 g/100 ml 0.2M HCl	5	ml		sterile filtered, 2°C - 8 °C
L-Phenylalanine (200x)	2g/100 ml 0.01M HCL	5	ml	warm up to dissolve	autoclaved, 15°C - 30°C
L-Proline 200x)	2 g/100 ml RWT1	5	ml		autoclaved, 2°C - 8 °C
L-Serine (200x)	2g/100 ml RWT1	5	ml		autoclaved, 2°C - 8 °C
L-Glutamic acid, monosodium salt, monohydrate	2,544g/100 ml RWT1	5	ml		sterile filtered, 2°C - 8 °C
L-Glutamine (200x)	4g/100 ml 1 M HCl	5	ml		sterile filtered, 2°C - 8 °C
Glycine (200x)	2g/100 ml RWT1	5	ml		autoclaved, 2°C - 8 °C
L-Threonine (200x)	4g/100 ml RWT1	5	ml		autoclaved, 2°C - 8 °C
L-Trypotophan (200x)	2g/100 ml 0.1 M NaOH	5	ml	protect from light (aluminium foil)	sterile filtered, 2°C - 8 °C
L-Tyrosine (200x)	2,884g/100 ml 1 M NaOH	5	ml	protect from light (aluminium foil)	sterile filtered, 2°C - 8 °C
L-Valine (200x)	2g/100 ml RWT1	5	ml		autoclaved, 2°C - 8 °C
L-Histidine monohydrochloride, monohydrate	2,7g/100 ml RWT1	5	ml		autoclaved, 2°C - 8 °C
L-Isoleucine (200x)	2g/100 ml RWT1	5	ml	warm up to dissolve	autoclaved, 2°C - 8 °C
L-Leucine (200x)	2g/100 ml RWT1	5	ml	warm up to dissolve	autoclaved, 15°C - 30°C
L-Lysine monochloride (200x)	2.498 g/100 ml RWT1	5	ml		autoclaved, 2°C - 8 °C
L-Methionine (200x)	2g/100 ml RWT1	5	ml		autoclaved, 2°C - 8 °C
Folic acid (min. 98%)	30 mg/6 ml 1 M NaOH	0.16	ml	protect from light (aluminium foil)	sterile filtered, 1ml aliq./-15°C - -25°C
Biotin (min. 99%)	10 mg/100 ml RWT1	2	ml	warm up to dissolve	sterile filtered, 12 ml aliq./-15°C --25°C
Niacinamide	40 mg/ 8 ml RWT1	0.2	ml		sterile filtered, 1.2 ml aliq./-15°C --25°C
D-Calcium pantothenate	80 mg/8ml RWT1	0.2	ml		sterile filtered, 1.2 ml aliq./-15°C --25°C
Para-aminobenzoic acid	8 mg/8 ml RWT1	0.2	ml		sterile filtered, 1.2 ml aliq./-15°C --25°C
Pyridoxal hydrochloride	40mg/8 ml RWT1	0.2	ml	protect from light (aluminium foil)	sterile filtered, 1.2 ml aliq./-15°C --25°C
Pyridoxine	40mg/8 ml RWT1	0.2	ml	protect from light (aluminium foil)	sterile filtered, 1.2 ml aliq./-15°C --25°C
Pyridoxamine, dihydrochloride	40mg/8 ml RWT1	0.2	ml	protect from light (aluminium foil)	sterile filtered, 1.2 ml aliq./-15°C --25°C
Thiamine hydrochloride	40mg/8 ml RWT1	0.2	ml	protect from light (aluminium foil)	sterile filtered, 1.2 ml aliq./-15°C --25°C
Cyanocobalamin	5 mg/10 ml RWT1	0.2	ml	protect from light (aluminium foil)	sterile filtered 1.2 ml aliq./-15°C --25°C
(-)-Riboflavin	50 mg /+ 06ML	20	ml		

Table A2: Preparation of CDM. RWT1: ultra-pure distilled water. SB: Sorenson phosphate buffer (see Table A3)

Substance	Concentration	for 1 l		Remarks	Aliquots / storage
	glacial acetic acid +400 ml RWT1			protect from light (aluminium foil)	sterile filtered, 120 ml flask/-15- -25°C
	dissolve, ad 500 ml mit RWT1			warm up to dissolve	
Guanine hydrochloride	0.4 g/100 ml 1 M HCl	5	ml		sterile filtered, 15°C - 30°C
Diadenine sulfate salt, dihydrate	0.4 g/100 ml 1 M HCl	5	ml		sterile filtered, 15°C - 30°C
Uracil	0.4 g/200 ml RWT1	10	ml	warm up to dissolve	autoclaved, 15° - 30°C
Calcium chloride, anhydrous	202.4mg/40ml RWT1	1	ml		sterile filtered, Rö.à 6 ml/-15°C --25°C
Sodium acetate, anhydrous		2.7126	g	dissolve in 100 ml SB	
Sodium phosphate dibasic, anhydrous		7.35	g	dissolve in 200 ml SB	
Sodium phosphate monobasic, monohydrate		3.195	g	dissolve in 100 ml SB	
Choline chloride		1	g	dissolve in 100 ml SB	
Sodium bicarbonate (min. 99%)		2.5	g	dissolve in 200 ml SB	
measure pH and adjust to 6.85					
Volume up to		1000	ml	in SB	
filter sterilization and storage at 4°C					
Use within three weeks					
The following substances					
have to be added by the user separately:					
Cystein	0.75g/10ml RWT1	10	ml	added by user	sterile filtered, 2°C - 8 °C
β-NAD	10mg/ml RWT1	0.25	ml	added by user	sterile filtered
Copper (II)sulfate	10mg/10ml RWT1	0.6	ml	added by user	sterile filtered, 0.5 ml aliq./-15°C --25°C
Manganese sulfate	125 mg/5 ml	1	ml	added by user	sterile filtered, 0.8 ml aliq./-15°C --25°C

Table A2 (continued): Preparation of CDM. RWT1: ultra-pure distilled water. SB: Sorenson phosphate buffer (see Table A3)

Sorenson phosphate buffer (pH 7) for 1 liter CDM:

Solution A:	
Potassium hydrogen phosphate (Merck 4873)	3.632 g in 400 ml distilled H_2O
Solution B:	
Sodium phosphate dehydrate (Merck 6580)	8.316g in 700 ml distilled H_2O
Sørensen buffer (pH 7):	
Solution A: 392 ml Solution B: 608 ml	

Table A3: Preparation of Sorenson phosphate buffer

Lacks culture medium [196, 197]

Component	Amount/litre
casein hydrolysat (difco vitaminfree casamino-acids)	5g
tryptophan	6 mg
cystine	35 mg
sodium acetate	2 g
K_2HPO_4	8.5 g
$MgCl_2$ x6 H_2O	0.5 g
$CaCl_2$	2.5 mg
$MnSO_4x4H_2O$	25 µg
$FeSO_4x7H_2O$	0.5 µg
$CuSO_4x5H_2O$	0.5 µg
$ZnSO_4x7H_2O$	0.5 µg
biotin	0.2 µg
nicotine acid	0.2 mg
pyridoxin HCl	0.2 mg
thiamin HCl	0.2 mg
riboflavin	0.1 mg
calcium-Pantothenat	0.6 mg
glucose	2 g
BSA 4 %	12 ml
catalase	3000 units
fresh yeast extract	30 ml

Table A4: Composition of Lacks culture medium

Declaration of Originality

Last name, first name: Küng Eliane

Matriculation number: 06-114-870

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date

Bern, 16/12/2013

Signature