Tampere University

ANNI SARALAHTI

A Zebrafish Model for Host-pathogen Interactions in Streptococcal Infections

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ACADEMIC DISSERTATION
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ABSTRACT

Two streptococcal species, *Streptococcus pneumoniae* and *Streptococcus agalactiae*, are among the leading causes of pneumonia, sepsis, and meningitis in young children. *S. pneumoniae* infections are estimated to be responsible for over a million child deaths each year, while *S. agalactiae* is a noteworthy cause of invasive neonatal diseases, preterm labor, and stillbirth. Both species have developed sophisticated mechanisms either to persist in their host as long-term colonizers or to overcome the barriers set by the human immune system and cause serious infections. Both pathogens also show high genetic variability and are rapidly evolving to escape current treatment and prevention methods.

To be able to fight against these pathogens, the host and the bacterial factors contributing to the outcome of the infection need to be thoroughly elucidated, and for this purpose, a proper animal model is an essential experimental tool. Although the traditionally used mammalian models have been informative in the study of the pathogenesis of *S. pneumoniae* and *S. agalactiae*, ethical issues and high costs limit their use in large-scale experiments. Therefore, I examined the potential of using another vertebrate, the zebrafish (*Danio rerio*), in the study of the host-pathogen interactions in *S. pneumoniae* and *S. agalactiae* infections. Zebrafish are small, and they can be easily handled and kept in large numbers. Also, they provide versatile opportunities for genetic manipulation. Importantly, the zebrafish immune system, with both the innate and adaptive arms, is highly similar to the human immune system, which makes zebrafish an attractive model for the study of infectious diseases.

In this thesis, both *S. pneumoniae* and *S. agalactiae* were shown to be able to cause an infection in zebrafish, with the pathology resembling human sepsis and meningitis. Additionally, the virulence of *S. pneumoniae* and *S. agalactiae* lacking common virulence factors was attenuated in zebrafish, emphasizing that these infections are promoted by the same pathogenic factors as in human infections. The zebrafish embryo model was also used for the closer analysis of the innate immune response to a *S. pneumoniae* infection in a whole-genome level transcriptome analysis and in a genetic screen. These studies revealed that, at the level of gene expression, the early host responses to *S. pneumoniae* in zebrafish are strikingly similar to human responses and include the activation of genes coding for pro-inflammatory

cytokines, chemokines, acute phase proteins, and antimicrobial peptides. Especially the expression of complement-related genes was highly induced upon infection suggesting an important role for the complement system in the innate immune response to a *S. pneumoniae* infection also in our model. Finally, in the genetic screen, the lack of the acute phase protein CRP (C-reactive protein), which has been shown to promote the phagocytosis of *S. pneumoniae* in humans, was identified as a potential predisposing factor to a severe *S. pneumoniae* infection in zebrafish.

Altogether, this thesis describes the conservation of the main host-pathogen interactions in *S. pneumoniae* and *S. agalactiae* infections in zebrafish. Due to the notable similarities with human infections, this model is suitable for the identification of novel virulence factors promoting streptococcal pathogenesis as well as for the evaluation of the host factors affecting the susceptibility to the infection. The unraveling of these factors may in the future provide us with important insights into the treatment and prevention of the devastating diseases caused by these pathogens.

TIIVISTELMÄ

Streptococcus pneumoniae (pneumokokki) ja *Streptococcus agalactiae* ovat maailman yleisimpiä keuhkokuumeen, sepsiksen ja aivokalvontulehduksen aiheuttajia erityisesti pienillä lapsilla. *S. pneumoniae*-bakteerin on arvoitu olevan vastuussa jopa yli puolen miljoonan lapsen kuolemasta vuosittain, kun taas *S. agalactiae* on merkittävä taudinaiheuttaja vastasyntyneillä sekä altistava tekijä ennenaikaiselle synnytykselle ja raskauden keskeytymiselle. Molemmat patogeenit ovat kehittäneet monipuolisia mekanismeja pystyäkseen oleilemaan isännässään pitkäaikaisina, oireettomina kommensaaleina tai toisaalta taas karatakseen isännän immuunipuolustukselta ja aiheuttamaan vakavia infektioita. Nämä streptokokit ovat myös osoittaneet laajaa kantojenvälistä vaihtelua sekä nopeaa mikroevoluutiota ja siten haastavat nykyisiä hoito- ja ehkäisykeinoja.

Jotta näitä merkittäviä patogeenejä vastaan voitaisiin kehittää tehokkaita hoitokeinoja, niiden taudinaiheuttamismekanismit pitäisi tuntea läpikotaisin. Tällaista tutkimusta varten tarvitaan oikeanlaisia eläinmalleja. Perinteisesti käytetyt nisäkäsmallit ovat auttaneet selvittämään streptokokkien sekä ihmisen välisiä vuorovaikutuksia, mutta ongelmat eettisyydessä sekä korkea hinta vaikeuttavat esimerkiksi hiirten käyttöä kokeissa, jossa tarvitaan suuria yksilömääriä. Sen vuoksi pyrin tässä väitöskirjassa tutkimaan toisen selkärankaisen, seeprakalan (*Danio rerio*), potentiaalia *S. pneumoniae* -ja *S. agalactiae* -bakteerien taudinaiheuttamismekanismien tutkimisessa. Seeprakalat ovat pieniä ja helposti käsiteltäviä, niiden ylläpitoon tarvitaan vain suhteellisen vähän tilaa ja ne omaavat monipuolisia geneettisen manipulaation mahdollisuuksia. Näiden ominaisuuksien lisäksi seeprakalan immuunipuolustus on pitkälle kehittynyt ja hyvin samankaltainen kuin ihmisellä, minkä vuoksi seeprakala onkin houkutteleva malli infektiotutkimuksiin.

Tämä tutkimus osoitti, että *S. pneumoniae* ja *S. agalactiae* aiheuttavat seeprakalassa infektion, jossa on samoja piirteitä kuin ihmisen sepsiksessä ja aivokalvontulehduksessa. *S. pneumoniae* -ja *S. agalactiae* -mutantit, joilta puuttui tunnettuja virulenssitekijöitä, olivat seeprakalassa taudinaiheuttamiskyvyltään villityypin bakteeria heikompia, josta päättelimme, että nämä bakteerit käyttävät samoja infektiomekanismeja sekä seeprakaloissa että ihmisissä. Tämän lisäksi käytimme seeprakalan poikasia *S. pneumoniae*-infektion aikaisen synnynnäisen

immuunivasteen tarkemmassa tutkimisessa. Tätä varten määritimme infektion aikana ilmentyviä geenejä sekä tutkimme *S. pneumoniae* -infektion alttiustekijöitä geneettisen seulonnan avulla. Nämä tutkimukset osoittivat, että seeprakalan synnynnäinen immuunivaste *S. pneumoniae*-infektiossa on hyvin samankaltainen kuin ihmisen vaste, koostuen mm. pro-inflammatoristen sytokiinien, kemokiinien, akuutin vaiheen proteiinien sekä antimikrobiaalisten peptidien tuotannosta. Analyysin mukaan *S. pneumoniae* aktivoi erityisesti komplementti-välitteiseen immuunivasteeseen liittyviä geenejä, mikä viittaa näiden mekanismien tärkeään rooliin infektion vastustamisessa. Geneettinen seulonta puolestaan paljasti, että kuten ihmisellä, C-reaktiivisen proteiinin (CRP) puutos saattaa altistaa seeprakalat vakavammalle *S. pneumoniae*-infektiolle.

Kaiken kaikkiaan, tämä väitöskirja osoittaa, että sekä patogeenien taudinaiheuttamismekanismit että isännän puolustusreaktiot *S. pneumoniae* -ja *S. agalactiae* -bakteereita vastaan ovat hyvin samankaltaisia seeprakalla ja ihmisellä. Tämän vuoksi seeprakalaa voidaan luotettavasti käyttää vaihtoehtoisena mallieläimenä tutkittaessa streptokokkien ja ihmisen välisiä vuorovaikutuksia. Mikä tärkeintä, seeprakalan avulla voidaan tulevaisuudessa saada tärkeää tietoa pneumokokin taudinaiheuttamismekanismeista sekä uusia lähtökohtia nykyistä tehokkaampien hoitokeinojen kehittämiseen.

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III **Saralahti A**, Piippo H, Parikka M, Henriques-Normark B, Rämet M, Rounioja S. Adult zebrafish model for pneumococcal pathogenesis. Dev Comp Immunol. 2014. Feb; 42(2):345-53.

IV **Saralahti A**, Harjula S-K, Uusi-mäkelä M, Rantapero T, Piippo H, Nykter M, Lohi O, Rounioja S, Parikka M, Rämet M. Characterization of the innate immune response to *Streptococcus pneumoniae* infection in zebrafish. *(Submitted manuscript 2018)*

ABBREVIATIONS

actb1	*actin, beta 1*
BBB	Blood-brain barrier
C3	Complement component 3
cfu	colony forming units
CovS/CovR	Two-component regulatory system CovS/CovR
Cps	Capsular polysaccharides
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
dpf	days post fertilization
e.g.	For example (exempli gratia)
ENU	Ethylnitrosourea
GFP	Green fluorescent protein
hpf	hours post fertilization
hpi	hours post injection/infection
Ig	Immunoglobulin
IL	Interleukin
LD50	A dose that kills 50 % of the individuals
LytA	Autolysin A
MBL	Mannose binding lectin
MO	Morpholino oligonucleotide
MyD88	Myeloid differentiation primary response 88
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCV	Pneumococcal conjugate vaccine
Ply	Pneumolysin

PPV23	Pneumococcal polysaccharide vaccine, 23-valent
PRR	Pattern recognition receptor
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
qRT-PCR	quantitative real-time polymerase chain reaction
Rag	Recombination activating gene
RD	Rhodamine Dextran
RNA	Ribonucleic acid
rpm	rounds per minute
SB	Splicing blocking
spi1b	*Spi-1 proto-oncogene b*
ST	Sequence type
T4	*S. pneumoniae* wild type strain TIGR4
T4R	unencapsulated mutant of T4
T4Δ*lyt*	Autolysin A deficient mutant of T4
T4Δ*ply*	Pneumolysin deficient mutant of T4
T4Δ*rlrA*	Pilus deficient mutant of T4
TB	Translation blocking
TLR	Toll-like receptor
TNF	Tumor necrosis factor
wasa	*Wiskott-Aldrich syndrome (eczema-thrombocytopenia) a*
wasb	*Wiskott-Aldrich syndrome (eczema-thrombocytopenia) b*
Wasp	Wiskott-Aldrich syndrome protein
wpf	weeks post fertilization
WT	Wild type
ZIRC	Zebrafish international resource center
ΔcovSR	Two-component regulatory system CovS/CovR deficient *S. agalactiae* strain NEM316
ΔcpsD	Capsule deficient *S. agalactiae* strain NEM316
ΔcylE	β-hemolysin deficient *S. agalactiae* strain NEM316

1 INTRODUCTION

The streptococcus genus forms an extremely versatile group of Gram-positive bacteria, which are adapted to several niches and hosts, and are capable of causing a wide spectrum of infectious diseases. In humans, streptococci are predominantly harmless commensals and a part of the human microbiota at the mucous membranes of the mouth, skin, intestine, genital tract, or the respiratory tract (Nobbs et al., 2015; Cole et al., 2008). However, streptococcal bacteria also form one of the most invasive groups of human pathogens that are capable of causing severe systemic infections such as bacteremic pneumonia, sepsis, and meningitis (Krzysciak et al., 2013).

From the almost one hundred different streptococci known to us, two opportunistic and highly invasive species, *Streptococcus pneumoniae* (also known as pneumococcus) and *Streptococcus agalactiae* (or group B streptococcus), are responsible for significant morbidity and mortality among infants and young children. *S. pneumoniae* is a common colonizer of the human nasopharynx from where it may occasionally spread to deeper parts of the body and cause life-threatening invasive infections. According to the World Health Organization, WHO, about 1 million children die of a pneumococcal disease each year, making it one of the most common causes of death at a young age (WHO 2014). *S. agalactiae*, on the other hand, is a typical commensal in the human intestine and in the lower genital tract of pregnant women, from where it may be transmitted to the unborn baby before or during labor. In infants, the transmission of *S. agalactiae* may manifest into a severe invasive infection, either during the first days (early-onset disease) or the first months (late-onset disease) of life. The typical clinical presentations of a *S. agalactiae* infection are pneumonia, sepsis, and meningitis, but in addition to the neonatal disease, *S. agalactiae* is also a notable cause of stillbirths and preterm labor. Altogether, *S. agalactiae* is estimated to cause over 150 000 infant deaths and stillbirths every year (WHO 2017).

The first *S. pneumoniae* infections were described as early as in the late 18th century, but until to the discovery of penicillin in 1929 (Fleming 1929), the treatment of these infections was only minimally successful. The great effectiveness of penicillin, and also other antibiotics, has later been overshadowed by the appearance of antibiotic resistant *S. pneumoniae* isolates. Since the description of the first penicillin resistant *S. pneumoniae* isolate in 1965 (Kislak et al., 1965), the prevalence of strains

resistant to antibiotics has rapidly increased and reached a proportion of as high as 40 % by 2008 (Woodhead et al., 2011). To overcome the obvious limitations of antibiotic treatments, two *S. pneumoniae* vaccine formulations are currently in global use. While proven effective, these vaccines are based on the serotype specific polysaccharide antigens, which only give protection against a small proportion of *S. pneumoniae* serotypes. As a consequence, the overall incidence of *S. pneumoniae* infections, as well as the prevalence of antibiotic resistant isolates have remained nearly the same (Neves et al., 2018; Camilli et al., 2017; Lee et al., 2017). In the case of *S. agalactiae*, a maternal antibiotic treatment is the first choice for the prevention of a neonatal disease. However, due to the inefficient screening methods for maternal colonization and no protective effect of intrapartum antibiotics on a late-onset *S. agalactiae* disease, novel treatments and preventive strategies are constantly being searched for. Currently, there is no *S. agalactiae* vaccine in clinical use.

For both *S. pneumoniae* and *S. agalactiae*, the use of novel drugs acting on the immune response instead of targeting the pathogen itself, is a promising strategy for treatment. Similarly, novel protein-based vaccine antigens might overcome issues concerning the current serotype-specific vaccines. To be able to modulate the specific factors at the interphase of the host and the pathogen, these interactions need to be known in detail. To gain this knowledge, studies using animal models are necessary, and previously these studies have mainly been carried out in mammalian models. To overcome the ethical and practical issues concerning the mammalian models, this doctoral thesis introduces a novel vertebrate model organism, the zebrafish (*Danio rerio*), for the study of host-pathogen interactions in *S. pneumoniae* and *S. agalactiae* infections.

2 REVIEW OF THE LITERATURE

2.1 The pathogenesis of streptococcal bacteria

The genus streptococcus consists of over 100 gram-positive species capable of inhabiting a variety of ecological niches. The flexibility and adaptability of streptococcal bacteria is emphasized by the number of different host species (e.g. human, cattle, fish) and tissues (oral cavity, respiratory tract, genital tract, skin), where they typically live as harmless commensals (Nobbs et al., 2015; Cole et al., 2008). On the other hand, streptococci also comprise one of the most infective group of pathogens causing diseases of varying severities in humans of all age groups (Krzysciak et al., 2013). Noteworthy, many streptococcal species can escape from the colonization site, invade deeper parts of the host's body, and cause life-threatening invasive infections (Krzysciak et al., 2013). When the bacteria succeed in invading the blood, lower respiratory tract, or brain, consequences can be highly dangerous or even fatal. By far the most important human pathogens within the streptococcus genus are *S. pneumoniae*, *S. agalactiae*, and *S. pyogenes,* all of which are frequently isolated from patients with invasive infections (Krzysciak et al., 2013). As they are the main focus areas of this thesis, the pathogenesis of *S. pneumoniae* and *S. agalactiae* are next described in more detail.

2.2 The epidemiology and pathogenesis of *Streptococcus pneumoniae*

S. pneumoniae was first identified in the 1880's and since then it has been a major cause of disease burden all around the world (Watson et al., 1993). Like other streptococci, *S. pneumoniae* is mostly an asymptomatic commensal bacterium, residing in the human nasopharynx (Henriques-Normark and Normark 2010). As far as is known, humans are the only natural hosts for *S. pneumoniae* and as much as 60 % of the human population is estimated to carry one or several *S. pneumoniae* strains and serotypes (Henriques-Normark and Normark 2010). Local upper respiratory tract

infections such as sinusitis and otitis media are the most common clinical manifestations of a *S. pneumoniae* infection. As many as 80 % of all children have at least one episode of otitis media during the first three years of their lives and in many cases children suffer from repeated infections at a young age (Laursen et al., 2017). Therefore, in addition to causing significant morbidity in young children, otitis media associated with *S. pneumoniae* is a major cause of economic burden and antibiotic consumption (Laursen et al., 2017; Weycker et al., 2010).

The harmless commensalism in the upper respiratory tract may occasionally turn into a more severe invasive *S. pneumoniae* disease. The prerequisite of the onset of an invasive disease is the escape of *S. pneumoniae* from the host's mucosal immune system and its spread into deeper parts of the body, including the lungs, the blood, or the cerebrospinal fluid, where it causes pneumococcal pneumonia, bacteremia or meningitis, respectively (Feldman and Anderson 2014; Ludwig et al., 2012; Lynch and Zhanel 2010). Half a million children of under 5 years are estimated to die annually of pneumococcal pneumonia alone (Becker-Dreps et al., 2017), while the other two clinical presentations are also associated with high mortality rates (on average 12 % for all the invasive *S. pneumoniae* infections) (Backhaus et al., 2016; Ludwig et al., 2012; Weycker et al., 2010). The incidence and the severity of *S. pneumoniae* infections are the highest at the extremes of age: in children of under 2 years and in the elderly of over 65 years (Ludwig et al., 2012; Lynch and Zhanel 2010). Another important group at risk includes individuals with an immunocompromised condition, as is exemplified by AIDS (acquired immunodeficiency syndrome) patients, among which the mortality rate of an invasive *S. pneumoniae* infection can be even 100 times higher than in other risk groups (Zhang et al., 2015). Since *S. pneumoniae* is transmitted by becoming directly in contact with respiratory secretions, crowded settings, like day care centers, schools, and hospitals, are the main sites for the spread of *S. pneumoniae* colonization (Lynch and Zhanel 2010).

2.2.1 Host-pathogen interactions in a *S. pneumoniae* infection

An infection is an outcome of the interplay between the host's immune system and the pathogen's virulence factors. The first interactions of *S. pneumoniae* with a human occur at the colonization site, the nasopharynx, where the progression of the infection is typically halted by the innate and adaptive mucosal immune responses, which prevent the uncontrolled replication and spread of the bacteria (Calbo and

Garau 2010). However, *S. pneumoniae* expresses a plethora of soluble and membrane bound molecules, which either hide the bacteria from the host, allowing longer colonization or blood stream survival, or promote the progression of an invasive disease. Together, these factors contribute to the success of each of the three stages of the infection, 1) the colonization and the attachment to the respiratory endothelium, 2) the transendothelial migration to reach the circulation, and 3) the invasive state of the disease (Henriques-Normark and Normark 2010; Kadioglu et al., 2008). Importantly, the expression of virulence factors is tightly controlled during the infection by the bacteria's regulatory systems to meet the requirements set by the environment and the state of the disease (Gómez-Mejia et al., 2017). Many of the *S. pneumoniae* virulence factors are also recognized by the immune system conferring protection against the pathogen. A brief introduction to some of the most important host and *S. pneumoniae* responses participating in the battle between the two, are given next.

2.2.1.1. The key components of the immune response to *S. pneumoniae*

The innate immunity forms the front line of defense against invading pathogens in every multicellular organism. The innate immune response comprises of the mechanical barriers and soluble components and immune cells providing unspecific and fast protection during the first minutes and hours of the invasion (Calbo and Garau 2010). Innate immune mechanisms are also responsible for activating the type specific adaptive immune response capable of maintaining an immunological memory for a previously encountered pathogen (Paterson and Mitchell 2006). In a natural infection, *S. pneumoniae* enters the body through the nasal and oral cavity and, therefore, the first **physical barriers** are set by the epithelial cells lining the upper respiratory tract. These cells produce a viscous mucus that acts by trapping the bacteria leaving them unable to move downwards in the respiratory tract (Stannard and O'Callaghan 2006). The mucus together with the entrapped *S. pneumoniae* are then removed from the respiratory tract by coughing and by the movement of cilia, the hair-like structures on the surface of epithelial cells (Stannard and O'Callaghan 2006). Human respiratory mucosa also produces the enzyme **lysozyme,** which actively degrades the peptidoglycan in the cell wall of *S. pneumoniae* (Henriques-Normark and Normark 2010).

Pattern recognition receptors (PRRs) are a diverse set of host molecules responsible for the early recognition of invaders and the initiation of the inflammatory response (Suresh and Mosser 2013). The group of PRRs include for

example Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectins, Scavenger receptors and various cytosolic DNA censors (Suresh and Mosser 2013). Of those recognizing *S. pneumoniae*, a few examples are given below. During an infection, these receptors recognize conserved molecules (microbe associated molecular patterns, PAMPs) on the surface of or released by the invading pathogens (Suresh and Mosser 2013). Upon activation, these receptors may trigger signaling cascades which lead to the production of soluble inflammatory mediators or activate the intake of bacteria by the cell. Three classes of **TLRs** have so far been shown to play a role in the recognition of *S. pneumoniae*: TLR2, TLR4, and TLR9 (Albiger et al., 2007; Malley et al., 2003; Yoshimura et al., 1999). TLR2 and TLR4 are expressed on the surface of numerous host cells, including the innate immune cells, epithelial cells, and a subtype of T cells, and they have been shown to recognize constituents of the pneumococcal cell wall, (e.g. lipoteichoic acid and peptidoglycan) and soluble pneumolysin, respectively (Malley et al., 2003; Schroder et al., 2003; Yoshimura et al., 1999). Unlike TLR2 and TLR4, TLR9 is an endosomal receptor and is known to recognize the DNA of phagocytosed *S. pneumoniae* (Albiger et al., 2007). In many cases, these three TLRs bind to an intracellular adapter protein **MyD88** (Myeloid differentiation primary response 88) which, through a signaling cascade, activates the transcription factor **NF-κB** (nuclear factor kappa-light-chain-enhancer of activated B cells) (Koppe et al., 2012; Albiger et al., 2005). NF-κB controls the expression of many host genes, including genes encoding the proinflammatory cytokines and chemokines, such as IL1B, IL6, IL8, and TNF, resulting in the recruitment of the innate and adaptive immune cells and the activation of a general inflammatory response to *S. pneumoniae* (Anderson and Feldman 2011).

Besides TLRs, other types of innate PRRs have been found to respond to *S. pneumoniae* related molecules. **NOD2** is a cytosolic NLR, which can recognize *S. pneumoniae* proteoglycans and activate the NF-κB dependent expression of pro-inflammatory genes (Davis et al., 2011; Opitz et al., 2004). Another NLR, **NLRP3**, binds to pneumolysin and contributes to the **inflammasome** mediated proteolytic activation of IL1B and IL18 (Witzenrath et al., 2011; McNeela et al., 2010). The cytosolic DNA sensor **AIM2** also acts through the inflammasome after recognizing endosomal *S. pneumoniae* DNA (Fang et al., 2011). As an example of the C-type lectins, mouse **SIGN-R1** is known to respond to *S. pneumoniae*, namely, to its polysaccharide capsule and to activate the intake and killing of the bacteria by macrophages (Lanoue et al., 2004). Similarly, a scavenger receptor **MARCO** (macrophage receptor with collagenous structure) on the surface of macrophages is shown to recognize and promote the intake of *S. pneumoniae,* via a yet unknown

ligand, and lead to the enhanced TLR2 and NOD2 -mediated signaling (Dorrington et al., 2013; Arredouani et al., 2004). A schematic presentation of the selected host receptors involved in *S. pneumoniae* recognition, is shown in **Figure 1**.

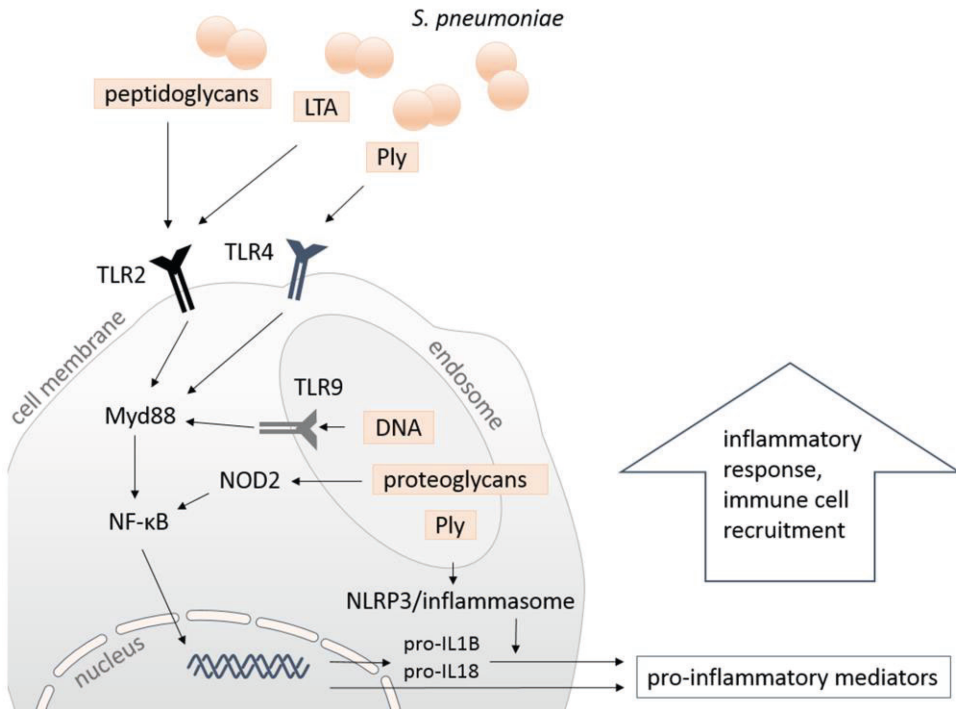

Figure 1. The host's pattern recognition receptors participating in the recognition of *S. pneumoniae*. The image is a simplified schematic representation of the selected host-*S. pneumoniae* interactions described in the text. Various bacterial factors (orange), are bound by the surface (TLR2, TLR4), endosomal (TLR9) or cytosolic (NOD2) receptors of host's antigen presenting cells. The recognition of *S. pneumoniae* cell wall components (LTA, peptidoglycans), pneumolysin, and endosomal DNA and proteoglycans leads to the activation of the transcription factor NF-κB which triggers the expression of pro-inflammatory genes. In addition, the pneumolysin of *S. pneumoniae* activates the inflammasome cascade leading to the proteolytic activation of Il1B and Il18. The image is out of scale. *TLR=toll-like receptor, LTA=lipoteichoic acid, Ply=pneumolysin.*

Macrophages are the resident innate immune cells in several tissues, including those of the lungs, and have therefore an important role in the clearance of *S. pneumoniae* during the early stages of an infection (Dockrell et al., 2003). Macrophage's main roles are to engulf and kill *S. pneumoniae* and, also to trigger an inflammatory response to recruit other immune cells (Calbo and Garau 2010). Another type of leukocytes, **neutrophils**, are soon recruited to the site of infection where they adopt the role of the primary cell type in the clearance of *S. pneumoniae*

(Dockrell et al., 2012; Calbo and Garau 2010; Kolling et al., 2001). Although phagocytosis and the following intracellular killing of the bacteria through the action of hydrolytic enzymes and reactive oxygen species are the main clearance mechanisms of neutrophils, these cells are also found to contribute to the entrapment of *S. pneumoniae*, through the formation of neutralizing NETs, the **neutrophil extracellular traps** (Papayannopoulos 2018; Beiter et al., 2006).

The phagocytic activity of neutrophils and macrophages is greatly enhanced by the action of the **complement system**, a complex network of over 30 proteins which can induce an efficient inflammatory and cytolytic reaction as a response to invading pathogens (Dunkelberger and Song 2010). In a *S. pneumoniae* infection, the effector functions of the complement system are initiated by the antibody, CRP (C-reactive protein) -or SAP (Serum amyloid P) -dependent **classical pathway**, through spontaneous activation by the **alternative pathway,** or the **lectin pathway** (Ali et al., 2012; Yuste et al., 2007; Brown et al., 2002). The activation of complement by either one of these mechanisms ultimately leads to the proteolytic cleavage of the component 3 (C3) into the proinflammatory mediator C3a and the opsonin C3b (Dunkelberger and Song 2010). As its main function during the infection, the complement system greatly enhances the phagocytotic activity of neutrophils by marking the *S. pneumoniae* surface with C3b (Dunkelberger and Song 2010). In addition, through the function of C3a, and many other anaphylatoxins, several immune cell types are recruited to the site of infection (Dunkelberger and Song 2010). However, due to the protective role of the thick polysaccharide capsule, complement does not seem to have a membrane attack complex -mediated cytolytic activity towards *S. pneumoniae* (Andre et al., 2017).

To completely eradicate a *S. pneumoniae* infection, the innate immune mechanisms represented above need to act in a synergy with the adaptive immune mechanisms. The adaptive immunity comprises of the cellular and humoral parts, characterized by the function of **T and B lymphocytes**, respectively (Murphy 2012). In general, two main classes of T cells exist, the cytotoxic CD8+ T cells which destroy the host's own harmful cells by inducing apoptosis and the helper CD4+ cells, which stimulate B cells and other leukocytes and regulate the immune response (Murphy 2012). B cells, on the other hand, secrete specific antibodies and are mainly responsible for the immunological memory (Murphy 2012). Traditionally, the adaptive immune response to *S. pneumoniae* has been thought to be mainly mediated by the B cells and the secreted antibodies (immunoglobulins, Ig) against the protein and the polysaccharide antigens of *S. pneumoniae* (Malley 2010; Lee et al., 2003). As explained above, the host's phagocytosing cells are an important part of the innate immune

response to *S. pneumoniae*, since they are able to engulf and destroy the attacking bacteria. However, these cells, mainly the macrophages and the dendritic cells, also act by linking the innate and adaptive responses by presenting specific peptide antigens derived from the engulfed *S. pneumoniae* on their surface (Murphy 2012). These antigens are bound to the **major histocompatibility complexes** (MHC) and interact with the T cell receptors on the surface of helper T cells. T cells, then, can activate the antibody production in B cells by straight interaction and by secreting stimulating cytokines. Since, the MHCs are only able to present the peptide antigens, another mechanism exists for the activation of the B cells by the polysaccharide antigens. This mechanism is **T cell independent** and occurs when the polysaccharides on the surface of *S. pneumoniae* bind directly to the antibodies on the B cells (Lee et al., 2003). More recently, another adaptive response to *S. pneumoniae* has been reported, an **antibody-independent T cell response** (Zhang et al., 2009; Lu et al., 2008). In this mechanism, the IL17A secreting CD4+ helper T cells are the important mediators, which recognize *S. pneumoniae* protein antigens and confer protection against the colonization through enhanced phagocytosis by neutrophils and macrophages (Zhang et al., 2009; Lu et al., 2008).

2.2.1.2 Examples of the *S. pneumoniae* virulence factors

During the long time that it has coexisted with its host, *S. pneumoniae* has developed sophisticated methods to escape from the above-mentioned traps set by the immune system. Some examples of these factors are given next. First, being more a characteristic than a virulence factor, multiple *S. pneumoniae* may form large communities, **biofilms**, which isolate the bacteria from the environment and confer longer persistence at the colonization site (Chao et al., 2015). More specifically, biofilms for example, hamper the binding of antibodies and other opsonins, but can also prevent the penetration of antibiotics to the bacterial surface (Chao et al., 2015; Steel et al., 2013). As another isolating factor, the *S. pneumoniae* cell wall is surrounded by the layer of polysaccharide chains which form a so-called **capsule**. The capsule has important roles in several steps of the infection and is therefore considered the most important virulence factor of *S. pneumoniae* (Geno et al., 2015). Moreover, the variation in the composition of capsule polysaccharides is the basis for the high variability among *S. pneumoniae*, dividing the species into more than 90 serotypes (Geno et al., 2015). During colonization, the capsule prevents the entrapment and clearance of the bacterial cell by the mucus and subsequently promotes the early steps of the infection (Nelson et al., 2007). During colonization, the capsule observes

a thinner, "transparent" form, which has been proposed to expose components of the cell wall that promote the binding of the bacteria to the endothelium (Weiser 1998; Weiser et al., 1994). The thin capsule also appears beneficial during the migration through the epithelial cell layer into the circulation (Steel et al., 2013). Along with promoting colonization, the main role of the capsule in the pathogenesis of *S. pneumoniae* is to prevent bacterial phagocytosis by the host's macrophages and neutrophils. Therefore, to achieve the best antiphagocytic activity during the invasive state, the capsule returns to its thick, or "opaque" form (Serrano et al., 2006; Kim et al., 1999; Weiser et al., 1994). By hampering the binding of immunoglobulin, complement, and CRP to the bacterial cell wall, the capsule prevents bacterial clearance by opsonophagocytosis (Hyams et al., 2010a; Mitchell and Mitchell 2010). On the host side, the capsular polysaccharides are bound by antibodies which promote the activation of complement and the phagocytosis of *S. pneumoniae* (Anderson and Feldman 2011). The antibodies recognizing the capsular structures are also the basis for the serotype specific protection and the development of an immunological memory against *S. pneumoniae* (Malley 2010). The great importance of the capsule in the pathogenesis of *S. pneumoniae* has been demonstrated in animal models where the unencapsulated bacteria have been shown to be avirulent (Morona et al., 2004; Briles et al., 1992).

S. pneumoniae produces a variety of surface exposed proteins may be divided into the classes of **LPXTG-anchored proteins, lipoproteins, choline-binding proteins and non-classical proteins** based on the motifs anchoring them on the bacterial surface (Mitchell and Mitchell 2010). These proteins serve diverse functions in the virulence of *S. pneumoniae* and in the modulation of host's responses. Some of them, mostly choline-binding proteins and non-classical proteins, are **surface adhesins** promoting the attachment of the bacteria to the epithelial cells, an action that determines the success of the infection (Hammerschmidt 2006). For example, two choline-binding proteins with particular relevance in vaccine development, are the **pneumococcal surface protein A** (PspA) and the **pneumococcal surface protein C** (PspC), which bind various glycoconjugates on host cells as well as lactoferrin and the epithelial polymeric immunoglobulin receptor, respectively (Zhang et al., 2000; Hammerschmidt et al., 1999; Hammerschmidt et al., 1997). Besides promoting adherence, PspA and PspC also have important immunomodulatory functions in *S. pneumoniae* virulence, namely, the disturbance of the activation of complement and subsequent opsonophagocytosis (Yuste et al., 2010; Dave et al., 2004; Jedrzejas et al., 2000; Janulczyk et al., 2000). As examples of the non-classical surface adhesins, with no recognized anchor motifs,

pneumococcal adherence and virulence factors A (PavA) and B (PavB) bind to the host's extracellular matrix, and this way, promote adherence and transendothelial migration (Bumbaca et al., 2004; Holmes et al., 2001). In addition to several cell surface adhesion proteins, some strains of *S. pneumoniae* also possess extended polymer structures, or **pili**, which contribute to the attachment of the bacterium to the respiratory epithelium, but also stimulate the intake of bacteria by macrophages (Orrskog et al., 2012; Barocchi et al., 2006).

Pneumolysin (ply) and autolysin LytA are other virulence proteins important in *S. pneumoniae* pathogenesis, during both the colonization and the invasive disease. Pneumolysin is a well-studied protein of *S. pneumoniae* with a wide array of different roles during the infection, both in promoting the virulence of *S. pneumoniae* and in inducing the host's immune response (Marriott et al., 2008). Pneumolysin is a toxin that in high concentrations can cause the lysis of host cells by forming pores on the cell surface (Marriott et al., 2008). Through its cytotoxic effect on the respiratory epithelium, for example, pneumolysin promotes the transendothelial migration of bacteria into the circulation (Anderson and Feldman 2011; Feldman et al., 1990). Pneumolysin also supports the infection by activating the complement system, in attempt to guide the complement to act further away from the infection, consume the complement components or exhaust the host with an excessive inflammatory response (Marriott et al., 2008; Alcantara et al., 2001; Paton et al., 1984). On the other hand, pneumolysin is a soluble and exposed protein and therefore frequently recognized by the host's immune system. By binding to macrophages through the TLR4, pneumolysin promotes the production of proinflammatory cytokines and the chemotaxis of neutrophils and CD4+ T cells to the site of infection (Koppe et al., 2012; Malley et al., 2003). Pneumolysin has also been found to interact with a type of NLR, the NLRP3 in macrophages, with the same consequence (Witzenrath et al., 2011; McNeela et al., 2010). **Autolysin LytA,** on the other hand, is another choline-binding protein and one of the many hydrolytic enzymes (LytA, LytB, LytC, CbpE) of *S. pneumoniae* (Mitchell and Mitchell 2010). LytA cleaves the peptidoglycan in the *S. pneumoniae* cell wall, and this way, takes care of the cell wall turnover and enables cell growth (Mellroth et al., 2012; Berry et al., 1989a). More importantly, the activity of LytA is responsible for the lysis of the bacterial cells, autolysis, and the release of inflammatory components and toxins, including pneumolysin, into the surroundings (Steel et al., 2013; Kadioglu et al., 2008).

2.3 The epidemiology and pathogenesis of *Streptococcus agalactiae*

S. agalactiae is a highly invasive streptococcus, which is not restricted to only humans but is also an important pathogen in goats, cows, fish, and seals (Delannoy et al., 2013; Brochet et al., 2006). Originally, in the late 1880's, *S. agalactiae* (or group B streptococcus, GBS) was discovered as an infectious agent in cattle and almost 50 years later it was isolated from human vaginal swaps (Le Doare and Heath 2013; Lancefield and Hare 1935). Nowadays, this pathogen is best known as the dominant pathogen in neonates. The vaginal tract of a pregnant woman is the main reservoir of *S. agalactiae* and, occasionally, it may cause an infection of the upper genital tract and placenta, or more rarely, bacteremia and pneumonia (Hall et al., 2017; Deutscher et al., 2011). An *S. agalactiae* colonization and infection in pregnant women is also associated with a higher risk of premature birth and stillbirth (Bianchi-Jassir et al., 2017). An average of 20 % of pregnant women worldwide are carriers of *S. agalactiae*, and the vertical transmission prior or during birth is estimated to occur in 50 % of cases, 1-2 % of which develop into a neonatal disease (Russell et al., 2017a; Russell et al., 2017b; Seale et al., 2017; Le Doare and Heath 2013). The clinical outcomes of a *S. agalactiae* infection in neonates can be classified into two groups, an early-onset and a late-onset disease, both being associated with high (9-15 %) mortality rates and a risk for long-term dysfunction (Seale et al., 2017; Le Doare and Heath 2013). The *S. agalactiae* infection presented during the first six days of life is termed as early-onset and the most common outcomes during this period are sepsis and pneumonia (Russell et al., 2017b; Le Doare and Heath 2013). A late-onset *S. agalactiae* infection in turn, occurs within the first three months after birth and in most cases, develops into meningitis (Le Doare and Heath 2013). Besides vertical transmission, a late-onset *S. agalactiae* infection may also be acquired from the hospital or community sources (Le Doare and Heath 2013). Altogether, *S. agalactiae* is the leading cause of sepsis and meningitis in neonates and accounts for almost 150 000 stillbirths and infant deaths annually (Seale et al., 2017). Moreover, meningitis caused by *S. agalactiae* causes moderate or severe neurological impairments in 20 % of the survivors (Kohli-Lynch et al., 2017).

2.3.1 Host-pathogen interactions in a *S. agalactiae* infection

2.3.1.1. The key components of the immune response to *S. agalactiae*

Due to the close relatedness and the structural similarities between *S. pneumoniae* and *S. agalactiae*, these two bacteria also share basic mechanisms of pathogenesis. Moreover, the host immune response employs the same components of the innate and adaptive immune system in the clearance of a *S. agalactiae* infection, as it does in a *S. pneumoniae* infection. These host-pathogen interactions are only briefly described in this review. *S. agalactiae* has been found to colonize multiple mucosal sites, including the intestine, genital tract, and respiratory tract where the **mucosal immune response** plays an important role in preventing the progression of the disease. These responses include the **mucus entrapment**, phagocytosis and intracellular killing by **resident macrophages**, and the specific **production of IgA** (Kolter and Henneke 2017; Vornhagen et al., 2017). When it is able to proceed into the circulation, *S. agalactiae* encounters effectors, such as phagocytosing neutrophils, the **complement system**, and Ig secreting B cells, which act in co-operation to prevent fulminant sepsis and the onset of meningitis (Kolter and Henneke 2017; Doran et al., 2016). Importantly, while *S. agalactiae* is mostly a harmless colonizer in humans, the early developmental stage of these responses, particularly the immaturity of phagocytes and the adaptive immune response during the first months of life, may explain the high prevalence and severe consequences of an invasive *S. agalactiae* disease in neonates (Basha et al., 2014).

Like in a *S. pneumoniae* infection, the innate immune response to *S. agalactiae* is triggered by the activation of PRRs on the surface of dendritic cells and macrophages. These PRRs include, for example, **TLR2** and **TLR7** that recognize *S. agalactiae* lipoproteins and RNA, respectively (Mancuso et al., 2009; Henneke et al., 2008). The activation of TLRs by *S. agalactiae* typically leads to the induction of proinflammatory mediators, such as IL1B, IL6, IL8, and TNF, in a **Myd88**-dependent manner, as well as the production of type I interferons (IFN) (Landwehr-Kenzel and Henneke 2014; Mancuso et al., 2009; Henneke et al., 2008). The production of IFN is also induced by the interaction of the cytosolic censors **STING** and **cGAS** with *S. agalactiae* DNA (Andrade et al., 2016; Charrel-Dennis et al., 2008). The inflammasome mediated activation of IL1B and IL18, on the other hand, is triggered by the recognition of the *S. agalactiae* RNA and β-hemolysin by **NLRP3** (Gupta et al., 2014; Costa et al., 2012). A schematic presentation of the selected host receptors involved in *S. agalactiae* recognition, is shown in **Figure 2**.

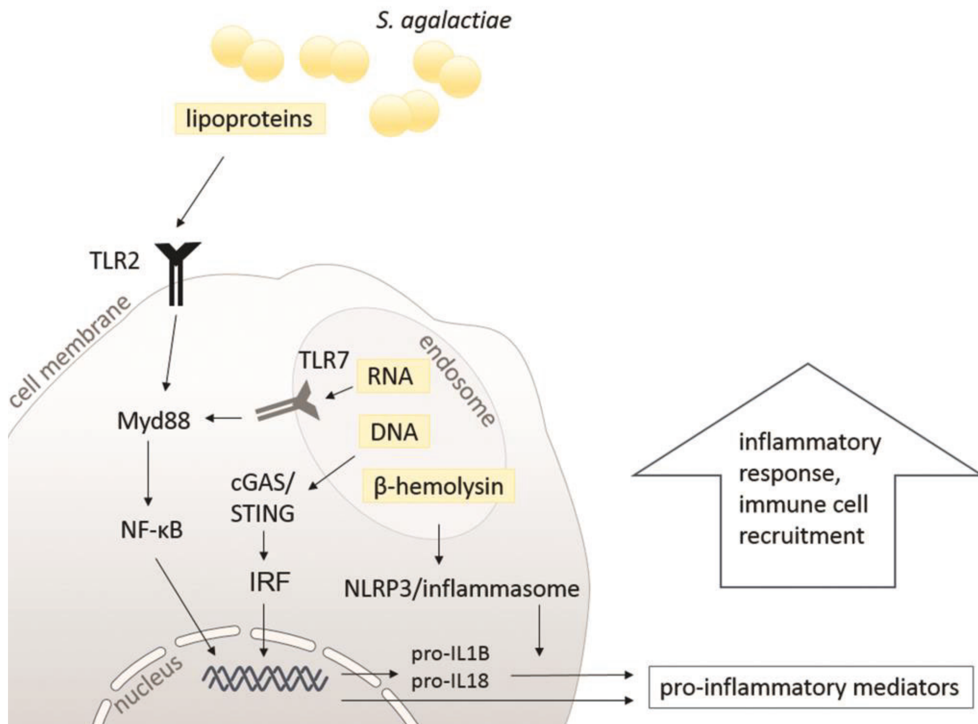

Figure 2. The host's pattern recognition receptors participating in the recognition of *S. agalactiae*. The image is a a simplified chematic representation of the selected host-*S. agalactiae* interactions described in the text. Various bacterial factors (yellow), are bound by the surface (TLR2), endosomal (TLR7) or cytosolic (SIGN/cGAS) receptors of host's antigen presenting cells. The recognition of *S. agalactiae* lipoproteins and endosomal nucleic acids leads to the activation of the transcription factor NF-ϰB which triggers the production of various cytokines, chemokines, and interferons. In addition, endosomal *S. agalactiae* β-hemolysin activates the inflammasome cascade leading to the proteolytic activation of Il1B and Il18. The *S. agalactiae* β-hemolysin also activates the histamine and cytokine production by mast cells. Ultimately the recognition results in the immune cell recruitment and an inflammatory response. The image is out of scale. *TLR=toll-like receptor. IRF=interferon regulatory factor.*

Upon recognition through various PRRs, macrophages recruit other immune cells to the site of infection. As with *S. pneumoniae*, **neutrophils** play a crucial role in the phagocytic clearance of *S. agalactiae* and the entrapment of bacteria by extracellular traps (NETs) (Vornhagen et al., 2017). Like in a *S. pneumoniae* infection, specific **IgA and IgG** antibodies against the polysaccharides of the *S. agalactiae* capsule are key components in the adaptive immune response to *S. agalactiae*. Additionally, various other surface structures, including the pilus and the surface proteins Ripb and alpha C, may trigger the production of specific antibodies and an immunological memory (Lachenauer and Madoff 1996; Stålhammar-Carlemalm et al., 1993). Yet another cell type, **mast cells**, have relatively recently been associated

with the initiation of the immune response against *S. agalactiae* in the lower genital tract. Although best known for their role in allergies, mast cells also act in the first steps of the immune response by recruiting neutrophils to the *S. agalactiae* colonization site (Gendrin et al., 2015). The mechanism of recruitment includes the degranulation of mast cells by the *S. agalactiae* β-hemolysin and the subsequent release of histamine and proinflammatory cytokines (Gendrin et al., 2015).

2.3.1.2 Examples of the *S. agalactiae* virulence factors

The development of an invasive *S. agalactiae* infection requires successful colonization, translocation, survival in the bloodstream and, in the case of meningitis, crossing of the blood-brain barrier (BBB), a layer of specialized endothelial cells separating the central nervous system from the blood (Doran et al., 2016; Landwehr-Kenzel and Henneke 2014). To overcome these challenges, *S. agalactiae* produces a diverse array of virulence determinants, which interfere with the host's clearance mechanisms and account for the specific interactions leading to the penetration of the barriers. In general, the adhesion and tissue penetration at distinct colonization sites, as well as at the BBB are mostly mediated by the same host-pathogen interactions (Vornhagen et al., 2017; Doran et al., 2016; Landwehr-Kenzel and Henneke 2014). First, the formation of a **biofilm** has been shown to be important also during *S. agalactiae* colonization, as the biofilm protects the bacterial community from the environment, and also facilitates the attachment of bacteria to epithelial cells (Rosini and Margarit 2015). Among the protein virulence factors of *S. agalactiae* (e.g. LPXTG-anchored proteins and lipoproteins), numerous **adhesion molecules** mediate the binding of *S. agalactiae* to the mucosal epithelium and the BBB, and some of them also promote passage through these barriers (Doran et al., 2016; Lindahl et al., 2005). For example, fibronectin-binding protein A (**FbsA**) and laminin-binding protein (**Lmb**) attach *S. agalactiae* to the extracellular matrix while another fibronectin-binding protein, **FbsB** contributes to the invasion into tissues (Schubert et al., 2004; Gutekunst et al., 2004; Schubert et al., 2002; Spellerberg et al., 1999). In addition, *S. agalactiae's* **Alpha C proteins** and serine rich proteins (**Srr**) also participate in attaching the bacteria to the extracellular matrix and the surface of epithelial cells (Seo et al., 2012; Seo et al., 2013; Samen et al., 2007; Bolduc and Madoff 2007; Bolduc et al., 2002). Like *S. pneumoniae*, *S. agalactiae* possess **pilus** structures which facilitate the binding of the bacteria to the epithelium, and also to the BBB (Doran et al., 2016; Konto-Ghiorghi et al., 2009; Dramsi et al., 2006). Importantly, another factor mediating adherence to the epithelial cells and the BBB

is the **hypervirulent GBS adhesin** (HvgA) (Tazi et al., 2010). Intriguingly, this surface exposed adhesion molecule is only expressed in *S. agalactiae* serotype III, the sequence type 17 (ST-17) clone, which is the most common isolate in meningitis associated with the late-onset neonatal disease (Tazi et al., 2010).

Like in *S. pneumoniae*, the cell wall of *S. agalactiae* is covered by a thick polysaccharide **capsule** (Vornhagen et al., 2017; Landwehr-Kenzel and Henneke 2014). This structure also shares the mechanisms with the *S. pneumoniae* capsule in promoting immune evasion during the invasive disease and in providing antigens for antibody-mediated recognition (Doran et al., 2016; Landwehr-Kenzel and Henneke 2014; Campbell et al., 1991). Based on the varying capsule composition and immunogenicity, *S. agalactiae* are divided into 10 serotypes (Doran et al., 2016). For these reasons and due to the avirulent nature of the capsule-deficient *S. agalactiae,* the capsule is thought to be the most important virulence factor of *S. agalactiae* (Rubens et al., 1987). Another virulence factor, **β-hemolysin** plays a role in many steps of the *S. agalactiae* infection. First, β-hemolysin is capable of modulating epithelial and endothelial cells at the intestinal, amniotic, and blood-brain barriers, and cause the translocation of bacteria through these barriers (Whidbey et al., 2013; Doran et al., 2003; Doran et al., 2002). Second, in order to cause severe tissue damage, β-hemolysin can also use its cytolytic activity against several types of host cells, including brain endothelial cells and primary neurons (Doran et al., 2016; Reiß et al., 2011). Moreover, β-hemolysin uses several strategies to resist or modulate the host's immune response, including the inhibition of complement, protection form intracellular killing in neutrophils, and induction of the expression of the anti-inflammatory IL10 (Doran et al., 2016; Landwehr-Kenzel and Henneke 2014; Sagar et al., 2013; Bebien et al., 2012; Liu et al., 2004).

Finally, the expression of the above-mentioned virulence factors in addition to many more bacterial components is highly regulated in the course of an infection to either maintain colonization or enable invasion (Landwehr-Kenzel and Henneke 2014). *S. agalactiae's* **two-component control system CovS/CovR** (control of virulence sensor/regulator) is one of the many systems responsible for such a regulatory mechanism (Jiang et al., 2005). This system consists of two domains: CovS, which responds to changes in environmental conditions and activates/inactivates CovR, the regulatory part of the system (Lamy et al., 2004). When activated, CovR regulates the expression of the other virulence factors of *S. agalactiae*, including the pilus and β-hemolysin (Lamy et al., 2004). In addition to disease progression, the regulated expression of *S. agalactiae* genes under different

environmental conditions most probably contribute to the high adaptability of this streptococci to multiple ecological niches (Rajagopal 2009).

2.4 The remaining challenges in the eradication of *S. pneumoniae* and *S. agalactiae*

The current treatment methods for *S. pneumoniae* and for *S. agalactiae* rely on antibiotics (Braye et al., 2017; Woodhead et al., 2011). In the case of suspected or diagnosed maternal *S. agalactiae* colonization, intrapartum antibiotic prophylaxis is recommended (Braye et al., 2017). In fact, systematic screens to detect maternal colonization are conducted in many countries using either of the two recommended strategies, the risk-factor based screening or the universal screening (Braye et al., 2017; Heath 2016). However, the efficiency of the screen may vary among countries, and also, both strategies may lead to false positive and false negative results and, consequently, to unnecessary or insufficient antibiotic treatments (Braye et al., 2017). Despite the downsides of the screening, the intrapartum antibiotic prophylaxis regime has been proved efficient in preventing the vertical transmission of *S. agalactiae* and the early-onset infection in neonates, but it has no effect on the late-onset disease (Toyofuku et al., 2017). In the case of *S. pneumoniae*, the discovery of penicillin in 1929 revolutionized the treatment and the prognosis of *S. pneumoniae* infections turning hopeless cases into treatable conditions (Chain et al., 1940; Fleming 1929). However, the great success of antibiotic treatments has later been overshadowed by an increase in the incidence of antibiotic resistant strains of *S. pneumoniae* (Kim et al., 2016; Liñares et al., 2010; File 2006). In 2008, the proportion of clinical isolates resistant to β-lactams, macrolides or tetracyclines, and importantly, also multidrug resistant strains, had reached a worryingly high incidence rate of as much as 40 %, and since that, the rate has remained nearly the same Neves et al., 2018; Camilli et al., 2017; Lee et al., 2017; Woodhead et al., 2011).

Due to the challenges in treating *S. pneumoniae* and *S. agalactiae* with antibiotics, preventive vaccination strategies hold more promise in the battle against these two streptococci. Currently, there is no vaccine for *S. agalactiae* in clinical use. The inefficient screening methods, concerns about the excessive use of antibiotics, and the limited methods for preventing the late-onset neonatal disease have, however, driven the development of vaccines to a point where several potential alternatives have made it into clinical trials (Heath 2016). These vaccine candidates are based on the capsular polysaccharides from the most relevant serotypes, which are conjugated

to a carrier protein (Heath 2016). In the case of *S. pneumoniae*, two vaccine formulations, also based on the immunogenicity of capsule polysaccharides, are currently in global use, the pneumococcal polysaccharide vaccine (PPV23) and the conjugate vaccine (PCV) (Falkenhorst et al., 2017; Feldman and Anderson 2014; Lynch and Zhanel 2010). The polysaccharide vaccine consists of plain polysaccharide antigens and, thus, its efficiency is poor in children under 2-years of age, due to their immature T cell independent immune response (Lee et al., 2003). The polysaccharides in the conjugate vaccine, on the other hand, are linked to an immunogenic carrier protein, which can also elicit a strong T cell dependent immune response and, therefore, provide better protection compared to PPV (Feldman and Anderson 2014; Lee et al., 2003). Consequently, since 2000, the licensing year for the conjugate vaccine PCV7, this vaccine type has been the first choice for vaccination and, together with the updated version of PCV13, has shown to decrease the incidence of *S. pneumoniae* infections remarkably (Becker-Dreps et al., 2017; Corcoran et al., 2017; Waight et al., 2015; Moore et al., 2015; Isaacman et al., 2010; Huang et al., 2009). For instance, in the United States, the incidence of invasive *S. pneumoniae* diseases dropped to almost zero after the introduction of PCV7, while the incidence of pneumonia decreased by 39 % in children (Pilishvili et al., 2010; Grijalva et al., 2007).

Despite the clear positive effect of *S. pneumoniae* vaccines, the high capsular variability among the species together with the serotype specific nature of protective antibodies has raised concerns about the global efficacy of the polysaccharide based vaccines (Geno et al., 2015). As mentioned previously, there are over 90 capsular variants (or serotypes) of *S. pneumoniae*, which differ in their ability to colonize and invade, and in their geographical distribution (Geno et al., 2015; Grabenstein and Musey 2014; Rodrigo and Lim 2014). Out of the over 90 serotypes, PCV7 provided protections against seven of the clinically most relevant serotypes and resulted in a clear decline in the prevalence of these serotypes (Huang et al., 2009). However, as the carriage of vaccine serotypes decreased, the emergence of the uncovered serotypes became evident and the new serotypes soon started to take the role of the most common infectious agents (Huang et al., 2009). To respond to the burden of newly emerged serotypes, the PCV13 vaccine, covering 13 serotypes, was introduced in 2009 (Geno et al., 2015). Although the positive impact of PCV13 has been evident, serotype replacement remains a major challenge in the prevention of *S. pneumoniae* infections and forces vaccine developers into a continuous race with the evolving *S. pneumoniae*.

As discussed, *S. pneumoniae* shows strong adaptability to the changing environment and can readily respond to the pressures introduced by antibiotics and vaccines to ensure better survival. Other selective pressures include competition for nutrients and interactions with host's immune system (Henriques-Normark et al., 2008). The wide spectrum of hosts and the original transition to humans, on the other hand, emphasize the similar adaptability of *S. agalactiae* (Flores et al., 2015). Although in a much larger scale for *S. pneumoniae*, both streptococci show notable genetic variation within the species, but also within serotypes (Flores et al., 2015; Henriques-Normark et al., 2008). This genetic variation explains the intraspecies differences for example in the pathogenicity, invasiveness, and target hosts and is caused by the evolution of the genomes of both streptococci (Nobbs et al., 2015; Straume et al., 2015). The mechanisms driving genome evolution include point mutations and larger rearrangement within the genome, but also the transfer of genetic material between bacteria form the same or different species (Straume et al., 2015; Nobbs et al., 2015). Since both the streptococci described in this thesis are efficient colonizers and share their niche with many other bacteria, the prolonged habitation of these sites provides favorable circumstances for the horizontal transfer of genes (Nobbs et al., 2015). With the horizontal transfer of genetic material, a bacterium may gain, for example, antibiotic resistance or change its serotype to one not covered by the current vaccines (Straume et al., 2015). Antibiotic resistance may also be acquired through the accumulation of point mutations in the genes encoding the target molecules of antibiotics, such as *S. pneumoniae* penicillin-binding proteins (Straume et al., 2015). With the same mechanism, bacteria may also gain useful changes in the structure and function of virulence factors and regulatory systems, which may help them escape the immune system or leads to improved invasiveness (Flores et al., 2015; Mitchell and Mitchell 2010).

To circumvent the detrimental effects of genome evolution to the treatment and prevention of *S. pneumoniae* and *S. agalactiae*, novel approaches are needed. In the case of *S. pneumoniae*, treatment strategies modulating the host's immune response, rather than targeting the bacteria directly, are being investigated (Zumla et al., 2016; Feldman and Anderson 2014). In addition, vaccines based on non-capsular antigens are constantly being developed to gain broader protection against *S. pneumoniae* and *S. agalactiae* serotypes (Heath 2016; Alderson 2016). So far, several proteins of both pathogens have proved to be potential vaccine antigens either alone or in combination, including the *S. pneumoniae*'s pneumolysin, PspA and PspC and the *S. agalactiae*'s Alpha C and Rib proteins, and the pilus (Kamtchoua et al., 2013; Margarit et al., 2009; Ogunniyi et al., 2007; Cao et al., 2007; Larsson et al., 1999).

2.5 An overview of the zebrafish as a model organism

Animal models have been for long utilized in biomedical research to gain a better understanding of human physiology or embryogenesis, but also to mimic biological responses to normal and pathological conditions. Through these studies, animal models have improved our knowledge about many diseases, and also contributed to the development of new drugs and vaccines. Regarding the complex nature of the human immune system, the comprehensive modeling of infectious diseases would not be possible without a proper animal model. The use of invertebrate models has provided important discoveries about the human immune system, exemplified by the Toll-like receptors originally found in the fruit fly *Drosophila melanogaster* (Lemaitre et al., 1996). In addition, invertebrate models have also been extensively used in the study of host-pathogen interactions during an infection (O'Callaghan and Vergunst 2010). However, due to similarities with human anatomy and physiology and, therefore, better translation of the results to humans, mammalian models, like mice, rats, rabbits, and non-human primates, have gained more popularity in the fields of immunology and infectious diseases than invertebrates (Andersen and Winter 2017). However, another vertebrate, the zebrafish (*Danio rerio*), has more recently shown its value by providing a practical and ethical alternative for mammals in the study of complex interactions between microbes and the host's immune system (Goldsmith and Jobin 2012; Meeker and Trede 2008).

Zebrafish are small teleost fish which are native to the Himalayan region where they commonly inhabit slowly-moving streams, ponds, and rice fields (Spence et al., 2008). Due to the small size of the adult fish (<6 cm), zebrafish can be maintained in laboratory conditions with a high population density and are thus more cost-effective compared to mammals (Bowman and Zon 2010; Meeker and Trede 2008). In addition, the embryonic development of zebrafish is fast (most of the internal organs are fully developed at 5 days post fertilization) and they reach the adulthood in a relatively short time (in 2-3 months). Because of the short generation time (2-3 months) and high fecundity, zebrafish are especially well-suited for studies requiring a large number of animals, like genetic or chemical screens (Bowman and Zon 2010; Meeker and Trede 2008). Importantly, the transparency of the embryos provides a unique opportunity for live imaging during embryogenesis, and the visualization of cellular and molecular functions in different conditions and genetic backgrounds (Tobin et al., 2012). Yet another beneficial characteristic of the zebrafish as a model organism is its relatively easy genetic manipulation and the availability of several genetic tools (discussed later).

While the ethical concerns, high costs, and small number of progeny are noteworthy disadvantages of most mammalian models, zebrafish also has its own limitations as a model organism. First, the clear anatomical differences in fish compared to humans, including gills instead of lungs, no limbs or lymph nodes, different sites of hematopoiesis, and a very different reproductive system limit the modeling of certain types of diseases (Tobin et al., 2012; Menke et al., 2011). Second, the zebrafish is a relatively new model organism, which affects the availability of some reagents and tools, including antibodies specific to surface markers, zebrafish cell lines, and a lack of a feasible homologous recombination mediated knock-in method (Tobin et al., 2012; Meeker and Trede 2008). Third, the teleost-specific genome duplication event during evolution together with the still limited gene annotation might pose challenges to comparative genomics and gene manipulation (Howe et al., 2014; Goldsmith and Jobin 2012). The sequencing of the zebrafish genome revealed that approximately 70 % of all human genes have an ortholog in fish. However, due to the duplication event, 50 % of these human genes correspond to several copies in the zebrafish (Howe et al., 2014). Finally, the environmental conditions of zebrafish are different from humans, as is exemplified by temperature. While zebrafish are raised at 28 °C, human specific pathogens have adapted to 37 °C, which might limit or change the nature of the pathogenicity of certain microbes in this model (Allen and Neely 2010). Despite the above-mentioned limitations, zebrafish do have great potential in disease modelling, drug discovery, and vaccine development. Importantly, a major characteristic driving the development of zebrafish disease models is the similarity of the immune systems of fish and humans. Therefore, the zebrafish immune system and its relation to the human immune system is reviewed next.

2.5.1 The zebrafish immune system and its relation to the human immune system

As vertebrates, zebrafish possess a highly evolved innate immune system. Due to the ancient genome duplication event and the following diversification, components of the zebrafish innate immunity show great diversity, providing perhaps a broader protection to invading pathogens compared to mammals (van der Vaart et al., 2012; Stein et al., 2007). Then again, the zebrafish adaptive immune response comprises of all the main components found in humans (Kanther and Rawls 2010). Therefore, this organism provides a suitable model for the study of the adaptive immune

response as well. As a unique characteristic, the development of the zebrafish immune system is graduated, the fully functional adaptive responses appearing as late as 4 weeks post fertilization (wpf) (Lam et al., 2004). This provides an attractive possibility to study the vertebrate innate immune system in zebrafish embryos and larvae without the influence of the adaptive immunity. However, when using the zebrafish embryo model to study host-pathogen interactions, the hematopoietic context into which the microbe is introduced should be carefully considered, since it might affect the outcome of the microbial challenge (Lohi et al., 2013; Kanther and Rawls 2010; Paik and Zon 2010).

In the developing embryo, the primitive wave of hematopoiesis begins already at 12 hours post fertilization (hpf) giving rice to the primitive phagocytosing cells: **macrophages** around 20 hpf and **neutrophils** around 30 hpf (Le Guyader et al., 2008; Herbomel et al., 1999). The primitive macrophages are able to phagocytose invading pathogens already at 30 hpf and are the dominant cells clearing bacteria in zebrafish during the first 2 days of development (Torraca et al., 2014; Colucci-Guyon et al., 2011; Herbomel et al., 1999). The phagocytosing activity of primitive neutrophils starts around 50 hpf, but at a seemingly lower level (Colucci-Guyon et al., 2011; Le Guyader et al., 2008). Neutrophils, however, are the main cell type responsible for bacterial clearance in larvae and adult zebrafish (Harvie and Huttenlocher 2015; Le Guyader et al., 2008). The second wave of hematopoiesis, the definitive wave, starts around 30 hpf and gives rise to multipotent myeloid and lymphoid progenitors, which around 4 days post fertilization (dpf) start to colonize the definitive hematopoietic tissues in adult fish, the kidney marrow and the thymus (bone marrow and thymus in humans) (Paik and Zon 2010; Jin et al., 2007; Murayama et al., 2006). In general, the definitive hematopoiesis in zebrafish produces all of the innate immune cell types observed in humans, except for basophilic granulocytes. Out of the three human granulocyte lineages, zebrafish have neutrophils and **eosinophils** (Balla et al., 2010; Le Guyader et al., 2008). Moreover, zebrafish **monocytes**, macrophages, **dendritic cells**, **mast cells**, and **Natural killer (NK) -like cells** have also been described (Lugo-Villarino et al., 2010; Dobson et al., 2008; Yoder et al., 2004; Yoder et al., 2001; Herbomel et al., 1999). Although the lymphopoiesis and the Rag-dependent rearrangements of lymphocyte receptors occur already at 4 dpf, the adaptive immune system with a protective antibody response is fully active only after 4 wpf (Lam et al., 2004).

Like humans, zebrafish possess an array of pattern recognition receptors (PRRs), which are bound by the conserved molecular structures derived from pathogens. The TLRs, NLRs, lectins, and the common adaptor proteins are conserved between

human and zebrafish at varying levels, while the group of mammalian cytosolic DNA censors seems to be absent (Li et al., 2017; van der Vaart et al., 2012). In general, zebrafish **cytokines** and **chemokines** are highly diverged in zebrafish compared to humans, although the counterparts for the most important proinflammatory cytokines, IL1B, IL6, and TNF, and the anti-inflammatory cytokine IL10, do exist with similar functionalities (Varela et al., 2012; van der Vaart et al., 2012; Stockhammer et al., 2009; Pressley et al., 2005). In contrast, the group of **interferons** mainly responsible for antiviral responses, seems to be more distinct in zebrafish than in humans (Yoon et al., 2016; Stein et al., 2007). While humans have three types of interferons, type I (IFNα, IFNβ), type II IFN (IFNγ), and type III IFN (IFNλ), zebrafish possess only type II IFNs with two members, IFNγ (or IFNγ2) and IFNγ-rel (or IFNγ1) (Yoon et al., 2016).

Zebrafish has homologous genes for the 10 human **TLR**s, except for TLR6 and TLR10 (Meijer et al., 2004). Due to the genome duplication, human TLR4, TLR5, and TLR8 have two counterparts in the zebrafish genome (Meijer et al., 2004). In addition, the zebrafish genome encodes for fish specific TLRs and TLRs found exclusively in fish, amphibians and birds (Li et al., 2017). The ligand specificity for some of the zebrafish TLRs is still only partly known, but recent studies have shown clear functional conservation with some of the TLRs, including TLR2, TLR5 and TLR9 (S. Yang et al., 2015; Yeh et al., 2013). Interestingly, this genomic conservation does not necessarily implicate functional similarity (e.g. TLR4), however, nor does the absence of a clear homolog in zebrafish genome always signify the loss of a homologous function in the species. The knowledge of the ligand specificity of the TLRs from other fish species suggests that, for example, the zebrafish TLR14 might be a functional counterpart for human TLR6 and TLR10, compensating for the absence of these human homologs in zebrafish (Hwang et al., 2011). As described for the TLRs, the common **adaptor proteins** (such as MyD88, Tirap, Ticam1 and Sarm) and the components of the TLR downstream signaling cascades leading to the activation of the NF-κB mediated proinflammatory response, as well as the components of interferon signaling, are also well conserved (Stein et al., 2007).

While the receptors of the TLR family in zebrafish show clear conservation with the mammalian TLR family supplemented with a few fish specific members, the family of **NLR**s in zebrafish seems to be highly expanded with more than 400 members compared to 23 NLRs in human (Li et al., 2017; Howe et al., 2014). Among this huge gene family, the homologs for *Nod1*, *Nod2*, and *Nod3*, to name a few, have been identified and also represent similar functionalities in the innate immune response as their human counterparts (Li et al., 2017; Howe et al., 2014). In contrast,

the functions of the hundreds of fish specific NLRs and their possible roles in pathogen recognition remain unknown (Li et al., 2017; Howe et al., 2014). Some C-type lectins, including the complement activating **mannose-binding lectin** (MBL) as well as **scavenger receptors** have been identified in zebrafish (Yang et al., 2014; Benard et al., 2014; Jackson et al., 2007).

As an important part of the innate immune response to pathogens, the **complement system** is also highly conserved in zebrafish and includes all three activation pathways (Boshra et al., 2006; Boshra et al., 2004). A genomic comparison between fish and humans revealed the existence of zebrafish genes for all of the more than 30 complement proteins in humans (Zhang and Cui 2014; Boshra et al., 2006). Although the structural similarity between the human and zebrafish complement system postulates also functional conservation, the roles of most zebrafish complement proteins have not yet been characterized. Importantly, as described for other components of the innate immunity, the zebrafish genes for the complement system show isotypic diversity, as is exemplified by the existence of eight copies of genes for the C3 in the zebrafish genome (Forn-Cuní et al., 2014; Gongora et al., 1998).

As described above, the zebrafish innate immune response shows a higher level of diversity and complexity compared to mammals. The zebrafish adaptive immunity, on the other hand, contains all the main components of the human adaptive immune response, but does not provide the same diversity for specific antigen recognition. To bridge the innate and adaptive immune responses, the antigen presenting cells (APCs) take in, process, and present the pathogen-derived molecules to the naïve T cells (Murphy 2012). The professional APCs in mammals include dendritic cells, macrophages and B cells, which all present the pathogen derived antigen through a type II MHC and the co-receptors. Similarly, the role of the **APC**s in zebrafish is carried out by the same cell types with the characteristic MHC II expression (Lewis et al., 2014; Renshaw and Trede 2012; Lugo-Villarino et al., 2010). Zebrafish dendritic cells are found in great numbers in the spleen and gut, and these organs are thought to serve as secondary lymphoid organs, the sites of antigen presentation (the spleen, lymph nodes and gut in mammals) (Renshaw and Trede 2012; Lugo-Villarino et al., 2010). Upon stimulation, the naïve CD4+ T cells specialize into distinct subsets, such as **Th1, Th2 and Th17** with characteristic cytokine repertoires and specialized functions, also found in zebrafish (Dee et al., 2016; Mitra et al., 2010). In addition to CD4+ T cells, the adaptive cellular immune response in zebrafish is substituted by other T cell types, **cytotoxic CD8+ cells** and **CD4+/CD25+ T regulatory cells** (Kasheta et al., 2017; Renshaw and Trede 2012).

The CD4+ helper T cells stimulate B cell maturation and proliferation leading to the production of antibodies. A notable difference between the zebrafish and mammalian adaptive immune response lies in the antibody classes and the diversity. In general, based on the B cell numbers, the zebrafish adaptive immune response is estimated to be 5 orders of magnitude simpler than in humans (Weinstein et al., 2009). In addition, although zebrafish undergo **Rag-dependent VDJ recombination**, the **somatic hypermutation** creating variability in the antigen recognition is less efficient than in mammals (Marianes and Zimmerman 2011; Weinstein et al., 2009). Importantly, in contrast to five antibody classes in humans (IgM, IgG, IgD, IgE, and IgA), zebrafish only expresses two mammalian classes (**IgM, IgD**) in addition to a fish specific **IgZ** class and do not undergo class switching (Hu et al., 2010; Danilova et al., 2005; Wakae et al., 2005). However, despite these differences and limitations, zebrafish have been shown to mount a protective antibody response upon immunization and have successfully been used in vaccination studies (Myllymäki et al., 2017; Oksanen et al., 2016; Oksanen et al., 2013; Cui et al., 2010; Lam et al., 2004).

Table 1. Comparison of the selected components of the immune systems of zebrafish and human

	HUMAN	**ZEBRAFISH**
innate cell types		
macrophages	yes	yes
granulocytes	eosinophils, neutrophils, basophils	eosinophils, neutrophils
dendritic cells	yes	yes
mast cells	yes	yes
Natural Killer cells	yes	yes
pattern recognition receptors		
Toll-like receptors	10 different (TLR1-TLR10)	homologs for the human genes encoding TLR1-TLR5 and TLR7-TLR9. Two copies of TLR4, TLR 5 and TLR8. Several zebrafish specific genes.
NOD-like receptors	23 members	over 400 members
complement system		
pathways	classical, alternative and lectin pathway	classical, alternative and lectin pathway
protein components	over 30 protein components	homologs for all the complement genes in humans. Several copies of each component.
adaptive immune cells		
antigen presenting cells	macrophages, dendritic cells, B cells	macrophages, dendritic cells, B cells
T cells	helper, cytotoxic and regulatory T cells, Th1, Th2 and Th17 responses	helper, cytotoxic and regulatory T cells, Th1, Th2 and Th17 responses
B cells	yes	yes
antibody response		
Ig classes	IgA, IgD, IgG, IgE, IgM	IgD, IgM, IgZ
VDJ recombination	yes	yes
class switching	yes	no
somatic hypermutation	yes	yes

2.5.2. Zebrafish toolbox

Both zebrafish embryos and adult zebrafish have been utilized in the study of infectious diseases due to the similarities with human immune system, as described above. Moreover, both models also provide unique possibilities for studies due to a toolbox full of practical methods for imaging, genetic manipulation, and experimental infections. An experimental bacterial infection in zebrafish larvae, for example, can be conducted through various routes to achieve either a systemic infection (e.g. the blood circulation valley or the caudal vein) or a local infection (e.g. the hindbrain, the otic vesicle, or the pericardial cavity) (Saralahti and Rämet 2015; Harvie et al., 2013; Prajsnar et al., 2008; van der Sar et al., 2003; Davis et al., 2002). Alternatively, a more natural infection can be accomplished by static immersion or through the alimentary tract (Harriff et al., 2007; Pressley et al., 2005). The yolk sac injection at 0-1 dpf represents a high-throughput and even automated method for an experimental infection, and can be conducted with slowly replicating bacteria, which then disseminate from the injection site into various tissues (Spaink et al., 2013). In adult zebrafish, two routes of infection are mostly used, the intraperitoneal route to cause a systemic infection, and the intramuscular route to follow the dissemination of bacteria from the local infection site (Miller and Neely 2004).

The transparency of the zebrafish embryos provides an additional benefit for the *in vivo* imaging of the infection dynamics and immune cell functions. Zebrafish are naturally non-pigmented until 2 dpf, after which the amount of pigmentation in the skin can be restricted either chemically or genetically to extend the imaging period (Antinucci and Hindges 2016; Karlsson et al., 2001). Through the genetic manipulation of the pigmentation genes, the advantages of imaging can also be extended to adult zebrafish (*casper* and *crystal* lines) (Antinucci and Hindges 2016; White et al., 2008). The *in vivo* imaging of zebrafish is even more enhanced by the availability of transgenic zebrafish lines, in which distinct cell lines or signaling molecules are marked by a fluorescent reporter. Nowadays, a fluorescent reporter lines for a myeloid cell population (Ward et al., 2003), macrophages (Ellett et al., 2011), neutrophils (Renshaw et al., 2006), T cells (Langenau et al., 2004), MyD88 (Hall et al., 2009), NF-κB (Kanther et al., 2011), and many others are available. When combined with fluorescent particles or labelled microbes, these lines have helped to reveal new aspects of phagocytosis, cell migration, cell-cell interactions, and chemotaxis, for example (Meijer and Spaink 2011). In addition, in the absence of zebrafish specific antibodies, the fluorescent reporter lines have proved practical in fluorescent-activated cell sorting (FACS). Finally, to utilize the vast imaging

properties of zebrafish larvae even more, bioassays with labelled sensors and probes to detect the production and the role of hydrogen peroxide, reactive oxygen species, and nitric oxide as part of the host's antimicrobial defense have been developed (Meijer et al., 2014).

Due to the unavailability of zebrafish embryonic stem cells and the inefficient homologous recombination methods, the zebrafish lacks a proper method for creating engineered mutations or knock-in models. On the other hand, the zebrafish provides a versatile toolbox for reverse and forward genetics, including methods based on morpholino oligonucleotides, targeted nucleases, or transposable elements and retroviruses (Huang et al., 2012). The use of antisense morpholino oligonucleotides (MO) to disrupt target mRNAs has gained popularity among zebrafish researchers due to its simplicity in design and applicability to almost any gene (Nasevicius and Ekker 2000). However, concerns regarding possible off-target effects in addition to the only partial and transient nature of the knockdown, have increased the interest in conventional knock-out methods. The current methods for creating targeted mutations into the zebrafish genome rely on nucleases that cause a double-strand break in the specific site of the DNA and subsequently mutate the site (Varshney and Burgess 2014; Hisano et al., 2014). Of the three methods available, the zinc finger nuclease method, the TALEN method, and the CRISPR/Cas9 method, CRISPR/Cas9 has proved to be superior compared to the other two due to its easiness of design, cost-effectiveness, larger number of possible target sequences, and the unique possibility to conduct multiplex gene targeting (Varshney and Burgess 2014). However, although the CRISPR/Cas9 method holds promise for the introduction of engineered mutations and knock-ins via homologous recombination, such an application has so far proved to be very inefficient in zebrafish (Albadri et al., 2017).

Possibilities for forward genetics in zebrafish are also numerous and several methods have been successfully used in large-scale screens to reveal novel genes associated with embryonic development (Golling et al., 2002; Haffter et al., 1996) genetic disorders (Haffter et al., 1996), infection susceptibility (Tobin et al., 2010), and immune cell development (Iwanami et al., 2016; Seiler et al., 2015), for example. Traditionally, chemical mutagenesis with ethylnitrosourea (ENU) has been used to introduce germline mutations in such screens (Driever et al., 1996; Haffter et al., 1996), however, the laborious identification of the mutated genes has tempted the use of retrovirus or transposon based insertional mutagenesis (Song and Cui 2013; Amsterdam et al., 2011; Sivasubbu et al., 2007). As an example of the insertional mutagenesis methods, the tol2 transposon from medaka can introduce relatively

large DNA transgenes in an unbiased manner and, therefore, has become particularly popular in mutagenesis and transgenesis in zebrafish (Kawakami 2007). One of the most sophisticated versions of the tol2 transposon is the introduction of a protein trap and a fluorescent reporter to the insertion site simultaneously silencing the gene and allowing the visualization of the expression of the disrupted gene (Clark et al., 2011). In addition, a Gal4/UAS -based method for the temporal and tissue specific expression of transgenes has been applied to zebrafish (Halpern et al., 2008).

3 AIMS OF THE STUDY

Despite the notable achievements in the eradication of *S. agalactiae* and *S. pneumoniae* infections through optimized antibiotic treatments and, in the case of *S. pneumoniae*, also effective vaccination programs, these streptococci remain a global health burden for humans. The serotypic diversity as well as the rapid microevolution of both streptococci present challenges for the treatment and prevention of *S. agalactiae* and *S. pneumoniae* infections when using conventional methods. However, in order to find novel drug targets and vaccine candidates for the fight against a bacterial infection, the pathogenic mechanisms associated with the infection need to be known in detail. In the search for host and bacterial factors affecting the outcome of the infection, a proper animal model, which is capable of reproducing the human pathology and can mimic the host-pathogen interactions seen in human infections, is essential. Although, mammalian models have been informative in the study of the pathogenic mechanisms of *S. pneumoniae* and *S. agalactiae*, these models are limited in their ethicality and practicality and are not, thus, suited for large-scale studies. Lower vertebrates, on the other hand, have the potential of serving as alternative models in the study of immune responses and infectious diseases. Therefore, the specific aims of this thesis were:

1) To test, whether the zebrafish (*Danio rerio*) can be used to model the host-pathogen interactions in human *S. pneumoniae* and *S. agalactiae* infections.

2) To study the role of innate and adaptive immune responses in a *S. pneumoniae* infection in zebrafish and to evaluate the suitability of this model for the search of novel vaccine candidates against *S. pneumoniae*.

3) To identify, through a genetic screen, host factors that affect the susceptibility to *S. pneumoniae* infections.

4 MATERIALS AND METHODS

4.1 Zebrafish and the ethical statements (I-IV)

The AB or TL (Tupfel long fin, $leo^{t1\ -/-}$, $lof^{dt2\ -/-}$) zebrafish were used as wild type reference lines in the study (obtained from ZIRC, Zebrafish international resource center, University of Oregon, Oregon, USA). Other zebrafish lines were the transgenic myeloid cell reporter line Tg(Spi:EGFP) (ZIRC), the Rag1 mutant zebrafish line ($Rag1^{hu1999}$) (ZIRC), and the $MyD88hu^{3568}$ mutant zebrafish line (van der Vaart et al., 2013) (a generous gift from Professor Annemarie Meijer, Leiden University). All the zebrafish lines were maintained according to the standard protocols (Nusslein-Volhard and Dahm 2002) and the guidelines set by the Ethical board in Finland. Briefly, the zebrafish embryos were grown at 28 °C on a Petri dish containing E3-medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 10^{-5} % Methylene Blue) until 6 dpf after which they were transferred to a filtered flow-through water system (28 °C) with a 14/10-hour light/dark cycle. The fish were fed once (adults) or twice (larvae) a day with fresh (Artemia) and dry food (SDS-400 or GEMMA micro). The well-being of the fish was monitored daily and the unwell fish were removed from the system and euthanized with an overdose of an anesthetic tricaine (0.08 % 3-aminobenzoic acid ethyl ester, pH 7.0) (Sigma-Aldrich, St. Louis, Missouri, USA). Infected embryos and adult fish were kept in isolation in corresponding conditions. In all the experiments, the adult fish and the embryos were anesthetized in 0.02 % tricaine and euthanized in 0.08 % tricaine. Humane-endpoint criteria was followed throughout the study, and the maintenance and the experiments conducted with the adult zebrafish were approved by the Animal Experiment Board in Finland with the following licenses: ESLH/2008/07610/Ym-23, STH761A, ESAVI/2010/08379/Ym-23, STH603A, ESAVI/2106/04.10.03/2011, ESAVI/6403/04.10.03/2012, ESAVI/6407-/04.10.03/2012, ESAVI/733/04.10.07/2013, ESAVI/8108/04.10.07/2015, ESAVI/10366/04.10.07/2016, ESAVI/10366/04.10.07/2016, ESAVI/2464-/04.10.07/2017, LSLH-2007-7254/ym-23, ESAVI/10079/04.10.06/2015.

4.2 Bacterial strains and culture (I-IV)

As pathogens capable of infecting humans, *S. pneumoniae* and *S. agalactiae* are classified as biosafety level 2 bacteria and are, therefore, handled with special safety, including the appropriate protective equipment and the inactivation of the contaminated water and equipment with an appropriate disinfectant. The wild type *S. pneumoniae* used throughout the studies was the wild type TIGR4 (T4) of serotype 4, ST205, originally isolated from a patient suffering from an invasive *S. pneumoniae* disease (Aaberge et al., 1995). Two wild type lines for *S. agalactiae* were used, the NEM316 (serotype II, ST-23) and the FIM314 (serotype II, ST-17) isolated from a septic neonatal patient. Other bacterial strains included the isogenic T4 and NEM316 mutants deficient in selected virulence determinants (**Table 2**).

Unless otherwise stated, all the bacterial strains were grown overnight on 5 % lamb blood agar plates (Tammer-Tutkan maljat Oy, Tampere, Finland) in 37 °C and 5 % CO_2. Bacterial cells were suspended in 5 ml Todd Hewitt broth (Becton, Dickinson and Company, New Jersey, USA) supplemented with 0.5 % Todd Hewitt Yeast extract (Becton, Dickinson and Company), and grown in 37 °C until they reached the mid-log phase of growth (OD_{620} 0.4) corresponding to 10^8 colony forming units (cfu) / 1 ml. The cells were harvested by centrifugation (10 min in 4000 rpm for *S. pneumoniae* and 7000 rpm for *S. agalactiae*) and resuspended in 0.2 M KCl to obtain the desired concentrations. The final bacterial concentrations were confirmed by quantitative plating; serial dilutions of the bacterial solution were plated on the lamb blood agar plates and grown overnight at 37 °C and 5 % CO_2.

Table 2. The bacterial strains used in the study.

Strain	Species	Serotype	Sequence type	Phenotype
T4	*S. pneumoniae*	4	205	Wild type
T4R	*S. pneumoniae*	4	205	Capsule deficient
T4Δ*lyt*	*S. pneumoniae*	4	205	Autolysin A deficient
T4Δ*ply*	*S. pneumoniae*	4	205	Pneumolysin deficient
T4Δ*rlrA*	*S. pneumoniae*	4	205	Pilus deficient
FIM314	*S. agalactiae*	II	17	Wild type
NEM316	*S. agalactiae*	II	23	Wild type
ΔcylE	*S. agalactiae*	II	23	β-hemolysin deficient
ΔcpsD	*S. agalactiae*	II	23	Capsule deficient
ΔcovSR	*S. agalactiae*	II	23	CovS/CovR deficient

4.3 Experimental infection in zebrafish embryos (I, IV)

4.3.1 Microinjection of zebrafish embryos

2 dpf zebrafish embryos were infected through an intravenous microinjection with a desired concentration of *S. pneumoniae*. Prior to the injection, zebrafish embryos were manually dechorionated, anesthetized, and laid on agar plates with wedged-shaped troughs. The microinjection needles were prepared from 1.0 mm x 0.78 mm borosilicate glass capillaries (Harvard Apparatus, Holliston, Massachusetts, USA) using the Sutter P-97 capillary needle puller (Sutter instruments, Novato, California, USA). The needle was filled with a desired injection solution labelled with 1 % of 70 kDa Rhodamine Dextran (RD) (Invitrogen, Carlsbad, California, USA) and calibrated to release a dose of 2 nl. Injections directed to the blood circulation valley were carried out using the micromanipulator (Narishige international, London, UK) and the PV830 Pneumatic PicoPump microinjector (World precision instrument, Sarasota, Florida, USA). The success of the injection was monitored by visually inspecting the dispersion of the labelled injection solution and by the temporal cardiac arrest of the injected embryos.

4.3.2 Survival assays in zebrafish embryos

In the *S. pneumoniae* survival assays, 2 dpf zebrafish embryos were intravenously infected with a desired concentration of wild type T4 or the isogenic T4 mutants as described above. The final bacterial count per injection was confirmed by quantitative plating before and after injecting each group. As a mock-infected control group, 2 dpf embryos were injected with 0.2 M KCl labelled with RD. The infected and control embryos were kept on 24-well plates, a single embryo in a separate well, and the mortality of the embryos was monitored once a day for 4 days. The lack of movement and heartbeat were considered as the signs of death. At the end of the experiment (6 dpf), the surviving embryos were euthanized and the Kaplan-Meier survival graphs were drawn to obtain the survival rates.

4.3.3 Determination of bacterial load in zebrafish embryos

To obtain the bacterial load in infected embryos at selected time points, 5 embryos per group were randomly selected and euthanized. Embryos were washed in 1x phosphate buffered saline (PBS) solution and manually homogenized in 200 µl 1x PBS with 1 % Triton-X (Sigma-Aldrich) by pipetting up and down. Each sample was homogenized evenly to achieve comparable bacterial counts. When properly dispersed, 10-fold serial dilutions were prepared in 1x PBS. The bacterial counts in the dilutions were determined by quantitative plating on the lamb blood agar plates containing 1 µg/ml erythromycin (Tammer-Tutkan maljat Oy).

4.4 Experimental infection in adult zebrafish (II, III)

4.4.1 Intraperitoneal infection

5-8 months old adult zebrafish were infected intraperitoneally with *S. pneumoniae* or *S. agalactiae* (Neely et al., 2002). Briefly, prior to injection, zebrafish were anesthetized and laid on a moist foam bed ventral side up. An omnican 30 G insulin syringe and needle (Braun, Melsungen, Germany) was used to administer 5 µl of the injection solution labelled with 0.3 mg/ml filtered phenol red (Sigma Aldrich) into the zebrafish abdomen. Special care was taken to avoid causing any harm for the fish through the handling or through the injection. Successfully injected fish were transferred to a tank containing fresh water and the recovery of the fish was followed before returning them into the water system. Any fish observed to be bleeding or otherwise suffering was immediately removed from the system and euthanized.

4.4.2 Intramuscular injection

5-8 months old adult zebrafish were used for intramuscular injection. For the injection, anesthetized fish were laid on a moist foam bed dorsal side up. The intramuscular injection was carried out using the microinjection instruments described in section 4.3.1 "Microinjection of zebrafish embryos" and the needle for the injection was prepared from 1.0 mm x 0.5 mm aluminosilicate glass capillaries (Harvard Apparatus) as described above. To carry out the injection, a needle was filled with 5 µl of the injection solution labelled with 0.3 mg/ml filtered phenol red,

after which the needle was positioned anterior to the dorsal fin at 45° angle in relation to the spine. The injection solution was administered to the dorsal muscle and the successfully injected and recovered fish were moved to the water system as explained for intraperitoneal injection.

4.4.3 Survival assays in adult zebrafish

In the survival assays of the adult zebrafish, groups of 15-20 zebrafish were randomly selected to be intraperitoneally or intramuscularly infected with desired concentrations of *S. pneumoniae* or *S. agalactiae*. The final bacterial count per injection was confirmed by quantitative plating before and after injecting each group. As a mock-infected control group, 15-20 adult fish were injected with 0.2 M KCl labelled with phenol red. The infected and control fish were kept in a stand-alone flow-through system, and their survival was checked twice a day for a 7-day period. Fish showing any signs of infection, including aberrant swimming behavior, increased rate of respiration, visible lesions, or cerebral edema were immediately euthanized and recorded as deceased. At the end of the experiment, surviving fish were euthanized and the Kaplan-Meier survival graphs were drawn to obtain the survival rates.

4.4.4 Determination of bacterial load from zebrafish blood

Following intraperitoneal infection, the bacterial counts were determined from adult zebrafish blood at certain time points during the 7-day follow up. Five randomly chosen zebrafish were sacrificed for the collection of blood samples. First, the fish were euthanized and the skin was dried and sterilized with 70 % EtOH to avoid the contamination of the sample. Thereafter, a sterile scalpel blade was used to cut the fish neckline and 5 µl of blood was collected from the wound by pipetting. The blood sample was quickly transferred to a tube containing 45 µl of 0.2 M KCl and mixed thoroughly to prevent coagulation. The bacterial counts in each sample were determined by quantitative plating of 10-fold serial dilutions in 0.2 M KCl on selective blood agar plates.

4.4.5 Determination of bacterial load from zebrafish brain

The brain from 5 intraperitoneally infected adult fish were dissected for the determination of bacterial counts at selected time points. For the dissection, fish were euthanized and tacked on a dissecting board dorsal side up and the fish skin was cleaned as described above. Using sterile scalpel blade and forceps, the skull plate was removed and the exposed brain collected. The collected tissue was transferred to a tube containing 200 µl of 1x PBS and homogenized using a sterile pestle. For the quantitation, 10-fold serial dilutions were prepared in 1x PBS and plated on selective blood agar plates.

4.5 Characterization of the zebrafish immune response to *S. agalactiae* and *S. pneumoniae* infection (I-IV)

4.5.1 Morpholino oligonucleotide gene knockdown (I)

The antisense morpholino oligonucleotides (MO) were used to silence the expression of *wasa* (previously *wasp2*, *Wiskott-Aldrich syndrome (eczema-thrombocytopenia*) a), *wasb* (previously *wasp1*, *Wiskott-Aldrich syndrome (eczema-thrombocytopenia) b)* and *spi1b* (previously *pu.1, Spi-1 proto-oncogene b*). The translation blocking (TB) and splice blocking (SB) MOs were obtained from the Gene Tools (Philomath, USA). The genes wasa and wasb were silenced in a single embryo by using two translation blocking (TB) MOs. One TB and one SB MO were used as a combination to silence the spi1b expression (**Table 3**). To prepare the MO injection solutions, the 1 mM MO stocks were heated at 65 °C for 5 minutes to reduce secondary structures and to dissolve the precipitated MO. The MO injection solutions were prepared in 0.2 M KCl with the following concentrations: for Wasp 100 µM + 100 µM (*wasa* + *wasb*) and for *spi1b* 130 µM + 130 µM (TB + SB). To visualize the success of the injection, 1 % RD was added to the solution. A total of 1 nl MO solution was microinjected into the yolk sac of 1-cell stage zebrafish eggs. As a control, a group of eggs were injected with random control MO (Gene Tools). After the injection, eggs were kept in E3-medium (28 °C) and the success of the injection was verified 24 hours post injection (hpi) by visualizing the embryos under the fluorescent microscope (Lumar VI.1; Carl Zeiss Microimaging GMbH, Cardiff, UK).

Table 3. The antisense morpholino oligonucleotide sequences. *MO=morpholino oligonucleotide, TB=translation blocking, SB=splicing blocking.* (The *spi1b* MO sequences were obtained from Clay et al., 2007)

Target gene	MO	MO sequence 5'→3'
wasb (ZDB-GENE-030131-7098)	TB	CCCTTTGCTTTTGCCTTTGCTCATC
wasa (ZDB-GENE-081104-419)	TB	CCTCATGGCCTCATACGCCGTCTAA
spi1b (ZDB-GENE-980526-164)	TB	CCTCCATTCTGTACGGATGCAGCAT
spi1b (ZDB-GENE-980526-164)	SB	GGTCCTTCTCCTTACCATGCTCTCC

4.5.2 Live imaging of phagocytosis in zebrafish embryos (I)

For live imaging of phagocytosis of *S. pneumoniae* in zebrafish embryos, transgenic Tg(spi:EGFP) embryos were injected with T4 labelled with *Bac*light™ Red bacterial stain (Invitrogen, Thermo Fisher Scientific). The bacteria were cultured as previously described and the labelling was carried out according to the manufacturer's instructions. The *S. pneumoniae* infections in zebrafish larvae were conducted as described earlier at 2 dpf. At 1-day post injection (dpi), randomly selected embryos were anesthetized and mounted onto 1 % low-melting-point agarose (Sigma-Aldrich) for imaging. The fluorescence imaging was carried out at 1-3 hpi using the Olympus BX51WI microscope (Olympus Corporation, Tokyo, Japan) with 40x or 100x water immersion objectives (Olympus Corporation). Image J (National institutes of Health, Maryland, USA) was used to assemble the stacks of obtained images and the final editing was done with Photoshop C2S (Adobe systems Inc., California, USA).

4.5.3 Quantitative analysis of the gene expression levels (I, II, IV)

4.5.3.1 Total RNA extraction

In original communication I, groups of 10-15 infected or control embryos were euthanized and pooled for RNA extraction 18 hpi. In original communication II, the adult zebrafish brain was isolated, as described above, for the RNA extraction at various time points. Total RNA from pooled embryos or the adult zebrafish brain were homogenized in TRI Reagent (MRC, Ohio, USA) using a pestle and the following RNA extraction was conducted according to the manufacturer's

instructions. After the extraction, the RNA concentrations were measured with the NanoDrop spectrophotometer (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA). In original communication IV, the RNA extraction from the pool of 5 embryos was conducted using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

4.5.3.2 Quantitative RT-PCR

The expression levels of *tnfa, il1b,* and *il6* were measured from the RNA extracted from the pooled embryos or the brain tissue. The qRT-PCR was performed using the iScript™ One-Step RT-PCR Kit with SYBR® Green (Bio-Rad Laboratories, Hercules, California, USA), according to the manufacturer's instructions. The expression levels were normalized to the expression of *actb1*. The primers for *tnfa*, *il1b*, and *actb1* are listed in the **Table 4**. The thermal cycling was performed using the Bio-Rad CFX 96 instrument (Bio-Rad Laboratories) and the following program with melt curve analysis: 1. 50 °C for 10 minutes, 2. 95 °C for 5 minutes, 3. 95 °C for 10 seconds, 4. 60 °C for 30 seconds (steps 3-4 40 times). CFX Software version 1.6 (Bio-Rad Laboratories) were used to analyze the results and the double delta Ct method (ΔΔCt) was used to calculate the relative gene expression.

Table 4. The primer sequences. (*il1b* and *actb1* primer sequences were obtained from Pressley et al., 2005)

Gene	Forward primer 5'→3'	Reverse primer 5'→3'
tnfa	GGGCAATCAACAAGATGGAAG	GCAGCTGATGTGCAAAGACAC
il1b	TGGACTTCGCAGCACAAAATG	GTTCACTTCACGCTCTTGGATG
actb1	ATGGATGAGGAAATCGCTG	ATGCCAACCATCACTCCCTG

4.5.3.3 mRNA sequencing

The RNA for sequencing was extracted from pooled (5x) KCl injected wild type, T4 injected wild type and T4 injected mutant94 embryos at 18 hpi. The RNA extraction is described in the section 4.5.2.1 "Total RNA extraction". The genomic DNA was removed from the samples using the RapidOut DNA removal Kit (Thermo Scientific, Thermo Fisher Scientific) according to the manufacturers' instruction, after which the concentration and the purity of the samples were measured using the NanoDrop spectrophotometer and the Qubit™ RNA BR Assay Kit (Invitrogen™,

Thermo Fisher Scientific). In addition, the integrity of the RNA sample was verified with the Fragment Analyzer (Advanced Analytical Technologies, Iowa, USA) using the Standard Sensitivity RNA Analysis Kit (Advanced Analytical Technologies) and the PROSize® 2.0 Data Analysis Software (Advanced Analytical Technologies).

The cDNA library preparation and the RNA sequencing was done by Novogene, Hong Kong. Briefly, the 250-300 bp cDNA library was sequenced with the 150 bp paired-end sequencing on the Illumina platform and with a sequencing depth of >20 million reads/sample. The obtained reads were aligned against the GRCz10 zebrafish reference genome using the STAR software and the read counts normalized using the DEseq2. The differential gene expression was determined by DEseq2. The 3-fold induction and 3-fold reduction in gene expression were set as limits for the differentially expressed genes.

4.5.4 A forward genetic screen for host genes affecting *S. pneumoniae* infection (IV)

4.5.4.1 Generating mutant fish lines for the forward genetic screen

The pGBT RP2-1 (addgene #31828) and the pt3TS-Tol2 (addgene #31831) plasmids for the gene-breaking tol2 transposon -based mutagenesis were obtained from Professor Stephen Ekker's Laboratory, Maya Clinic, USA (Clark et al., 2011). To produce the Tol2 transposase mRNA for the mutagenesis, the pt3TS-Tol2 plasmid was linearized with Fast Digest restriction enzyme BamHI (Thermo Scientific, Thermo Fisher Scientific) and *in vitro* transcribed with mMESSAGE mMACHINE SP6 transcription Kit (Invitrogen™, Thermo Fisher Scientific) according to the manufacturers' instructions. The mRNA was purified with lithium chloride precipitation and dissolved in RNase free water. For the mutagenesis, 1 nl of the injection mixture containing 12,5 ng/µl of the pGBT RP2-1 and 12,5 ng/µl Tol2 transposase mRNA was microinjected into the cell of early 1-cell stage AB zebrafish eggs. The injected embryos were screened for the successful injection by the mosaic expression of GFP (green fluorescent protein) under the Zeiss Lumar V12 or the Nikon AZ100 (Nikon Corporation, Tokyo, Japan) fluorescence microscope. The GFP positive embryos were selected to be grown into adulthood and for subsequent crosses as F0 generation.

The mosaic F0 fish were crossed to TL fish to obtain the heterozygous F1 fish carrying multiple inserts. A single F1 fish served as a founder for a specific mutant

line named according to the running number given for the F1 fish (for example mutant94). Each F1 fish was individually crossed with TL fish to obtain the heterozygous F2 offspring. The F2 fish of a certain mutant line were in-crossed to gain homozygous F3 fish for the experiments. In every step of the crosses (**Figure 3**), the germline transmission of the inserts was verified by observing the embryos under the Zeiss Lumar V12 or the Nikon AZ100 fluorescent microscope. Only positive embryos were selected for the crosses and the phenotypic analyses. The phenotype of each mutant line was analyzed by survival assays (described in the section 4.3.2 "Survival assays in zebrafish embryos") and if a phenotype was observed, also by determining bacterial burden (described in the section 4.3.3 "Determination of the bacterial load in zebrafish embryos").

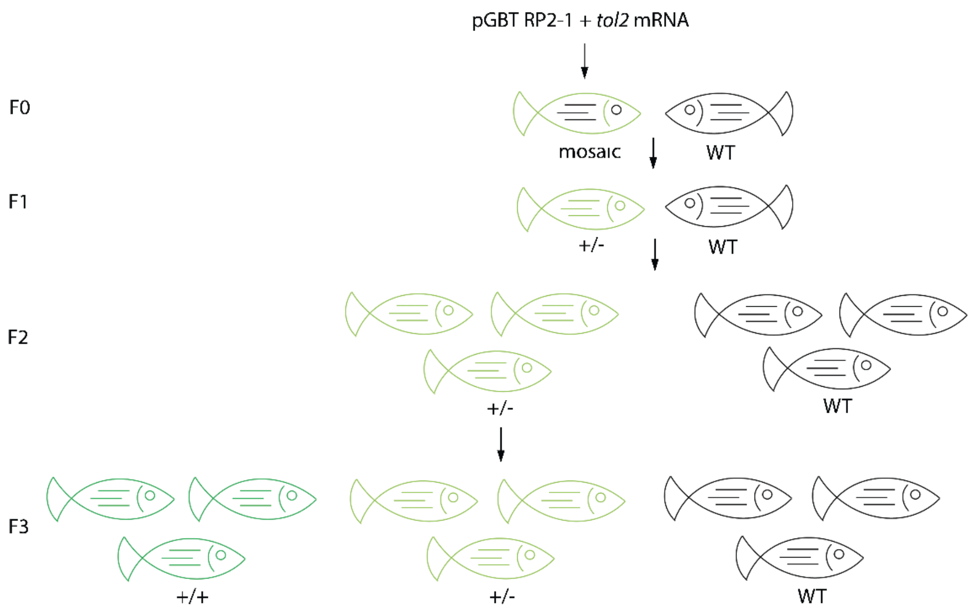

Figure 3. Breeding zebrafish for mutagenesis screen. WT=wild type, +/- = heterozygous, +/+ = homozygous.

4.5.5 Immunization assay (III)

To determine the induction of immunological memory of adult zebrafish against *S. pneumoniae*, groups of 20-30 fish were immunized with heat-killed T4 ($1x10^4$ colony forming units, cfu), sublethal dose of live T4 ($5x10^4$ cfu), or live T4R ($1.5x10^6$ cfu). To prepare the heat-killed bacteria, T4 was cultured and the cells harvested as

described above. The bacterial cells were resuspended in 500 µl of 0.2 M KCl and then autoclaved to inactivate the bacteria. The fish were immunized intramuscularly and infected with 2.5×10^6 cfu of T4 through the same route 2 or 3 weeks after immunization. The infection dose was verified as above. The two control groups received KCl immunization or no immunization. The mortality of the infected fish was followed for 14 days and the deaths recorded, after which the surviving fish were euthanized.

4.6 Statistical analyses

GraphPad Prism software (version 5.02) was used to carry out the statistical analyses for the survival assay, relative cytokine expression, and the comparison of bacterial counts. The survival rates were analyzed with the Mantel-Cox Log Rank test. The data for bacterial counts and relative cytokine expression was analyzed either using the one-way ANOVA/Kruskal-Wallis test with Dunn's multiple comparison post-test (multiple samples) or the two-tailed Student's t-test (two samples). A p-value of <0.05 was considered statistically significant.

5 SUMMARY OF THE RESULTS

5.1 *S. pneumoniae* causes a fulminant, dose-dependent infection in zebrafish (I, III)

Since to my knowledge, the zebrafish model had not been described in the context of a *S. pneumoniae* infection, we first tested the ability of this human-specific pathogen to cause an infection in zebrafish. Since the differences in the natural environmental temperatures of *S. pneumoniae* (37 °C) and the zebrafish (28 °C) were evident, we also tested the ability of the *S. pneumoniae* strain T4 and its isogenic mutants to grow at 28 °C *in vitro*. This experiment showed no differences in the growth rates of the *S. pneumoniae* strains incubated at 28 °C and 37 °C.

In original communication I, the infectivity of the wild type *S. pneumoniae* strain T4 was tested in 2 days old zebrafish embryos by survival assays with various doses (100 cfu, 1000 cfu and 10000 cfu) of the bacterium. After an intravenous injection of T4 into the zebrafish embryos, the infection progressed rapidly, the highest mortality occurring by 48 hpi (**Figure 4A**, Figure 1A in I). Importantly, the outcome of the infection was dose-dependent with an LD50 dose of ~100 cfu (combined mean survival 49 %). In original communication III, the corresponding dose-dependent disease progression was observed in adult zebrafish after intraperitoneal and intramuscular infections (**Figure 4B**, Figures 1A-B in III). The infection of adult zebrafish with a moderate dose of T4 (2.5×10^6 cfu) resulted in increased survival rates after the intramuscular infection (66 %) compared to the intraperitoneal infection (44 %) (Figures 1A-B in III). The highest mortality rate was also reached at a later time point for the intramuscular (48-72 hpi) than for the intraperitoneal infection (24-48 hpi) indicating an overall delayed disease progression in the local infection route compared to systemic administration. With both infection routes, the fish showed common signs of a severe infection; edema at the infection site, aberrant swimming behavior and increased respiration. Moreover, in a local intramuscular injection, fish developed hypopigmented lesions and severe tissue necrosis at the dorsal muscle, previously also observed in other streptococcal infections (Neely et

al., 2002). Intriguingly, some of the challenged fish also had visible cerebral edema, a sign of meningitis (Figure 4 in III).

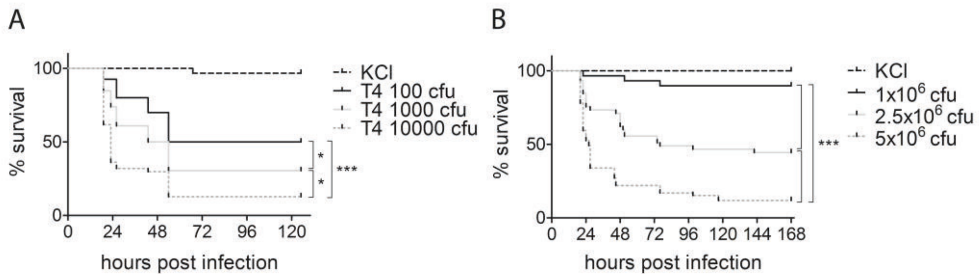

Figure 4. *S. pneumoniae* causes a fulminant systemic infection in zebrafish. A) The survival of 2 dpf zebrafish embryos from a *S. pneumoniae* infection is dose-dependent. B) The survival of adult zebrafish after an intraperitoneal injection of *S. pneumoniae* is dose-dependent. The graphs represent the collated results from three individual experiments (n=24 in A, n=15 in B) and the asterisks depict the statistically significant difference of $*p<0.01$ and $***p<0.0001$. *cfu=colony forming units*. Figure A modified from original communication I, and B from original communication III.

Since the survival assays showed that zebrafish could tolerate the small and moderate doses of *S. pneumoniae*, we further examined the ability of the embryos and adult fish to clear the bacteria after the challenge. For this, we followed the change in the bacterial burden in both models during the infection. When the bacterial counts in dead and live embryos were determined at various time points after the infection (original communication I), the rapid replication of *S. pneumoniae* in the dying embryos was observed during the 48-hour follow-up (**Figure 5A**, Figure 2A in I). Importantly, the bacterial counts in surviving embryos started to decrease at 24 hpi and approached 0 cfu by 48 hpi. Similarly, the bacterial counts in the blood of adult zebrafish, after an intraperitoneal infection (original communication III), reached their maximum at 6 hpi and decreased to 0 cfu by 48 hpi in live fish (**Figure 5B**, Figure 1C in III).

S. pneumoniae is one of the leading causes of bacterial meningitis in humans (Doran et al., 2016). As mentioned above, we observed visible signs of meningitis in adult zebrafish during the survival assay and, therefore, we wanted to test whether *S. pneumoniae* can infiltrate from the blood to the brain in zebrafish. For this, we extracted the whole brain from intraperitoneally infected ($2.5x10^6$ cfu) fish and determined the bacterial loads at several time points. As a result, we noticed the presence of bacteria in the brain already at 2 hpi, after which the bacterial loads increased until 12-24 hpi (**Figure 5B**, Figure 5 in III). Noteworthy, the bacterial burden in the brain correlated with the bacterial burden in the blood. From these

experiments concerning the infectivity of *S. pneumoniae* in zebrafish, we concluded that this human pathogen is able to cause a dose-dependent infection in both zebrafish embryos and adult fish. The infection is characterized by fulminant bacteremia and the dissemination of bacteria into the brain. Importantly, zebrafish are also capable of restricting the replication of *S. pneumoniae* by some surveillance mechanisms, leading to the clearance of bacteria from the bloodstream and the survival of the fish.

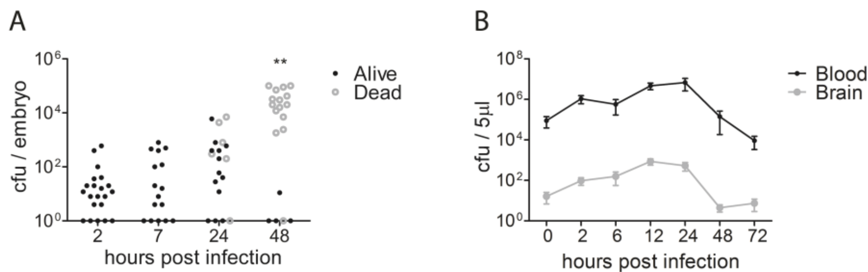

Figure 5. The bacterial burden in zebrafish embryos and adult zebrafish after a *S. pneumoniae* infection. A) The surviving embryos are able to clear *S. pneumoniae* by 48 h post an intravenous injection of 100 cfu. A dot represents the bacterial load in single live (black dot) or dead (grey circle) embryos. The asterisks depict the statistically significant difference between the live and dead fish. B) The bacterial burden in the brain of adult zebrafish correlates with the bacterial burden in the blood after an intraperitoneal injection of 2.5×10^6 cfu of T4. The figure represents the mean (+standard deviation) bacterial burden at each time point. Both graphs represent the collated results from at least three individual experiments (n=5 in A, n=10 in B). *cfu=colony forming units*. Figure A modified from original communication I, and B from original communication III.

5.2 The pathogenesis of *S. pneumoniae* in zebrafish is mediated by known virulence factors (I, III)

The first steps in the progression of an infectious disease are determined by the interplay between the host's innate immune response and the pathogen's virulence factors. Several *S. pneumoniae* virulence determinants have been shown to mediate specific interactions with the host and contribute to the progression of the infection (Kadioglu et al., 2008). To examine whether the same interactions play a role in the progression of a *S. pneumoniae* infection in zebrafish, we first compared the virulence capacity of the wild type (WT) *S. pneumoniae* (T4) and the T4 mutants lacking the capsule (T4R), pneumolysin (T4Δ*ply*), autolysin A (T4Δ*lyt*), or the pilus (T4Δ*rlrA*) in zebrafish embryos (original communication I) and adult zebrafish (original communication III). The polysaccharide capsule of *S. pneumoniae* appears essential

during the infection due to its antiphagocytic activity, and therefore, the unencapsulated strains of *S. pneumoniae* have been shown to be avirulent in several animal models (Hyams et al., 2010a; Morona et al., 2004; Briles et al., 1992). Similarly, the capsule deficiency led to an almost complete loss of virulence in both of our models, with the survival percentages of 97 % for T4R and 41 % for T4 in embryos (**Figure 6A**, Figure 3 in I) and 73 % for T4R and 38 % for T4 in adults (Figure 2 in III). In addition, in contrast to the wild type T4, the unencapsulated mutants of *S. pneumoniae* failed to induce TNF-α mediated inflammation in zebrafish embryos (**Figure 6B**, Figure 4B in I).

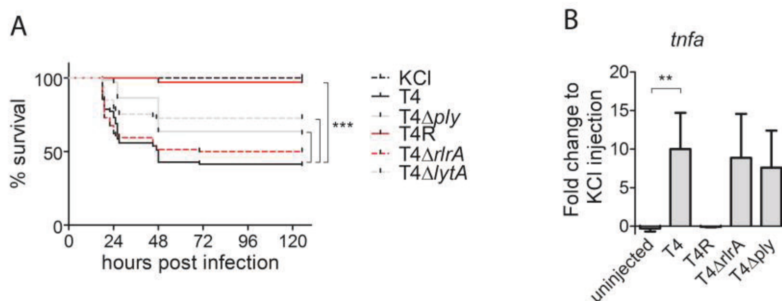

Figure 6. *S. pneumoniae* pathogenesis is mediated by conserved host-pathogen interactions. A) *S. pneumoniae* mutants lacking the capsule (T4R), the pneumolysin (T4Δ*ply*), and the autolysin LytA (T4Δ*lytA*) are attenuated in zebrafish embryos compared to the wild type *S. pneumoniae* (T4). The pilus mutant (T4Δ*rlrA*) shows virulence levels comparable to wild type. In the survival assay, 2 dpf zebrafish embryos were infected with 100 cfu of wild type T4 or the mutants. B) The expression of *tnfa* is induced 18 h post a T4 but not a T4R challenge. The *tnfa* expression was measured with qRT-PCR from a pool of 10 zebrafish embryos infected 2 dpf with 100 cfu. The *tnfa* expression was normalized to the expression of *actb1* and the figure represents the mean (+ standard deviation) fold change in the expression compared to KCl injected embryos. The graphs represent the collated results from three individual experiments (n=24 in A and n=3 in B) and the asterisks depict the statistically significant differences of ***p<0.0001 and **p<0.001. Figures modified from original communication I.

Similar to the unencapsulated *S. pneumoniae* mutants, the virulence of the mutants deficient in pneumolysin (T4Δ*ply*) and autolysin A (T4⊿*lyt*) was attenuated in zebrafish. The observed mean survival percentages were 62 % for T4Δ*ply* and 73 % for T4⊿*lyt* compared to 41 % for T4 in embryos, (**Figure 6A**, Figure 3 in I) and 46 % for T4Δ*ply* and 73 % for T4⊿*lyt* compared to 38 % for T4 in adult fish (Figure 2 in III). Another virulence factor of *S. pneumoniae*, the pilus, has been shown to mediate the adhesion of the bacteria to the epithelium during colonization (Barocchi et al., 2006), but to also stimulate bacterial intake by macrophages during the invasive disease (Orrskog et al., 2012). In our models of a systemic *S. pneumoniae* infection, the pilus seems to have a minor or dispensable role, since the injection of the pilus

deficient mutant or the wild type T4 resulted in equivalent survival rates in zebrafish embryos (T4 41 % versus T4Δ*rlrA* 50 %) and adult fish (T4 38 % versus T4Δ*rlrA* 42 %). Overall, however, these results indicate that a *S. pneumoniae* infection follows a conserved pathogenic mechanism in zebrafish.

5.3 The innate immune response to a *S. pneumoniae* infection in zebrafish embryos is well conserved (I, IV)

5.3.1 The clearance of *S. pneumoniae* in zebrafish embryos is dependent on myeloid cells and phagocytosis (I)

The observed conserved mechanisms of *S. pneumoniae* pathogenesis in fish and mammals tempted us to test whether a similar conservation could also be seen in the host response. Therefore, we started to examine the innate immune response to *S. pneumoniae* in zebrafish embryos. In humans, macrophages and neutrophils are the first immune cells to encounter invading pathogens during the infection and are the main cells responsible for the phagocytosis and intracellular killing of *S. pneumoniae* (Dockrell et al., 2012). To characterize the role of these cells in zebrafish during a *S. pneumoniae* infection, we manipulated the myeloid cell number and function in zebrafish embryos with the antisense morpholino oligonucleotide (MO) gene knockdown method. The MO knockdown of *spi1b* (previous name *pu.1*), previously shown to deplete myeloid cells (Rhodes et al., 2005), predisposed the zebrafish embryos to a significantly more severe *S. pneumoniae* infection with a faster disease progression (most deaths occurring by 24 hpi, compared to 48 hpi for WT embryos) and higher mortality (final survival percent 15 % compared to 51 % for WT) (**Figure 7A**, Figure 5 in I). A similar survival pattern (most deaths by 24 hpi, final survival percent 30 %) was observed in zebrafish embryos where *wasa* and *wasb* (previous names *wasp2* and *wasp1,* respectively) were knocked down, which has previously been shown to hamper the migration of immune cells and phagocytosis, due to a defect in the function of the actin cytoskeleton (Cvejic et al., 2008).

Since the MO studies gave us a clear indication of the importance of phagocytosing cells in the clearance of *S. pneumoniae* in zebrafish, we next visualized the function of myeloid cells during a *S. pneumoniae* infection *in vivo*. For this, we employed the transgenic zebrafish reporter line Tg(Spi1:EGFP), which expresses GFP under the promoter for the myeloid transcription factor gene *Spi1b* (Ward et

al., 2003). By using fluorescently labelled *S. pneumoniae*, we were able to visualize the interaction between the myeloid cells and bacteria *in vivo* in real-time. At 1-3 hours post an intravenous infection, *S. pneumoniae* were seen to co-localize with myeloid cells in the circulation (**Figure 7B-C**, Figure 4C-D in I). More importantly, myeloid cells with cell wall protrusions were observed to reach out for the bacteria while some bacteria were found ingested by the myeloid cells. Together, these studies provided proof for the existence of a specific interaction between *S. pneumoniae* and zebrafish myeloid cells and emphasized the conservation of the important role of phagocytosis in the clearance of *S. pneumoniae* during a septic infection.

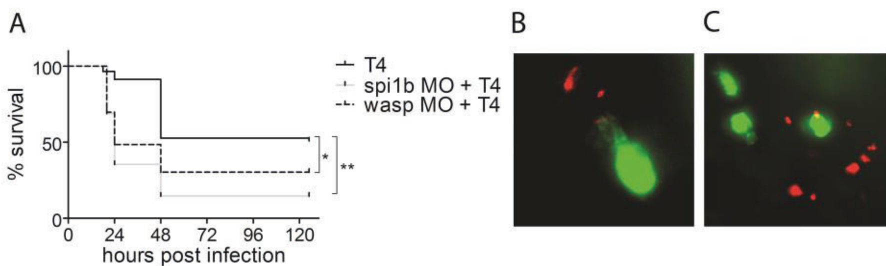

Figure 7. The survival of zebrafish embryos from a *S. pneumoniae* infection is dependent on myeloid cells and phagocytosis. A) The survival of zebrafish embryos is decreased upon the morpholino knockdown (MO) of *spi1b* or *wasa/b* compared to wild type embryos. MOs were injected into 1-cell stage zebrafish eggs, after which the morphant and the wild type embryos were intravenously infected at 2 dpf with 50 cfu of T4. The graph represents the collated results from three individual experiments (n=24 in each) and the asterisks depict the statistically significant differences of *p<0.01 and **p<0.001. B-C) Myeloid cells (green) are seen to reach (B) for the *S. pneumoniae* (red) and engulf (C) the bacteria 1-3 hpi. A live fluorescence image was taken of Tg(spi1:EGFP) transgenic zebrafish embryos infected with 100 cfu of Baclight™ Red stained T4. The experiment was repeated at least three times. Figures A and C modified from original communication I. Figure B from original communication I (Rounioja et al., 2011).

5.3.2 A transcriptome analysis reveals well-conserved innate immune response to *S. pneumoniae* in zebrafish embryos (IV)

To characterize the zebrafish innate immune response to *S. pneumoniae* in more detail, we carried out a whole genome transcriptome analysis (RNA sequencing) for wild type zebrafish embryos infected with 100 cfu of T4. Altogether, among the 129 differentially expressed genes at 18 hours post a *S. pneumoniae* infection, 57 protein coding genes were upregulated and 33 protein coding genes were downregulated at least 3-fold in wild type embryos compared to a KCl injection (Table 1, Table S1, and Table S3 in IV). Importantly, a notable proportion (26/57 genes) of the

upregulated protein coding genes were associated with the innate immune response, falling into the categories of the complement system (9/26 genes), acute phase response (3/26 genes), antimicrobial peptides (4/26 genes), immune signaling and regulation (4/26), cell migration and chemotaxis (3/26 genes), and macrophage function (3/26 genes) (Figure 1 and Table 1 in IV). On a smaller scale, the expression of genes associated with metabolic processes (13/57 genes), other functions (6/57 genes), or unknown function (12/57 genes) were also induced upon the infection. Of the downregulated protein coding genes, 7/33 genes have previously been associated with development and reproduction, 1/33 with metabolic processes, and 5/33 with other processes. For 20/33 downregulated protein coding genes, on the other hand, no functional prediction was found.

In particular, the expression of genes associated with the complement system was induced at the chosen time point suggesting their important role in the innate immune response to *S. pneumoniae* also in this organism. The induced complement-related genes coded for four C3 variants (*c3a.1, c3a.2, c3a.3, c3a.6*), a putative counterpart for human C2 (*si:ch1073-280e3.1*), C1r (*c1r*), Complement factor B (*cfb*), and two negative regulators, Factor H like 5 (*cfhl5*) and the C1 inhibitor (*serping1*) (**Figure 8**, Figure 2 in IV). The other induced genes associated with the activation of complement were the *crp* and *crp3*, the two zebrafish variants for the human CRP gene.

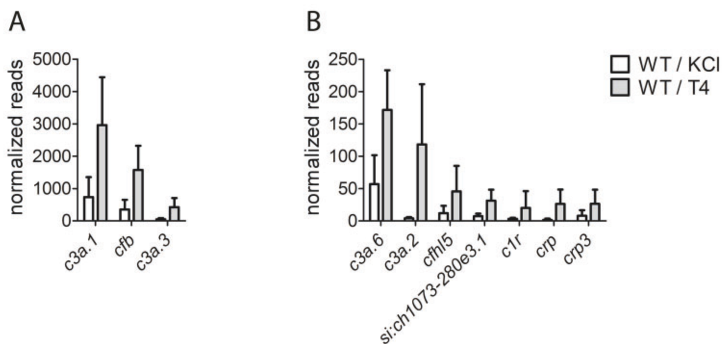

Figure 8. Complement -related genes induced at 18 hours post a *S. pneumoniae* infection in zebrafish embryos. A+B) The figures represent the normalized read counts in T4 or KCl injected wild type (WT) embryos for the selected genes. The bars represent the mean read counts from 3 replicates (+ standard deviation) obtained by mRNA sequencing. The 3-fold increase in the expression was set as a threshold for induction. *c3a.1=complement component 3, variant 1; c3a.2=complement component 3, variant 2; c3a.3=complement component 3, variant 3; c3a.6=complement component 3, variant 6; si:ch1073-280e3.1=a putative homolog for human complement component 2*; *C1r= complement component 1, r subcomponent; cfb=complement factor B; cfhl5=complement factor H like 5; and serping1= serpin peptidase inhibitor, clade G (C1 inhibitor), member 1*. Figures modified from original communication IV.

5.3.3 The lack of CRP is a potential predisposing factor for a severe *S. pneumoniae* infection in zebrafish embryos (IV)

To identify zebrafish genes important in the defense against *S. pneumoniae* in an unbiased manner, we carried out a forward genetic screen (original communication IV). In the screen, a gene-breaking Tol2 transposon -based mutagenesis (Clark et al., 2011) was used to create 126 fish lines carrying random mutations, after which these lines were screened for their altered susceptibility to a *S. pneumoniae* infection by survival assays. In the assays, 100 cfu of T4 was intravenously injected into mutant and WT zebrafish embryos at 2 dpf and the mutant lines showing significantly decreased survival compared to WT were selected for further study.In this study, we chose 3 mutant lines with decreased survival to be analyzed more thoroughly. The survival results for the mutant lines with decreased survival (mutant94, mutant14, mutant460) and a line with the survival rate equivalent to WT (mutant286) are shown in **Figure 9A**. One of the lines, mutant94, had a particularly strong phenotype with a 28 % survival compared to the WT survival of 83 % upon infection with T4. The survival rates for the other mutant lines were 45 % for mutant14, 39 % for mutant460, and 80 % for mutant286. To test whether the hampered survival of the hypersusceptible mutant lines was due to the defects in the clearance mechanisms or to the overall weakness, we measured the bacterial burden in these lines during the infection. The results showed that the mutant94 embryos had significantly elevated bacterial counts at 18 hpi compared to the WT, while the bacterial burdens in the mutant14 and the mutant460 embryos were equivalent to those of the WT (**Figure 9B**). Therefore, we concluded that, in contrast to the mutant14 and mutant460 which likely had decreased tolerance to infection, the mutant94 embryos had hampered defense mechanisms against T4 and thus developed a more severe *S. pneumoniae* infection.

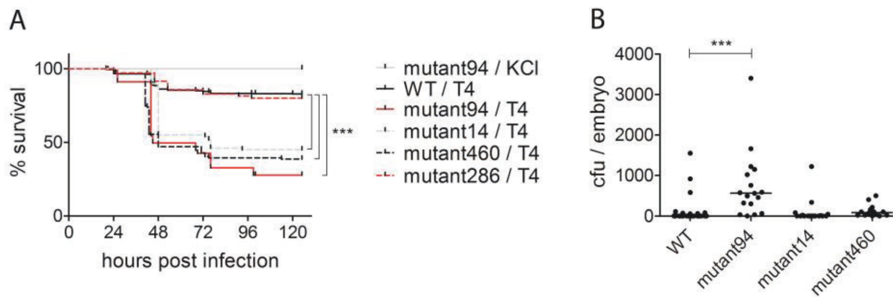

Figure 9. *S. pneumoniae* causes a more severe infection in mutant94 embryos compared to wild type. A) The survival of mutant94 embryos is impaired in a *S. pneumoniae* infection. In the assay, 2 dpf embryos were intravenously injected with 100 cfu of T4. The survival of mutant 94 was compared to the wild type (WT) and to other mutant lines (mutant460, mutant14, and mutant286). B) The mutant94 embryos have an increased bacterial burden at 18 hpi compared to wild type embryos. The zebrafish embryos were intravenously infected with 100 cfu of T4 and the bacterial counts were determined at 18 hpi. A dot represents the bacterial count in a single embryo and the line depicts the median. In both figures, the graphs represent the collated results from two or three individual experiments (n=24-48 in A and n=5 in B) and the asterisks depict the statistically significant difference of ***p<0.001. *cfu=colony forming units*. Figures modified from original communication IV.

To identify the factors predisposing mutant94 to a more severe infection, we used total RNA sequencing to identify the differentially expressed genes in mutant94 embryos upon a *S. pneumoniae* infection. Overall, of the 57 protein coding genes upregulated in *S. pneumoniae* infection, 9 genes were downregulated at 18 hours post a T4 infection in the mutant94 embryos compared to the infected WT embryos (Table S5 in IV). As the most striking difference, the expression of *crp* showed an 83-fold reduction in mutant94 compared to the WT. The other two of the most downregulated genes were the zebrafish specific genes *BX548011.1* and *si:dkey-9c18.3* with 43-fold and 29-fold reduction in expression, respectively. In contrast, the rest of the downregulated genes showed more subtle changes in expression (from 3.5- to 7-fold). Of these differences, the defect in the production of Crp, shown to predispose mice to a more severe *S. pneumoniae* infection (Simons et al., 2014), appeared as the most probable explanation for the decreased resistance of mutant94 embryos.

In humans, the expression of *CRP* by the hepatocytes is induced by the pro-inflammatory cytokines IL6, IL1B, and TNF (Slaats et al., 2016; Gruys et al., 2005; Zhang et al., 1995). Therefore, to further test our hypothesis, we checked the expression levels of *il6*, *il1b*, and *tnfa* from our transcriptome data. We noticed that all the genes were induced in T4 infection in WT embryos and that their expression levels were even more elevated in mutant94 embryos compared to the WT (2-fold increase in *il6*; 3-fold increase in *il1b*; 4-fold increase in *tnfa* compared to WT)

(**Figure 10**, Figure 4 in IV). These observations indicate that the lack of *crp* production in the mutant94 embryos was not due to the defect in the general inflammatory response and that the slight increase in the expression of these mediators may be due to a compensatory response to the hampered *crp* expression.

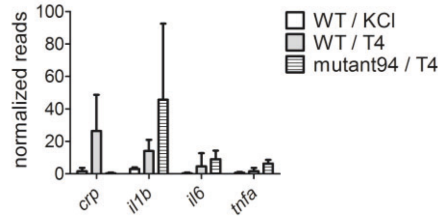

Figure 10. Mutant94 embryos have defects in *crp* expression. The figure represents the normalized read counts for *crp*, *il1b*, *il6*, and *tnfa* in T4 or KCl injected wild type (WT) embryos or T4 infected mutant94 embryos. The bars represent the mean read counts from 3 replicates (+ standard deviation) obtained by mRNA sequencing. The figure is modified from original communication IV.

In conclusion, to study the innate immune response to *S. pneumoniae* in zebrafish we utilized forward and reverse genetics, *in vivo* imaging of immune cells, and a whole genome transcriptome analysis. Overall, our findings indicate a well conserved host response to *S. pneumoniae* in zebrafish embryos and validates the use of this model in the closer study of the defense mechanisms against the pathogen. To sum up, the methods used for the characterization of the innate immune response to *S. pneumoniae* in zebrafish embryos and the obtained results are collected into **Figure 11**.

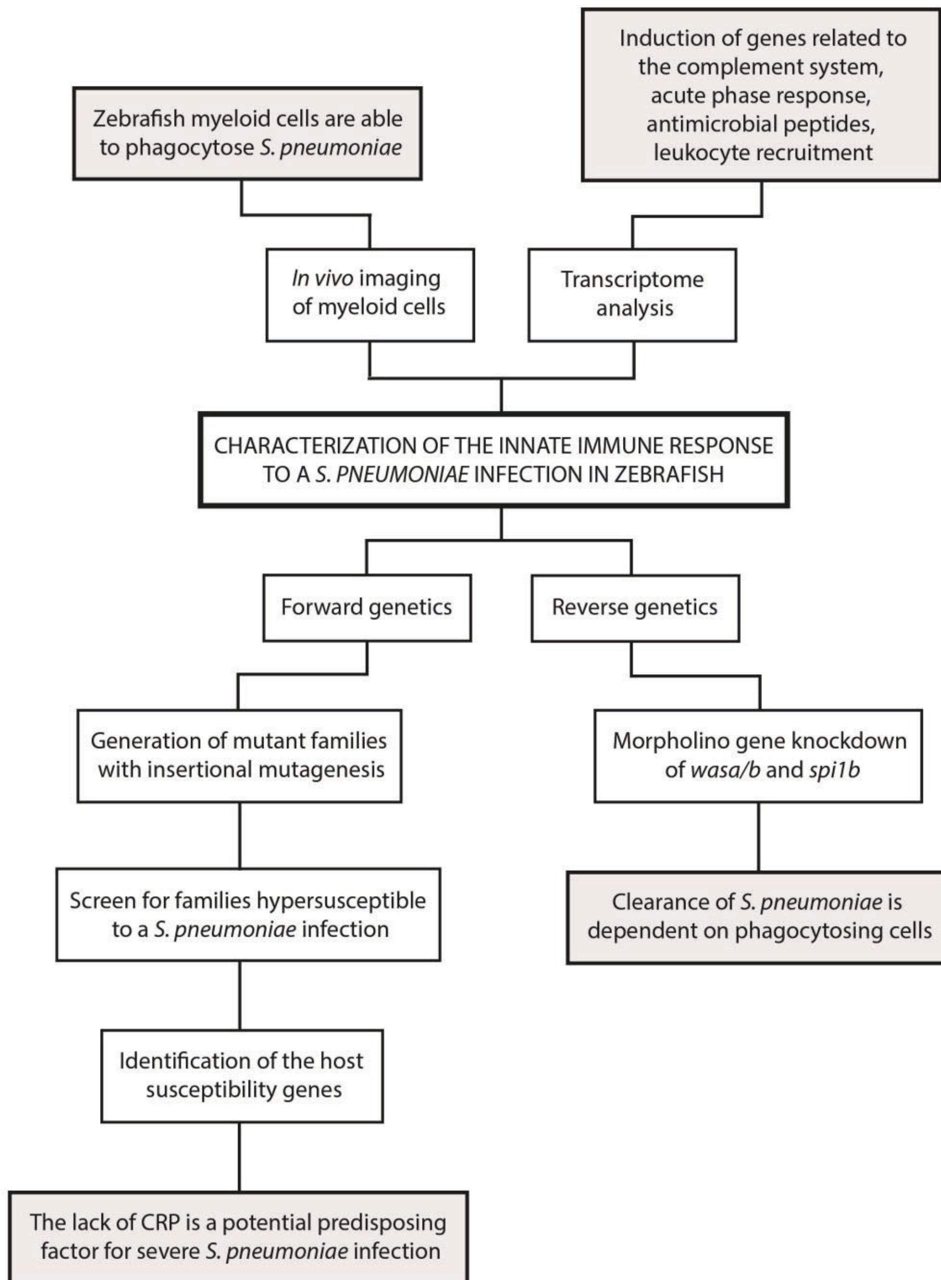

Figure 11. Characterization of the innate immune response to a *S. pneumoniae* infection in zebrafish embryos. A summary of the methods and results.

5.4 The adaptive immune response does not seem to play a role in the defense against *S. pneumoniae* in zebrafish (III)

A major benefit of adult zebrafish as an infection model comes from the highly developed adaptive immune system with functional lymphocytes and the ability to mount an immunological memory. Moreover, zebrafish has been proven to serve as a suitable model for the evaluation of vaccine candidates (e.g. Oksanen et al., 2013; Lam et al., 2004). Therefore, the protective role of the adaptive immune response against *S. pneumoniae* in adult zebrafish was investigated by infecting Rag1$^{-/-}$ mutant zebrafish, which lack active T and B lymphocytes. Due to the defective adaptive immune response, the Rag1 deficiency in humans has been shown to be linked to a higher susceptibility to infections and similar results have been obtained from the studies using Rag$^{-/-}$ mice (e.g. Ribes et al., 2016; Geier et al., 2015; Blair et al., 2005). However, when the survival of the Rag1$^{-/-}$ fish was compared to the WT fish after an intraperitoneal injection of *S. pneumoniae*, we saw no differences in the survival of these two lines (final survival of 56 % for Rag1$^{-/-}$ and 59 % for WT) (**Figure 12A**, Figure 7A in III). Moreover, the immunization of adult zebrafish with heat killed T4, live T4, or live T4R three weeks prior to the infection failed to confer any significant protection (**Figure 12B**, Figure 7B in III). From these studies, we then concluded that, unlike in mammals, the defense against a *S. pneumoniae* infection in zebrafish is mainly dependent on the innate arm of immunity, while the adaptive arm plays a minor or no role in our model of a *S. pneumoniae* infection.

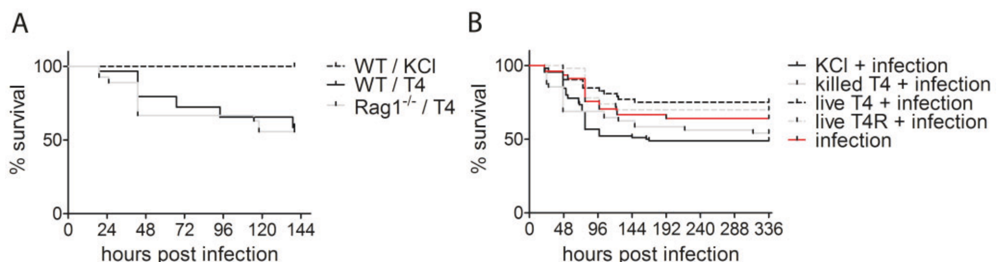

Figure 12. The adaptive immune system has a limited role in the host response against *S. pneumoniae* in zebrafish. A) The zebrafish Rag1$^{-/-}$ mutants which are deficient in VDJ recombination and thus lack an adaptive immune response survived comparably to wild type (WT) fish. In the survival assay, wild type adult zebrafish or the Rag1$^{-/-}$ mutant fish were intraperitoneally infected with 2.5x10^6 cfu of T4. B) The immunization of adult zebrafish does not confer protection against a *S. pneumoniae* infection. The fish were immunized by intramuscular immunization with heat-killed T4 (1x10^4 cfu), a sublethal dose of live T4 (5x10^4 cfu), or the unencapsulated mutant T4R (1.5x10^6 cfu), and infected intraperitoneally 3 weeks post immunization with 2.5x10^6 cfu of T4. Control fish received KCl immunization or no immunization prior to infection. The graphs represent the collated results from three individual experiments (n=20 in each). Figures modified from original communication III.

5.5 A *S. agalactiae* infection in adult zebrafish is characterized by fulminant bacteremia and the dissemination of bacteria into the brain (II)

Meningitis is a typical clinical manifestation of a neonatal *S. agalactiae* infection and is usually preceded by an overwhelming sepsis. To investigate the pathology of a *S. agalactiae* infection in zebrafish, the wild type FIM314 strain of *S. agalactiae* was administered into adult zebrafish with either an intraperitoneal or an intramuscular injection. Both routes of infection caused a rapidly progressing disease, which ultimately led to death in a dose-dependent manner. The infection kinetics were similar to *S. pneumoniae*, most of the deaths occurring by 48 h post an intraperitoneal infection and by 72 h post an intramuscular infection (**Figure 13A**, Figure 1A-B in II). The FIM314 strain appeared extremely virulent in zebrafish with a LD50 dose of ~100 cfu (56 % survival) for an intraperitoneal infection and ~10000 cfu (53 % survival) for an intramuscular infection. After an intraperitoneal injection of 100 cfu of FIM314, the high mortality was accompanied by an increasing level of bacteremia until 48 hpi (Figure 1C in II). The visible signs of the infection included damage at the injection site, common signs of illness, as well as swollen brain cavities and cerebral edema indicating high bacterial loads in the brain (Figure 2 in II).

To examine the ability of *S. agalactiae* to invade the brain in zebrafish, we measured the bacterial loads in the blood and brain over time. After an intraperitoneal injection of $1x10^5$ cfu of FIM314, bacteria were found in the blood immediately after the injection, while significant numbers of bacteria were detected in the brain at 6 hpi (**Figure 13B**, Figure 3A in II). In general, the level of bacteremia correlated with the bacterial counts in the brain, with a rapid increase in both from 6 hpi onwards. Bacterial meningitis is usually associated with an overwhelming inflammatory response in the brain also responsible for the severe tissue damage and sequelae (Barichello et al., 2013). Similarly, we observed an induction in the expression of *il1b* (15-fold increase) at 48 hpi and *il6* (9-fold increase) at 24 hpi in the zebrafish brain upon a FIM314 infection compared to KCl injected fish (Figure 3B-C in II). The gradual increase in the *il1b* expression during the 48h follow-up is shown in **Figure 13C**. With these results, we concluded that the pathology of a *S. agalactiae* infection in adult zebrafish resembles the neonatal *S. agalactiae* infection leading to a fulminant bacteremia and acute bacterial meningitis associated with high bacterial loads in the brain and the characteristic meningeal inflammation.

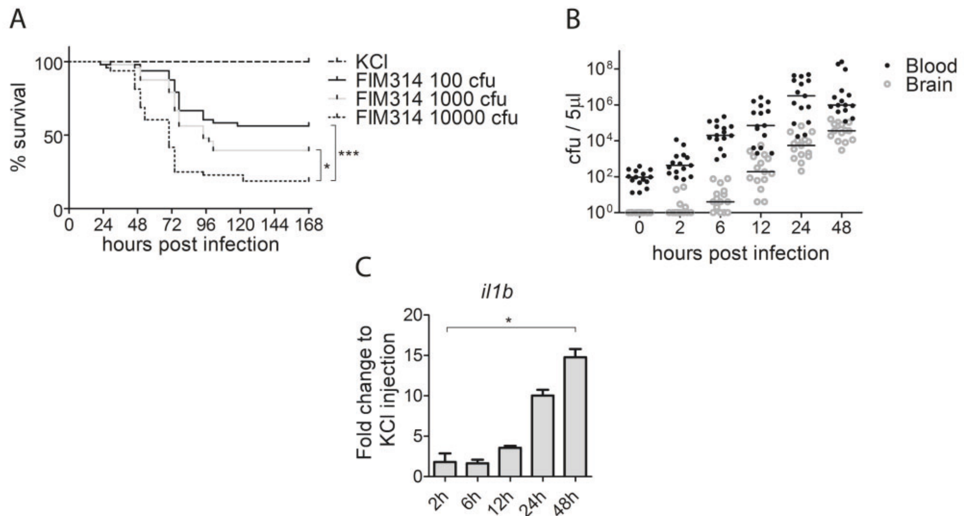

Figure 13. *S. agalactiae* causes fulminant bacteremia and meningitis in adult zebrafish. A) The survival of adult zebrafish from a *S. agalactiae* infection is dose-dependent. In this survival assay, adult zebrafish were intraperitonealy infected with various doses of the FIM314 strain of *S. agalactiae*. B) *S. agalactiae* is able to disseminate from the blood into the brain in adult zebrafish. Fish were intraperitoneally infected with $1x10^5$ cfu of FIM314 after which the blood samples and the whole brain were collected. A dot represents the bacterial load in 5 µl of blood (black dots) or brain homogenate (grey circles) from single fish. The line depicts the median bacterial count. The graph shows the collated results from three individual experiments (n=5 in each). C) The expression of *il1b* is induced in the brain of adult zebrafish by 48 h post a *S. agalactiae* infection. The fish were intraperitoneally infected with $1x10^5$ cfu of FIM314 and the expression levels were measured from dissected brains with qRT-PCR. The *il1b* expression levels were normalized to *actb1* expression. The graph shows the mean fold change (+standard deviation) in expression compared to KCl injected fish from three individual experiments. All of the graphs represent the collated results from three individual experiments (n=15 in A, n=5 in B, and n=3) and the asterisks depict the statistically significant differences of *p<0.01 and ***p<0.0001. *cfu=colony forming units*. Figures modified from original communication II.

5.6 *S. agalactiae* virulence factors contribute to the development of meningitis in zebrafish (II)

The two determining factors in the onset of meningitis are the survival of the bacteria in the bloodstream and the ability to penetrate the blood-brain barrier, and several specific interactions between the host epithelial and immune cells and the bacteria have been found to contribute to these events (Barichello et al., 2013). Thus, we next asked whether the interactions known to promote the early steps in the onset of meningitis in rodents would also affect the outcome of the infection in zebrafish.

For this, we compared the virulence capacity of three *S. agalactiae* mutants, lacking either the antiphagocytic polysaccharide capsule (ΔcpsD), the cytolytic β-hemolysin (ΔcylE), or the CovS/CovR regulatory system (ΔcovSR) to the corresponding WT strain NEM316. After an intraperitoneal injection of $1x10^6$ cfu of each strain, we noticed a significant increase in the survival of zebrafish infected with ΔcpsD (84 % survival), ΔcylE (82 % survival), and ΔcovSR (89 % survival) compared to the WT NEM316 (64 % survival) (**Figure 14A**, Figure 4 in II). Consistently, the bacterial amounts recovered from the blood and brain at 24 hpi were significantly lower in zebrafish infected with these mutants compared to the fish infected with WT *S. agalactiae* (**Figure 14B**, Figure 5A in II) indicating a hampered ability to survive in the blood and/or cross the BBB. Therefore, we concluded that the same interaction at the host-pathogen interphase in the blood and at the BBB promote the pathogenesis of S. agalctiae in zebrafish as has previously been detected in other animal models of *S. agalactiae* bacteremia and meningitis.

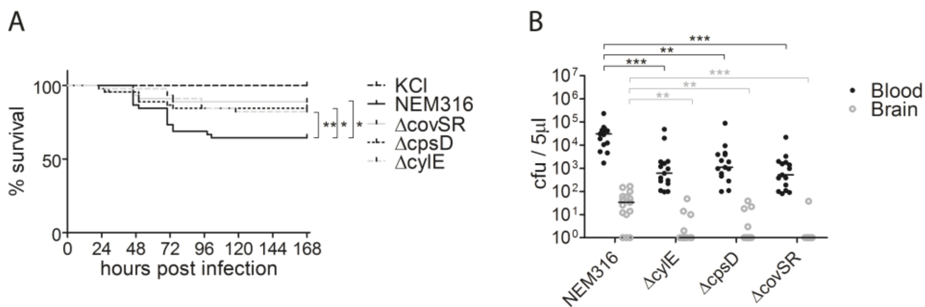

Figure 14. Known virulence factors of *S. agalactiae* contribute to the severity of bacteremia and meningitis in zebrafish. A) *S. agalactiae* mutants lacking the β-hemolysin (ΔcylE), the capsule (Δcps*D*), or the CovS/CovR regulatory system (ΔcovSR) are attenuated in their virulence capacity in adult zebrafish compared to the corresponding wild type strain NEM316. In the survival assay, adult zebrafish were intraperitoneally infected with $1x10^6$ cfu of wild type or the mutant bacteria. B) The *S. agalactiae* mutants cause lower levels of bacteremia and less dissemination of bacteria to the brain compared to the wild type *S. agalactiae*. Fish were intraperitoneally infected with $1x10^6$ cfu of NEM316 or mutants and at 24 hpi the blood samples and whole brains were collected. A dot represents the bacterial load in 5 µl of blood (back dots) or a brain homogenate (grey circles). The line depicts the median bacterial count. The graph shows the collated results from three individual experiments (n=15 in A, n=5 in B) and the asterisks depict the statistically significant differences of *p<0.01, **p<0.001, and ***p<0.0001). *cfu=colony forming units*. Figures modified from original communication II.

6 DISCUSSION

6.1 Perspectives on the suitability of the zebrafish model for studying streptococcal infections

Due to their high invasiveness and associated mortality, streptococcal bacteria are undoubtedly one of the most important groups of human pathogens. Despite the intensive study of the pathogenic mechanisms of streptococci in the past and present, much remains to be elucidated. The bacterial determinants contributing to the pathogenesis are strictly regulated and their expression restricted to certain tissues and stages of the infection. In addition, multiple host cells and soluble mediators participate in the host response in a coordinated way. Together, the host and the bacterial factors form a complex network of interactions which cannot be replicated in an *in vitro* experimental setting. In contrast, an appropriate animal model is needed to provide more natural conditions for the characterization of these factors, as well as for testing new drugs and vaccine antigens.

For most streptococci, including *S. agalactiae* and *S. pneumoniae*, the most commonly used animal models are rodents, particularly the mouse. As mammals, mice exhibit a relatively high level of genetic homology, and share the basics of anatomy and physiology with humans, and therefore, are well suited for the study of most human diseases. The inbred mouse strains, with minimum individual variation and thus more uniform responses to experimental treatments, provide a practical research tool with reproducible results and high statistical power. In contrast, zebrafish cannot be inbred for several generations and provide a more heterogenous population, mimicking the natural variation in humans. In the case of *S. pneumoniae* and *S. agalactiae* infections, mice have been successfully used to reproduce the complete disease spectrum of these streptococcal bacteria, including otitis media, urinary tract infections, pneumonia, sepsis, and meningitis (Huang et al., 2016; Leclercq et al., 2016; Gendrin et al., 2015; Ulett et al., 2010; Chiavolini et al., 2008; Doran et al., 2003; Melhus and Ryan 2003; Saeland et al., 2000). In addition, factors promoting colonization in the nasopharynx (Krone et al., 2013; Balachandran et al., 2002) and in the genital tract (Baker et al., 2017; Patras et al., 2015; Sheen et al., 2011) as well as the subsequent events leading to invasion have also been elucidated. Mice

have also been utilized in the analysis of post-infectious sequelae associated with meningitis, including brain damage and hearing loss (Grandgirard et al., 2007). However, depending on the bacterial strain used in the study, a high variation in the outcome of the infection has been observed in mice. Wild type mice seem to be relatively resistant to some *S. pneumoniae* strains and the infectivity of clinical isolates does not always recapitulate the virulence in humans (Sandgren et al., 2005; Briles et al., 1992). In general, however, mice models have provided invaluable information about the host and bacterial factors related to *S. pneumoniae* and *S. agalactiae* infections, and this knowledge has led to the development of current treatments or vaccines against both streptococci.

Despite the obvious positive impact on human health, mammalian models also have issues concerning their cost and ethicality and are therefore not the best choice for experiments requiring large numbers of animals. It is also important to note that no animal model is perfect in mimicking the host-pathogen interactions in a human infection, and that it is the combination of different models that will provide us with the most comprehensive view of the pathogenesis. In this thesis, I have described a novel vertebrate model, the zebrafish, for the study of *S. pneumoniae* and *S. agalactiae* infections, that provides a more ethical but also practical, easy, and cost-effective alternative for the traditional rodent models. Among the most beneficial characteristics of this model are the unique methodologies for genetic manipulation and *in vivo* imaging as well as the suitability for large-scale experiments, such as chemical and genetic screens. These characteristics were also utilized in this thesis, especially in the form of a medium-scale genetic screen described in original communication IV. In addition, during this project, the choice between the zebrafish embryo and adult fish was weighted, since both models provide special benefits for the study of infectious diseases, namely the practicality and the versatile methodology of the embryos and the adaptive immune system of the adults.

For the zoonotic *S. agalactiae*, which naturally infects both fish and humans, the zebrafish model is a rather logical choice for an animal model. In fact, other fish species, including other teleosts, have frequently been used in the elucidation of the host response to *S. agalactiae* (Zhu et al., 2017; Peng et al., 2017), as well as *S. agalactiae* virulence factors (Barato et al., 2016; Li et al., 2014), and vaccine candidates for aquaculture purposes (Cai et al., 2017; Zhang et al., 2017; Ma et al., 2017; Liu et al., 2016). The adaptation of this pathogen to both fish and humans may also reflect the conserved host-pathogen interactions in zebrafish. In contrast, *S. pneumoniae* is currently known to be a human-specific pathogen in nature and may lack some species-specific interactions required for the pathogenesis in zebrafish, due to

different environmental conditions, for example. However, several previous examples of other human pathogens, such as *S. pyogenes* (Neely et al., 2002), *Staphylococcus aureus* (Prajsnar et al, 2008), and *Salmonella typhimurium* (van der Sar et al., 2003), as well as the study represented in this thesis highlight that the zebrafish infection models are not restricted to fish pathogens only. In the early studies of this project, both *S. agalactiae* and *S. pneumoniae* were shown to be able to cause an infection in zebrafish (original communications I, II, III). Importantly, the survival of the fish was dose-dependent indicating the presence of some sort of a defense mechanism that can recognize and clear the streptococci. This formed the basis for the further characterization of the zebrafish model for the study of the pathogenic mechanisms of these two streptococci.

6.1.1 Studying the pathophysiology of *S. agalactiae* and *S. pneumoniae* infections in zebrafish

The human mucosal sites at the respiratory tract, gut and the genital tract are the typical niches of *S. agalactiae* and *S. pneumoniae* and they also serve as the sites for bloodstream invasion. In the infection models presented in this thesis, the bacteria were mainly administered to the blood, by intravenous injections into embryos (I, IV) or an intraperitoneal infection (direct access to blood) in adult fish (II, III), bypassing the natural entry route at the mucosa. The benefit of the systemic administration of bacteria is the induction of comparable levels of bacteremia in every individual, giving a controlled experimental setting for the study of host and bacterial factors affecting bacterial clearance in the blood. Similarly, the dissemination of bacteria into the brain, and the interactions required for the penetration of the blood-brain barrier can be specifically investigated (II, III). To study the factors required for colonization and invasion, the crucial steps in the development of a natural streptococcal infection, other infection routes should be used. Since zebrafish lack the respiratory and the reproductive systems equivalent to humans, the mucosal surfaces at the gastrointestinal tract or the gills might provide streptococcal entry sites comparable to the human or mouse nasal, oral, or vaginal cavity. In general, the cellular architecture and the inflammatory response upon a bacterial or chemical stimulus in the zebrafish gills and gut have been shown to resemble those of humans (Progatzky et al., 2016; Brugman 2016; Shan et al., 2015; Rombout et al., 2014; Galindo-Villegas et al., 2012; Harriff et al., 2007) providing potential experimental colonization sites also for streptococci. In fact, *S. pneumoniae*

has been found to colonize the respiratory epithelium in the zebrafish gills leading to a lethal infection after bacterial immersion (Diez-Martinez et al., 2013), although the ability to invade the bloodstream from this site has not yet been evaluated.

Streptococcal bacteria are found to cause an array of pathological conditions in humans, including for example, respiratory tract, skin, deep tissue, meningeal, and systemic infections (Cole et al., 2008). Although the anatomical differences between fish and mammals limit the modelling of certain infectious diseases, such as pneumonia, the pathology of the streptococcal infections in zebrafish described in this thesis and by others are strikingly similar to those in humans and mice (Saralahti and Rämet 2015; Phelps et al., 2009). A study by Neely et al. (2002) gave the first proof for successfully reproducing human streptococcal infections in zebrafish. The zoonotic pathogen *S. iniae* and the human-specific *S. pyogenes* were shown to cause an acute infection in adult zebrafish, with the clinical presentation resembling an invasive streptococcal infection in humans and local necrotizing fasciitis, respectively (Neely et al., 2002). In this thesis, I have shown the feasibility of using the zebrafish model in the study of the pathogenesis of *S. pneumoniae* and *S. agalactiae*. In zebrafish embryos, an intravenous injection of *S. pneumoniae* caused an acute systemic infection with dose-dependent lethality (I). The bacteria replicated fast during the first 24 hpi and induced a common inflammatory response (increased expression of *il1b* and *tnfa*) (I). Similarly, in adult zebrafish, the intraperitoneal administration of either *S. pneumoniae* (III) or *S. agalactiae* (II) caused a fulminant infection within the first 48 hpi. According to the significantly lower LD50 dose (~100 cfu), adult zebrafish appeared more susceptible to a *S. agalactiae* infection compared to *S. pneumoniae* (LD50 ~$2.5x10^6$ cfu). Nonetheless, both infections in adult fish were associated with the rapid replication of bacteria in the blood and the subsequent dissemination of bacteria into the brain accompanied with cerebral edema as a visible sign of meningeal inflammation (II, III). In mice and humans, the detrimental effects of meningitis are in part caused by an overwhelming inflammation and the infiltration of large numbers of leukocytes into the nervous system (Hoffman and Weber 2009). Consistently, the *S. agalactiae* infection was shown to induce an acute inflammatory response in the adult zebrafish brain as was exemplified by the increased expression of *il1b* and *il6* (II). Later, supporting data by Jim et al. (2016) and Kim et al. (2015) demonstrated the ability of *S. pneumoniae* and *S. agalactiae*, respectively, to invade the brain of zebrafish embryos, and in the case of *S. pneumoniae* also the infiltration of neutrophils, were observed (Jim et al., 2016; Kim et al., 2015).

6.1.2 Studying the bacterial factors that promote a streptococcal infection in zebrafish

One of the most challenging characteristics of streptococci is the variation and the poor predictability of the clinical outcome associated with certain strains and serotypes. It is well recognized that of the over 90 *S. pneumoniae* serotypes and the 10 *S. agalactiae* serotypes, some are more often associated with colonization, while others predominate in invasive infections. The different virulence capacity of the *S. pneumoniae* serotypes is in part explained by the extensive sequence variation at the genomic locus containing the genes for capsule biosynthesis (Wen et al., 2016; Shainheit et al., 2014; Morona et al., 2004). However, another level of variation is created by the allelic diversity of the genes coding for other virulence factors, such as the adhesion proteins and toxins (Gámez et al., 2018; Cornick et al., 2017; Jefferies et al., 2007; Silva et al., 2006; Iannelli et al., 2002). In general, by analyzing the allelic variation at multiple loci as well as the protein expression profiles of different strains at different stages of the infection, the mechanisms promoting virulence are found to be multifactorial and complementary. Consequently, the molecular background for the virulence capacity of different strains remains largely unknown.

Again, rodents have been frequently employed for the study of the pathogenic mechanisms of streptococci. By comparing the virulence of different serotypes, sequence types, as well as bacterial mutants lacking specific molecules, the importance of multiple membrane-bound and soluble virulence factors has been proved. One of the aims of this thesis was to find out whether the zebrafish model could be used in the search for novel virulence factors and their mechanisms of action in *S. agalactiae* and *S. pneumoniae* infections. The zebrafish model has been successfully used by others to verify the role of known virulence factors in the pathogenesis of streptococci, such as the capsule of *S. iniae* (Harvie et al., 2013; Lowe et al., 2007; Miller and Neely 2005; Neely et al., 2002), *S. pyogenes* (Kizy and Neely 2009), and *S. agalactiae* (Kim et al., 2015). In addition to the classical virulence factors, others have also used the zebrafish model to unravel the role of novel streptococcal virulence determinants, including the M protein and the transcriptional regulator CpsY of *S. iniae*, the Iron uptake (Siu) transporter of *S. pyogenes*, and the regulatory protein CpsA of *S. agalactiae* (Hanson et al., 2012; Allen and Neely 2011; Locke et al., 2008; Montanez et al., 2005). Moreover, as a great example of the practicality of the zebrafish model for the identification of previously unknown virulence factors, large-scale signature-tagged mutagenesis screens were conducted in adult zebrafish for *S. iniae* and *S. pyogenes* (Kizy and Neely 2009; Miller and Neely 2005).

By testing the ability of *S. agalactiae* and *S. pneumoniae* mutants lacking known virulence factors to cause an infection in zebrafish, we noticed that the main bacterial factors affecting the severity of sepsis and meningitis are conserved from zebrafish to humans. In both a *S. agalactiae* and a *S. pneumoniae* infection, the virulence was impaired in mutants lacking the antiphagocytic polysaccharide capsule (I, II, III), recapitulating the results obtained from rodents (Hyams et al., 2010a; Morona et al., 2004; Marques et al., 1992). Similarly, the cytolytic enzymes of both streptococci, as well as the CovS/CovR regulatory system of *S. agalactiae* and the autolysin A of *S. pneumoniae* were recognized as important mediators of pathogenesis in zebrafish, again supporting the previous studies in rodents (Lamy et al., 2004; Doran et al., 2003; Berry et al., 1989a; Berry et al., 1989b). Later, similar observations by Kim et al. (2015) and Jim et al. (2016) gave more evidence for the conserved mechanisms of pathogenesis in the onset of meningitis in *S. pneumoniae* and *S. agalactiae* infections. Our results, supported by those of others, indicate the existence of specific, and importantly, conserved interactions between the host and the studied streptococci in zebrafish. These interactions promote the survival of bacteria in the bloodstream and their transmission into the central nervous system, providing us with a useful model for the study of these important actions during a streptococcal infection in humans.

6.1.3 Using the zebrafish model in the development of *S. pneumoniae* vaccines

As was discussed in section 2.4 "The remaining challenges in the eradication of *S. pneumoniae* and *S. agalactiae*" of this thesis, global concerns regarding the efficacy of the current *S. pneumoniae* vaccines, as well as the absence of a *S. agalactiae* vaccine in clinics are constantly driving the search for novel vaccine candidates forward. As in most vaccine research, the development of a streptococcal vaccine for humans is dependent on mammalian models, and thus cannot be carried out using efficient and large-scale vaccine antigen screens. Zebrafish, on the other hand, have been proven to be suited for this task, as is exemplified by the successful screen conducted for *Mycobacterium marinum*, a model bacterium for *Mycobacterium tuberculosis*, antigens (Myllymäki et al., 2017; Oksanen et al., 2013). Having a potential model organism in our hands, we wanted to test the ability of the zebrafish to contribute to the development of a vaccine against *S. pneumoniae*.

As in humans, the zebrafish adaptive immune system comprises of T and B lymphocytes that serve the same immune functions as their human counterparts (see section 2.5.1 "The zebrafish immune system and its relation to the human immune system"). Although their capacity to produce antibodies with varying specificity is limited compared to humans, zebrafish are able to mount a protective antibody response against several bacteria and viruses upon immunization (Tandberg et al., 2017; Cui et al., 2010; Novoa et al., 2006). However, in original communication III, the infection of zebrafish devoid of active lymphocytes, due to a defect in Rag-dependent VDJ recombination, resulted in similar survival rates compared to the wild type fish. Moreover, the immunization of zebrafish with live or killed *S. pneumoniae* did not confer protection against a following infection. From these results, we concluded, that the *S. pneumoniae* infection model described in this thesis is not suitable for vaccine development. This is also consistent with an earlier study with the zebrafish model for *S. iniae* infection (Neely et al., 2002) and was also seen with *S. agalactiae* in our unpublished study.

The limited role of the adaptive immunity in our model of *S. pneumoniae* infection might be explained by the acute nature of the infection which is cleared rabidly through the innate immune system. In contrast, *M. marinum*, for example, causes a chronic disease in zebrafish and is slow enough to engage the adaptive immunity and is therefore amenable also for vaccine development. In order to thoroughly address the role of adaptive immune response to *S. pneumoniae* infection, other experimental settings should be tested. For example, instead of the intraperitoneal and intramuscular administration of the bacteria, another, milder infection strategy might be adequate for mounting an adaptive immune response. Supporting this, a recent research by Membrebe et al. reported that immunization with killed *S. iniae* protected zebrafish from a following subcutaneous infection (Membrebe et al., 2016). In addition, trying a different immunization and infection schedule or using a noninvasive pneumococcal strain could be necessary to detect the interaction between the *S. pneumoniae* and the adaptive immune system of the zebrafish.

6.1.4 Using the zebrafish model to study the innate immune response to *S. pneumoniae*

As was described in section 2.5.1 "The zebrafish immune system and its relation to the human immune system" of this thesis, the innate immune system of the zebrafish is very similar to the human immune system and has counterparts for most of the

human innate immune cells, receptors, and soluble effectors, such as cytokines, chemokines, and complement components (Zhang and Cui 2014; Kanther and Rawls 2010; Stein et al., 2007; Meijer et al., 2004). Despite the high level of conservation in the immune response between zebrafish and humans, relatively few studies have concentrated on the host response to a streptococcal infection in zebrafish. So far, the role of phagocytosing leukocytes in the eradication of streptococci has been the main target of interest in zebrafish studies. The innate immune system of zebrafish, including the innate leukocytes, arises by 2 days post fertilization, allowing the study of these host responses to a bacterial infection at the very early stages of development (Kanther and Rawls 2010; Le Guyader et al., 2008; Herbomel et al., 1999). In addition, since the adaptive immune response is active only after 4 weeks of development, the innate immune mechanisms can be studied without the influence of these factors. As another unique characteristic for a vertebrate, transgenic zebrafish lines can be used to study the function of immune cells *in vivo* in real-time during the bacterial invasion. Nowadays, a fluorescent reporter line for almost any immune cell type exists and has been used to analyze their function during a bacterial invasion (Ellett et al., 2011; Renshaw et al., 2006; Langenau et al., 2004; Ward et al., 2003). In original communication I, the transgenic reporter line for myeloid cells was used to visualize the localization and function of these cells in a *S. pneumoniae* infection. At 3 hpi, myeloid cells were seen to migrate to the site of infection, form protrusions towards, and ingest the injected bacteria. Similarly, the important role of the myeloid cells in the host response to a streptococcal infection has also been demonstrated by others (Vincent et al., 2017; Kim et al., 2015; Harvie et al., 2013). As yet another beneficial characteristic, there are multiple opportunities for reverse genetics in zebrafish embryos, for studying specific immune functions in the defense against bacteria. In this thesis the transient silencing of genes with the antisense oligonucleotide method was utilized to further verify the importance of myeloid cells in the host defense against *S. pneumoniae* in zebrafish embryos (I).

Along with the functional studies on the myeloid cells in a *S. pneumoniae* infection in zebrafish, we characterized the zebrafish innate immune response to *S. pneumoniae* in a larger scale through a whole genome level transcriptome analysis (IV). Importantly, the analysis revealed that many of the activated protein coding genes were associated with a common innate immune response, including genes coding for complement components, acute phase proteins, innate immune signaling molecules, and mediators of leukocyte migration and function. Since phagocytosis is the primary clearance mechanism for *S. pneumoniae* also in zebrafish (as shown in original

communication I), it was not surprising that the genes postulated to be associated with enhanced phagocytosis were enriched among the upregulated genes. For example, the most enriched functional group of proteins was formed by the complement components. The phagocytosis and the clearance of *S. pneumoniae* in mammals is highly dependent on the complement system which functions as opsonin to aid the recognition and intake of bacteria by the host's phagocytosing cells (Ali et al., 2012; Hyams et al., 2011; Kerr et al., 2005; Brown et al., 2002; Xu et al., 2001). Moreover, complement also serves other important immunomodulatory functions during a *S. pneumoniae* infection, including the recruitment of leukocytes and the activation of adaptive immune responses (Andre et al., 2017; Dunkelberger and Song 2010). In a *S. pneumoniae* infection, the complement system can be activated by any of the three activation pathways, the classical, the alternative, or the lectin pathway, the first of which seems to be the dominating pathway in a *S. pneumoniae* infection (Ali et al., 2012; Brown et al., 2002).

The importance of complement in the defense against *S. pneumoniae* is also emphasized by the number of pathogenic strategies developed to disturb its function (Andre et al., 2017). Again, the most important of these factors is the capsule, and the ability to resists the complement mediated immunity is in part linked to the capsular serotype (Hyams et al., 2010a; Hyams et al., 2010b; Melin et al., 2010). In addition, several protein virulence factors of *S. pneumoniae*, including PspC, PspA, pneumolysin, and Autolysin A also interact with the complement components and contribute to the resistance (Ramos-Sevillano et al., 2015; Mukerji et al., 2012; Dave et al., 2004; Alcantara et al., 2001; Tu et al., 1999). The mechanisms of complement resistance are numerous and include, for example, the direct binding to C3 or the derivative opsonins (e.g. PspC and PspA), the recruitment of inhibitors of the complement system (e.g. PspC, PspA, Autolysin A), depletion of the complement components (pneumolysin), or the inhibition of the binding of opsonin to its corresponding receptor (capsule) (Ramos-Sevillano et al., 2015; Mukerji et al., 2012; Dave et al., 2004; Alcantara et al., 2001; Tu et al., 1999). In fact, the pathogenic mechanism of each of these factors in mice has been strongly linked to their ability to inhibit complement. Similarly, the highly induced complement-related genes upon a *S. pneumoniae* infection and the attenuated nature of the capsule, the pneumolysin and the autolysin A deficient mutants in zebrafish embryos, suggests the presence of an analogous mechanisms for pathogenesis in our model as well.

The transcriptome data in original communication IV indicated the increased expression of genes specifically associated with the classical and alternative pathways, including the genes for Crp, known to activate the classical pathway of complement,

as well as the C1 complex subcomponent C1r and the complement factor B required for the activation of the C3 convertase in the classical and the alternative pathway, respectively (Dunkelberger and Song 2010). Importantly, the central complement component in every pathway, C3, was also highly upregulated upon infection. As was described earlier in this thesis, the zebrafish innate immune system is highly diversified compared to mammals, and this was also seen in our transcriptome data. For example, the most induced genes included four of the eight zebrafish C3 variants and a set of zebrafish genes with no putative homolog in mammals. With the still limited gene annotation and the lack of functional studies, the biological significance of the variants and the zebrafish specific genes remains unknown. However, the mechanisms of the innate immune response are in general well conserved across species, as is exemplified by the existence of complement in organisms as low as lancelets and sea urchins (Dunkelberger and Song 2010; Al-Sharif et al., 1998). Therefore, the complement system in zebrafish is likely to have retained its basic functions in the innate host response and, as has been proposed for other components of the innate immune system, the diversification might allow broader target specificity and the recognition of micro-organisms compared to mammals. Moreover, analogous gene synteny and sequence as well as the evidence obtained from other fish species (Zhang and Cui 2014; Boshra et al., 2006), strongly suggests also the functional conservation of the complement system in zebrafish.

6.2 The genetic screen for host factors affecting *S. pneumoniae* susceptibility in zebrafish

In addition to bacterial factors, differences in the clinical outcome may also be a result of the genetic variation within the human population. The host factors affecting the outcome of a *S. pneumoniae* infection have been searched for through numerous genetic association studies in humans and mice. While polymorphisms in the genes coding for common innate immune components, such as the inflammasome (Geldhoff et al., 2013), NF-kB signaling (Chapman et al., 2010; Chapman et al., 2007; Ku et al., 2007), and the complement system (Jönsson et al., 2005; Roy et al., 2002) have been associated with a susceptibility to pneumococcal infections in humans, the predisposing host factors are still poorly known. The evaluation of these factors has frequently been carried out in mice, through knock-out studies or by comparing the host response in various inbred strains (Alper et al., 2016; Jonczyk et al., 2014; Endo et al., 2012; Sun et al., 2011; Kerr et al., 2002).

To add our own contribution to the effort, we carried out a medium-scale forward genetic screen for host factors affecting the susceptibility to *S. pneumoniae* in zebrafish (original communication IV). Zebrafish are ideal models for such a screen since they develop fast and can be kept in big numbers in a relatively small space. From the large pool of different methods for forward genetics in zebrafish, we chose to use gene-breaking transposon-based insertional mutagenesis (Clark et al., 2011). As an advantage over more traditional chemical mutagenesis strategies, the disrupted genomic locus can be more easily identified. In addition, using a fluorescent marker in the mutagenesis construct, the mutated individuals can be specifically chosen for the crosses and the phenotypic analyses. Finally, the role of novel genes obtained from the screen can be studied using the practical reverse genetic tools that are available for zebrafish, including antisense morpholino knockdown or CRISPR/Cas9 targeted mutagenesis.

The practicality of this model complemented with the high level of conservation between the human and zebrafish innate immune systems provides an intriguing opportunity for studying the genetic susceptibility associated with a *S. pneumoniae* infection in humans. Overall, while zebrafish have frequently been utilized in screens for genes associated with development and human disorders (Golling et al., 2002; Haffter et al., 1996), relatively few screens have concentrated on the function and development of the immune system. The successful screens for T cell development (Iwanami et al., 2016; Seiler et al., 2015) and the innate susceptibility to a *M. marinum* infection (Tobin et al., 2010), however, will certainly pave the way for other screens uncovering unknown immune functions.

The screen carried out in our group was the first to study the innate susceptibility to a *S. pneumoniae* infection in zebrafish. Through the screen we found several mutant zebrafish lines with an increased susceptibility to a *S. pneumoniae* infection. One of the most susceptible lines showed hampered defense mechanisms against *S. pneumoniae* and thus the uncontrolled systemic growth of bacteria (IV). By analyzing the differentially expressed genes at the whole genome level in a *S. pneumoniae* infection in wild type and mutant embryos, the associating factor for a more severe *S. pneumoniae* infection was found to be the decreased expression of the gene homologous for human *CRP*. CRP is an acute phase protein whose production is rapidly induced by the pro-inflammatory cytokines IL1B, IL6 and TNF as a response to infection, injury or trauma (Slaats et al., 2016; Gruys et al., 2005; Zhang et al., 1995; Castell et al., 1989; Gauldie et al., 1987). Human CRP can specifically recognize and bind the C-polysaccharide on the surface of *S. pneumoniae* and promote the complement-mediated phagocytosis of *S. pneumoniae* (Holzer et al., 1984; Kaplan and

Volanakis 1974). Due to its rapid production upon infection, CRP may provide an immediate way of recognizing bacteria and promoting phagocytic clearance, as opposed to antibodies, which are produced in a more delayed manner. In addition to its ability to activate the classical pathway of complement (Kaplan and Volanakis 1974) CRP binding can also directly stimulate the uptake of bacteria by phagocytes and lead to the production of inflammatory mediators (Thomas-Rudolph et al., 2007; Mold and Du Clos 2006).

Much evidence for the importance of CRP for the innate immune response to *S. pneumoniae* has been gained from *in vitro* studies. The lack of a proper animal model for the biological role of CRP is mainly due to functional differences (e.g. between humans and mice) and the lack of cross-reactivity between the human CRP and complement components in other species (Torzewski et al., 2014; Black et al., 2003; Whitehead et al., 1990; Volanakis and Kaplan 1974). However, although CRP is not an acute phase protein in mice, human CRP is able to protect mice from severe bacteremia and mortality in a complement mediated way after an infection with *S. pneumoniae* (Suresh et al., 2006; Szalai et al., 1996; Yother et al., 1982; Mold et al., 1981). Quite recently, the importance of the CRP for the innate defense against *S. pneumoniae* was also shown in a knock-out mice model (Simons et al., 2014). As in humans, the endogenous CRP has been shown to activate complement in rats and trout indicating the conservation of this important biological function across species (Padilla et al., 2003; Nakanishi et al., 1991). According to the current knowledge, zebrafish has seven Crp isoforms (Bello-Perez et al., 2017a; Bello-Perez et al., 2017b; Chen et al., 2015). However, due to the missing comprehensive functional analysis of the isoforms, the exact biological role of these proteins in a *S. pneumoniae* infection remains unknown. The induction of zebrafish Crp genes in *S. pneumoniae* (IV) and other bacterial infections (Bello-Perez et al., 2017b; Benard et al., 2014) as well as the postulated association between Crp deficiency and an increased susceptibility to *S. pneumoniae* infections in zebrafish, clearly indicate the retained function of Crp as an important part of the innate immune response in this species.

To verify the role of CRP and the mechanisms of action in the host response to a *S. pneumoniae* infection in zebrafish, further evaluation through reverse genetics is required. However, the finding of such a component, with a previously shown strong association with a *S. pneumoniae* infection, as a potential predisposing factor to an infection in zebrafish, proves the value of our model and the screen in the study of the host factors associated with this pathogen. Noteworthy, besides the mutant line described in this thesis, several other mutant lines with impaired survival in a *S. pneumoniae* infection were discovered in the screen. Future studies will show, whether

these mutants and the corresponding candidate genes will help us reveal novel host factors associated with the innate susceptibility to a *S. pneumoniae* infection.

7 SUMMARY AND CONCLUSIONS

The zebrafish has a long history as a model organism for studying embryonic development, and more recently it has gained popularity also in the study of cancer, cardiovascular disease, neurological disease, and immune function. In many cases, the results obtained from zebrafish studies complement discoveries made in higher model organisms, such as rodents, surprisingly well. In the field of immunology, the mechanisms of pathogenesis of a continuously increasing number of infectious agents, including bacteria, viruses and parasites, have been successfully studied in zebrafish. These micro-organisms include both human-specific and fish-adapted species, thus indicating the conservation of the basic mechanisms of pathogenesis in fish and humans.

This study focused on the evaluation of the potential of the zebrafish to act as an infection model for the zoonotic pathogen *Streptococcus agalactiae* and the human-specific *Streptococcus pneumoniae*. Although they were recognized as major human pathogens already about a century ago, the complex mechanisms of the pathogenesis as well as the relevant host susceptibility factors of *S. pneumoniae and S. agalactiae* remain largely unclear. In the early stages of this study both pathogens were shown to cause a fulminant, dose-dependent infection when introduced systemically to zebrafish embryos (*S. pneumoniae*) or adult zebrafish (*S. pneumoniae and S. agalactiae*). The infections progressed rapidly and were associated with an inflammatory response (production of IL1B and TNF, IL6) and dissemination of the bacteria into the brain. The importance of known virulence factors of *S. pneumoniae* (the capsule, pneumolysin, and autolysin A) and *S. agalactiae* (the capsule, β-hemolysin, and the CovS/CovR system) for the pathogenesis of these streptococci was shown in zebrafish by testing the virulence capacity of the corresponding mutant bacteria. Besides the bacterial factors promoting the infection, the conservation of the host's response to a *S. pneumoniae* infection was evaluated in zebrafish. First, the importance of the phagocytic clearance of *S. pneumoniae* in zebrafish was discovered by analyzing the effect of depleting myeloid cells on the survival of the zebrafish and by imaging the behavior of myeloid cells during the infection. Second, a genome level transcriptome analysis of the infected zebrafish embryos revealed the overall similarity of the innate immune response to a *S. pneumoniae* infection in zebrafish and

in humans. Finally, further support for the conserved host responses to a *S. pneumoniae* infection in zebrafish came from the forward genetic screen in which the lack of *crp* expression was identified as a potential susceptibility factor for severe bacteremia. In contrast to the conservation of the innate immune response to *S. pneumoniae* in zebrafish, the adaptive immunity did not seem to play a significant role in combating the infection in our model. For this reason, this model is not a propriate model for the development of pneumococcal vaccines.

In conclusion, our studies indicate that both the infection models represented in this thesis are capable of reproducing the pathology of human streptococcal sepsis and meningitis. The zebrafish provides a practical model in which the important pathological features of human infections, including the bacterial counts and the inflammatory response, can be easily analyzed. In addition, the studies conducted in zebrafish embryos highlight the benefits of the unique methodologies available for this model, including the tracking of immune cell migration and function in real-time and the ease of reverse and forward genetics. Due to the conserved host-pathogen interactions in zebrafish and humans, this model shows great potential in revealing novel host factors associated with the clearance of bacteria from the bloodstream as well as the bacterial factors promoting immune evasion during *S. pneumoniae* and *S. agalactiae* infections. Importantly, zebrafish also provide a valuable model for the elucidation of the specific interactions promoting the penetration of the BBB and the onset of meningitis associated with both pathogens. Although there is no doubt that the traditional mammalian models will still provide important knowledge of the pathophysiology of human streptococcal infections, the zebrafish will serve as a beneficial alternative and also a superior model for large-scale experiments, such as genetic and drug screens.

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10 ORIGINAL COMMUNICATIONS

PUBLICATION
I

Defense of Zebrafish Embryos Against *Streptococcus pneumoniae* Infection is Dependent on the Phagocytic Activity of Leukocytes

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Defense of zebrafish embryos against *Streptococcus pneumoniae* infection is dependent on the phagocytic activity of leukocytes

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ABSTRACT

Severe community acquired pneumonia caused by *Streptococcus pneumoniae* is the most common cause of death from infection in developing countries. Serotype specific conjugate vaccines have decreased the incidence of invasive infections, but at the same time, disease due to non-vaccine serotypes have increased. New insights into host immune mechanisms against pneumococcus may provide better treatment and prevention strategies. Zebrafish is an attractive vertebrate model for studying host immune responses and infection biology. Here we show that an intravenous challenge with pneumococcus infects zebrafish embryos leading to death in a dose dependent manner. Survival rates correlate with the bacterial burden in the embryos. The production of proinflammatory cytokines is induced in zebrafish after pneumococcal exposure. Importantly, morpholino treated embryos lacking either myeloid cells or the ability to phagocytose bacteria have lowered survival rates compared to wild type embryos after pneumococcal challenge. These data suggest that the survival of zebrafish embryos upon intravenous infection with *S. pneumoniae* is dependent on the clearance of the bacteria by phagocytosing cells. Additionally, we demonstrate that mutant pneumococci lacking known virulence factors are attenuated in the zebrafish model. Our data demonstrate that zebrafish embryos can be used for study innate immune responses as well as virulence determinants in pneumococcal infections.

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1. Introduction

Streptococcus pneumoniae (pneumococcus) is a major cause of respiratory tract infections such as otitis media, sinusitis, and pneumonia as well as invasive diseases (septicemia and meningitis). Children, the elderly and immuno-compromised patients suffer from severe pneumococcal diseases (van der Poll and Opal, 2009). It is estimated that half a million children under five years of age die of pneumococcal pneumonia every year (Yu et al., 2003). For decades, penicillin was the drug of choice for treating pneumococcal infections, but increasing levels of penicillin resistance has resulted in the use of alternative antibiotics (Yu et al., 2003). It is known that the ability of *S. pneumoniae* to cause invasive disease depends on its ability to colonize the nasopharynx, traverse the respiratory epithelium, and eventually enter the bloodstream across the vascular endothelium (Tuomanen et al., 1995). During this process pneumococci will launch an inflammatory response, mediated by the host's innate immune system. The inflammatory response is triggered by the recognition of microbial molecular patterns in the bacterial cell wall or in the cytosol of lysed bacteria (Ishii et al., 2008). Toll-like receptors (TLR) have been shown to play a central role in the initiation of cellular innate immune responses against pneumococci. Mice deficient in the common TLR-adaptor protein MyD88 are more susceptible than wild type mice to pneumococcal disease (Albiger et al., 2005). Also children with a genetic MyD88 deficiency have been shown to have an increased susceptibility to invasive pneumococcal disease (von Bernuth et al., 2008). Moreover, the clearance of pneumococci from the blood has been shown to be dependent on complement components, another important part of the innate immune system (Brown et al., 2002). The recognition of pneumococci by innate immune receptors leads to the induction of inflammatory cytokines and chemokines, which recruit other inflammatory cells to the site of the infection eventually leading to the activation of the adaptive immunity (Tuomanen et al., 1995).

Abbreviation: WASP, Wiskott–Aldrich syndrome protein.

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Pneumococci express a variety of virulence factors, which enable the colonization and invasion of the host (Jedrzejas, 2001). The bacterial capsule is the most important virulence factor (Nelson et al., 2007b). It is strongly antiphagocytic and unencapsulated pneumococci are considered avirulent (Hyams et al., 2010; Morona et al., 2006). Recently, it was discovered that pneumococci may express pilus-like structures on their surface, promoting adhesion and bacterial spread (Barocchi et al., 2006; Nelson et al., 2007a). Also, several other bacterial factors have been shown to affect pathogenicity in experimental animal models (Hirst et al., 2004; Iannelli et al., 2004; Paterson and Mitchell, 2006).

The zebrafish (*Danio rerio*) has been used to study embryogenesis and organ development for decades. The use of zebrafish in studies of host–pathogen interactions during infection is more recent (van der Sar et al., 2004). As a teleost, zebrafish possesses both an innate and adaptive immunity (Yoder et al., 2002). Many aspects of its immune responses correspond to those of mammals. In zebrafish, TLRs have been characterized and as many as 24 putative variants have been described (Jault et al., 2004). Sequencing of the zebrafish genome has led to the identification of homologs of mammalian genes encoding cytokines, and interferons (Levraud et al., 2007; Roca et al., 2008). Also, studies indicate that T and B cells, antigen-presenting cells, and phagocytic cells are present in zebrafish (Yoder et al., 2002). Several bacterial infection models have been established in zebrafish (Clay et al., 2007; Miller and Neely, 2004; Stockhammer et al., 2009; van der Sar et al., 2006). Even pathogens that do not naturally infect fish (*Staphylococcus aureus* and *Pseudomonas aureginosa*) have been successfully used to trigger immune responses in zebrafish (Clatworthy et al., 2009; Prajsnar et al., 2008).

In this study, we show for the first time that zebrafish embryos may be used as a model for host immune responses in systemic pneumococcal infections. We demonstrate that the inoculation of pneumococci into the circulation of zebrafish embryos causes a fatal infection, which is dependent on the host's immune cells and their ability to clear the bacteria from the blood. We found that pneumococci may evade clearance in the host by interfering with phagocytic functions. Moreover, isogenic pneumococcal mutants harboring defects in important virulence factors were attenuated in this *in vivo* model system.

2. Materials and methods

2.1. Bacterial strains

S. pneumoniae TIGR4 belongs to a clone (ST205) with a high invasive disease potential. It is an encapsulated serotype 4 (ATCC BAA-334) originally isolated from a patient in Norway (Aaberge et al., 1995). The insertion–deletion mutagenesis used for most strains is described elsewhere (Barocchi et al., 2006). Briefly, approximately 1 kB of the upstream and downstream fragments of the relevant ORF were amplified by PCR and ligated to the ends of an erythromycin-resistance cassette (Erm^R, Genbank accession AB057644). The resulting construct was transformed into the appropriate wt pneumococcal strain and plated on blood agar plates with erythromycin (1 μg/ml). Mutants were confirmed by PCR, sequencing, and immunogenicity.

Unless stated elsewhere, T4 and the isogenic mutants were grown overnight on blood agar plates (at 37 °C and CO_2). Cells were suspended in 10 ml Todd Hewitt broth (Becton, Dickinson and Company, BD) supplemented with (0.5%) yeast extract (THY) and incubated at 37 °C until they reached an OD620 of 0.4. Bacteria were harvested by centrifugation (4000 rpm, 10 min) and the cell pellet was washed and re-suspended in 0.2 M KCl-buffer to obtain the desired dilutions.

2.2. Zebrafish embryos

Wild type AB zebrafish embryos were maintained according to standard protocols. During the experiments embryos were kept in E3 medium at 28.5 °C. The Tg(spi1:EGFP) mutant strain was obtained from ZIRC (http://zfin.org/zirc).

2.3. Preparation and microinjection of zebrafish embryos

Zebrafish embryos were dechorionated at 48–50 hpf and anaesthetized with buffered 0.02% tricaine (Sigma–Aldrich). A 70 kDa rhodamine dextran (Invitrogen/Molecular Probes) tracer was added to the bacterial suspension before injections. Injections of 1 nl each, were directed into the blood circulation valley so that a group of 24–30 embryos were injected with the same glass microcapillary needle filled with bacterial suspension. The output of each needle was controlled by plating the injection dose before and after the injections.

2.4. Following the mortality of infected embryos

After infection, embryos were kept in 24-well plates at 28.5 °C, a single embryo in each well. Embryos were followed frequently and dead embryos in every group were counted at each time point to obtain survival rates. Lack of movement and heartbeat was interpreted as a sign of death.

2.5. Determination of bacterial growth in infected embryos

Five embryos from each group were randomly collected at regular time points (2 h, 7 h, 20 h, and 48 h) and anaesthetized with tricaine. Individual embryos were then homogenized in 1.5 ml centrifuge tubes with 200 μl PSB and 1% triton-X by pipetting up and down. Thereafter, the homogenates were serially diluted in PBS and plated onto selective blood agar plates. After incubating overnight at (37 °C and CO_2), bacteria were counted on each plate.

2.6. Generation of morphants

The morpholinos used in the study were designed to block translation (TB) or pre-mRNA spilicing (SB). The gene silencing morpholinos targeting zebrafish WASP1 5′-CCCTTTGCTTTTGC CTTTGCTCATC3′ (ZDB-GENE-030131-7098) and WASP2 5′-CCTCATGGCCTCATACGCCGTCTAA-3′ (ZDB-GENE-081104-419), Pu.1 (ZDB-GENE-980526-164) 5′-CCTCCATTCTGTACGGATGCAG CAT3′ (TB) and 5′-GGTCCTTCTCCTTACCATGCTCTCC-3′ (SB), were obtained from Gene Tools (Philomath, OR). For Pu.1 and WASP a mix of both morpholinos was used. The morpholino concentration for Pu.1 was 130 μM + 130 μM (TB + SB) and for WASP 100 μM + 100 μM (WASP1 + WASP2). A total of 1 nl morpholinos in 0.2 M KCl was injected into the yolk sacs of fertilized eggs at the 1–2 cell stage. A standard control mismatch morpholino from Genetools was used in each experiment.

2.7. Real time qRT-PCR analysis

A total of 10–15 embryos from each group were pooled and total RNA extraction was done using standard procedures. qRT-PCR for mRNA expression levels of selected genes were obtained using the QuantiTect SYBR green RT-PCR kit (Qiagen) and ABI7000 instrument (Applied Biosystems). Primers were: *TNF-α* upstream 5′-GGG CAA TCA ACA AGA TGG AAG-3′ and downstream 5′-GCA GCT GAT GTG CAA AGA CAC-3′, *IL-1ß* upstream 5′-TGG ACT TCG CAG CAC AAA ATG-3′ and downstream 5′-GTT CAC TTC ACG CTC TTG GAT G-3′, *ß-Actin* upstream 5′-ATC GAT GAG GAA ATC GCT G-3′ and downstream 5′-ATG CCA ACC ATC CCC TG-3′. The primers

for *IL-1ß* and *ß-Actin* were obtained from a study carried out by Pressley et al. (2005).

2.8. Live fluorescent microscopy

For live imaging, Tg(spi1:EGFP) embryos were injected with Baclight™ (Invitrogen) labelled T4. Briefly, the bacteria were grown in THY medium to log-phase and washed with KCL. Washed bacteria were incubated according to the manufacturers instructions with Baclight red stain at room temperature. Thereafter, the infection procedure was carried out as described earlier. One hour after injection, living embryos were anesthetized and mounted onto 1% low-melting-point agarose. The interaction between red fluorescent bacteria and GFP expressing myeloied cells was captured from the circulation valley area. Microscopy was carried out using 40× or 100× (Olympus BX51) water immersion objectives. Stacks of obtained figures were assembled using ImageJ (National Institutes of Health) and the final editing was done with Photoshop CS2 (Adobe systems Inc.).

2.9. Statistical analysis

Survival rates were analyzed using the Mantel-Cox Log Rank test. The data for colony forming units and cytokine expression levels were analyzed using one-way ANOVA with Bonferroni's post test or the *T*-test. A *p*-value of <0.05 was considered statistically significant. All data was analyzed using the Graph Pad Prism 5.0 software.

3. Results

3.1. S. pneumoniae infection in zebrafish embryos

The completely sequenced, pathogenic pneumococcal strain TIGR4 (T4), previously used in mice model infections, was chosen to be intravenously infected into zebrafish embryos (Aaberge et al., 1995; Sandgren et al., 2005). To study the possible virulence capacity of pneumococci, various doses of the T4 strain were injected into the blood stream of zebrafish embryos, whose survival was then monitored. Regardless of the dose, mortality was highest during the first 48 h post injection. Fig. 1A shows the dose dependent mortality post infection. The combined mean survival proportion with a 100 cfu/e inoculum was 49%, with 1000/e it was 15% and with 10,000 cfu/e 3% ($p < 0.0001$). The highest dose (10,000 cfu/e) was associated with the most rapid disease progression. No deaths occurred in the control group. These results demonstrate that pneumococci may cause a fatal infection in zebrafish embryos after injection into the blood stream.

In addition to intravenous injections, a set of yolk sac injections was also carried out. In this setting, all the embryos tended to die regardless of the number of bacteria injected (data not shown). This is likely due to a lack of circulating immune cells inside the yolk, which leads to unlimited growth of the bacteria.

To monitor the expansion of bacteria in the host after intravenous injection, embryos were pooled (24 per group) and lyzed at various time points for bacterial counts. As shown in the Fig. 1B and C, pneumococcal growth in zebrafish embryos was logarithmic with inputs of both 100 cfu (Fig. 1B) and 1000 cfu (Fig. 1C) per embryo. These data show that pneumococci are able to expand in zebrafish embryos following an intravenous injection.

To investigate whether zebrafish embryos are able to combat and kill pneumococci, single embryos were collected and lysed to obtain bacterial counts. Both surviving and dying embryos were analyzed. As shown in the Fig. 2A, the survival of the embryos is associated with clearance of the bacteria. After 48 h, embryos had either cleared the infection or died. Importantly, the number of bacteria following the intravenous injection increased in dying embryos, while in the surviving individuals the bacterial load decreased ($P < 0.001$). Noteworthy, this clearance mechanism was not seen after a yolk sac injection of pneumococci as the bacterial counts increased gradually over time (Fig. 2B). In summary, these results show that zebrafish embryos may combat pneumococcal infections although this is not the case in the yolk sac.

3.2. Pneumococcal virulence factors contribute to pathogenicity in zebrafish embryos

The host environment such as the temperature (+28 °C) in zebrafish is not optimal for the human adapted pathogen *S. pneumoniae*. However, when growing the bacteria in THY medium at +28 °C we did not see a significant difference in the growth curves compared to growth at +37 °C (data not shown). Importantly, our data indicate that pneumococci are able to grow and replicate when injected into the blood stream of zebrafish. Thus, we sought to determine if pneumococcal virulence factors contributed to the pathogenicity in this model. The pneumococcal capsule is essential during all steps of disease progression, adherence, invasion and dissemination (Nelson et al., 2007b) and it helps the bacteria to evade phagocytosis (Hyams et al., 2010). To test if the capsule is required for virulence, the non-encapsulated mutant derivative T4R was used in the zebrafish model. In agreement with findings in mouse models, T4R appeared avirulent in zebrafish embryos following an intravenous injection (Fig. 3). Pneumolysin, a cytotoxin expressed by pneumococci, has been shown to be lytic to host cells, to impair respiratory burst of phagocytic cells and to induce the production of cytokines and chemokines (Gilbert et al., 1999; Hirst et al., 2004). A pneumolysin mutant of T4, T4Δ*ply*, showed higher survival rates (46%) compared to the wild type bacterium (70%) ($P = 0.01$), indicating that pneumolysin influences pneumococcal pathogenicity in zebrafish embryos (Fig. 3). Autolysin is another virulence factor, shown to hamper a range of host immune responses in pneumococcal infections (Berry et al., 1989a; Martner et al., 2009). An autolysin mutant, T4Δ*lytA*, showed significantly higher survival rates (72%) compared to wild type pneumococci ($P = 0.001$). Finally, the T4Δ*rlrA* mutant, lacking the genetic locus encoding pneumococcal pili showed lowered pathogenicity compared to the wild type strain ($P = 0.001$) in this model for systemic infection. Pili have been shown to be important for adhesion and for the dissemination of pneumococci from local sites into the blood stream (Barocchi et al., 2006; Nelson et al., 2007a). These results show that zebrafish embryos can be used as a model to identify and study the virulence factors of *S. pneumoniae*.

3.3. Clearance of pneumococci is dependent on myeloid cell recognition and phagocytosis

At the developmental stage of 2 dpf, immune defenses of zebrafish are dependent on innate immune responses, and especially on phagocytosing cells. When encountering bacteria, leukocytes recognize pathogen-associated patterns or molecules by pattern recognition receptors. This leads to signaling events that induce inflammatory mediators like cytokines. To examine innate immune responses of zebrafish embryos in pneumococcal infections, we used qRT-PCR to measure the expression levels of two important pro-inflammatory mediators, tumor necrosis factor (TNF)-α and interleukin (IL)-1ß, after pneumococcal challenge. For cytokine expression analyses, we used a time point when the embryos started to show the first signs of severe infection (lack of movement, lowered heart rate). Fig. 4A shows that both TNF-α and IL-1ß expression is highly induced after a pneumococcal infection ($P < 0.001$). The data show that the zebrafish immune system is able to respond to pneumococci. In addition, the avirulent strain

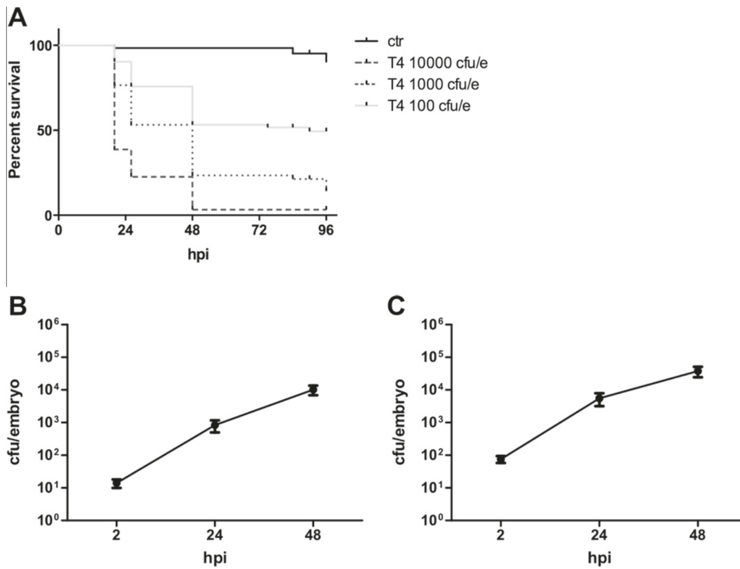

Fig. 1. *Streptococcus pneumoniae* infection in zebrafish embryos. (A) Survival rates of 2 dpf embryos after injection of *S. pneumoniae* (wild type, TIGR4, T4) with the indicated doses. The data comprise three separate experiments with 24 embryos in each group. $P < 0.0001$ (B and C). The amount of live *S. pneumoniae* increases in zebrafish embryos during the first 48 h after injection with 100 (B) or 1000 (C) bacteria per embryo. CTR = Control treated with KCl buffer. Hpi = hours post injection. Cfu = colony forming units.

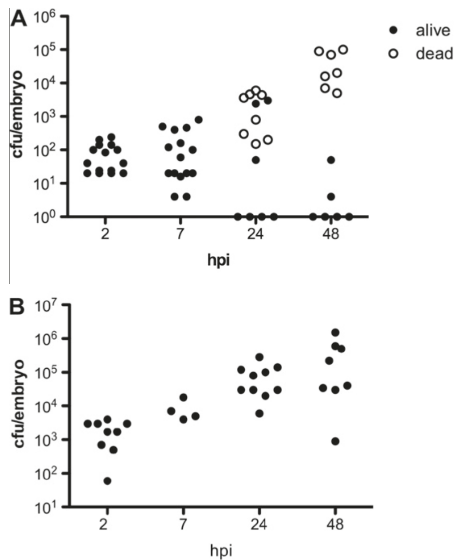

Fig. 2. Bacterial burden in zebrafish embryos during pneumococcal infection. Bacteria in embryos were enumerated at indicated time points post-injection (*S. pneumoniae* T4 strain). Each circle represents an individual embryo. (A) Expansion of pneumococcus (inj.100 cfu/e) in the blood. Open circles indicate the amount of live bacteria recovered from dead embryos, whereas solid circles represent bacterial loads in live embryos $P < 0.001$ (B). Expansion of pneumococcus (100 cfu/e) in the yolk. Only live embryos (solid circles) were analyzed. The figures are representative of three or more individual experiments. Hpi = hours post injection, cfu = colony forming units.

T4R failed to induce cytokine response (Fig. 4B) correlating to severity of infection at chosen time point.

Next, making use of the optical transparency of zebrafish embryos we visualized host–pathogen interactions using live

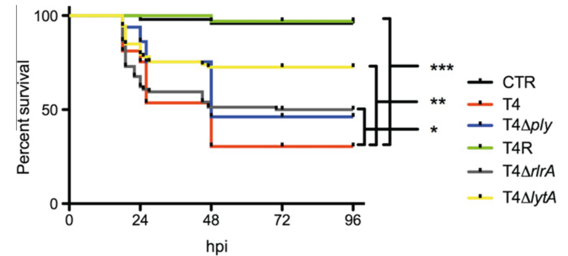

Fig. 3. The pathogenicity of pneumococcus is attenuated in avirulent mutant strains. Survival curves of 2 dpf embryos challenged with wild type pneumococcus (T4), and with its isogenic mutant derivates lacking pneumolysin (T4Δ*ply*), capsule (T4R), pilus structure (T4Δ*rlrA*), and autolysin (T4Δ*lytA*). In each group, 100 cfu/e. were injected into the circulation. The data represent three individual experiments. $^{*}P = 0.01$, $^{**}P = 0.001$, $^{***}P = 0.0001$. CTR = Control treated with KCl buffer.

fluorescence microscopy. Red fluorescent labeled T4 pneumococci were injected into the bloodstream of Tg(Spi:EGFP) embryos, in which the myeloid cells express GFP. Also wild type embryos were used. Images were taken during the early steps (1–3 h post injection) of infection. At this time point, most of the bacteria were still present in the blood stream. Importantly, some red fluorescent-labeled pneumococci co-localized with green fluorescent myeloid cells (Fig. 4B–D). As shown, phagocytosing cells reaching out for pneumococci (Fig. 4B) as well as leukocytes with ingested bacteria (Fig. 4C and D), were detected. This is indicative of direct recognition and phagocytosis of pneumococci by myeloid cells.

To further evaluate the roles of different phagocytic cells and other components of the innate immunity for defending against pneumococci we used a morpholino gene silencing technique (Fig. 5). First we knocked down the transcription factor Pu.1, which has been shown to cause myeloid cell depletion (Hsu et al., 2004). The lack of myeloid cells was confirmed using Tg(Spi1:EGFP) embryos, where no GFP expressing cells could be found after morpholino treatment (data not shown). When using morpholino

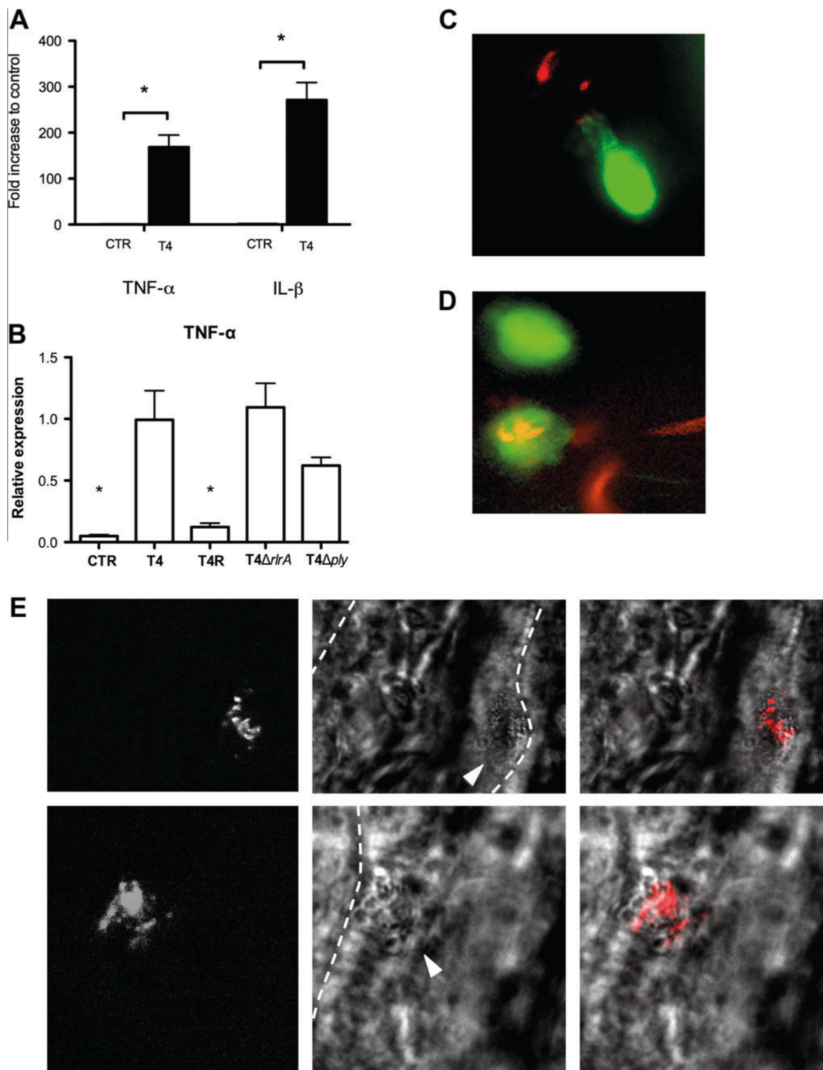

Fig. 4. *S. pneumoniae* induces an in innate immune response in zebrafish embryos. (A) Proinflammatory cytokine expression 18 h post-inoculation of pneumococcus T4 (100 cfu/e). Relative mRNA levels of TNF-α and IL-1β were measured using quantitative RT-PCR. The results were normalized to embryos injected with buffer (KCl 0.2 M, CTR). 10–15 Embryos were pooled for each experiment. Data are the mean ± standard error of the mean of three individual experiments. The asterix (*) depicts a statistically significant difference compared to the control, $P < 0.001$. (B) TNF-α espression induced by pneumococcal mutants. The values are correlated to wild type T4 expression level (=1.0). $P < 0.0001$. (C) Live microscopy with a zebrafish embryo showing a myeloid cell (expressing green fluorescent protein, GFP) reaching for red fluorescent bacteria (T4). (D) A live microscopy image showing a zebrafish phagocyte (expressing GFP), which has ingested live pneumococci (orange). (E) Image montage showing co-localization of fluorescent bacteria (first images on the left) inside a phacytosing cell (arrowhead). Dashed line represents endothelial lining of a blood vessel. Images were obtained from anesthetized zebrafish embryos 1–3 h post-inoculation.

injections to knock down Pu.1, embryos became more susceptible to infections with T4 pneumococci as compared to controls (mean survivals 15% and 50%, respectively, $P < 0.01$). The mortality rates were very high despite the size of the inoculum, suggesting a failure in the mechanisms needed to overcome pneumococcal infection rather than defective wound healing. To further investigate the importance of leukocyte phagocytosis for resistance against pneumococci, a morpholino targeting the Wiskott–Aldrich syndrome protein (WASP) was designed. WASP is a key regulator of the actin cytoskeleton and thus needed for immune cell migration and phagocytosis (Badour et al., 2003). The survival of embryos treated with a WASP-morpholino was significantly weaker compared to control morpholino treated (30%, and 50%, respectively, $P < 0.05$) embryos (Fig. 5), further arguing for the importance of phagocytic immune cells for defending against *S. pneumoniae* in this model.

4. Discussion

In recent years the zebrafish embryo has been established as a new model host for human pathogens. The gram-positive

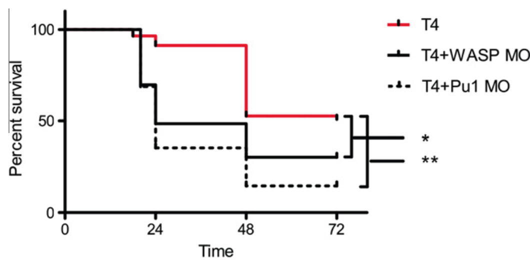

Fig. 5. The survival of zebrafish embryos upon pneumococcal challenge is dependent on myeloid cells and their phagocytic function. The graph presents Kaplan–Meier survival curves of morpholino-treated (MO) 2 dpf embryos after pneumococcal (T4) challenge (50 cfu/e). The data represent three independent experiments. *P = 0.05, **P = 0.01.

streptococci *S. pyogenes* and *Streptococcus iniae* have previously been studied in adult zebrafish (Miller and Neely, 2004). The most significant human streptococcus *S. pneumoniae*, however, has not been studied in this model until now. In the present study, we demonstrate for the first time that zebrafish embryos can be used to study both innate immune responses against pneumococci and pneumococcal virulence factors.

In this study, we show dose dependent mortality after injection of *S. pneumoniae* into to the blood stream of zebrafish embryos. A dose of 100 cfu/e caused approximately 50% mortality, whereas 10,000 cfu/e caused 100% mortality in 48 h. Importantly, there was a drastic difference between the two different sites of injection used in this study, the yolk sac and the blood circulation. There were no surviving individuals in the yolk-injected group when yolks were infected with 100 cfu/e (data not shown). This is analogous to earlier studies with other non-host pathogens such as *S. aureus* (Prajsnar et al., 2008) and suggests that there are no potent immune mechanisms in the yolk sac. Notably the doses used in these experiments are relatively high compared to, for example, mouse studies (Albiger et al., 2005). On the other hand, the doses are comparable to other studies with human pathogens in zebrafish embryos (Prajsnar et al., 2008; Wiles et al., 2009).

The immune system in zebrafish is remarkably similar to that of mammals although at the developmental stage of 50 hpf defense mechanisms are restricted to innate immune responses (Traver et al., 2003). At this point, both granulocytes and macrophages are present and functional (Lieschke et al., 2001). This stage is ideal for investigating the role of leukocytes for microbial resistance and was thus used in this study.

Previously it has been reported, that the survival of zebrafish after systemic bacterial inoculation is strongly dependent on phagocytosing cells (Brannon et al., 2009; Prajsnar et al., 2008). In the present study, we demonstrate analogous data for a pneumococcal infection. Importantly, bacterial counts increased in dying individuals over time, while in surviving embryos the bacterial burden decreased. This suggests that surviving individuals are able to clear the bacteria from the circulation. Although the embryos were monitored up to 96 hpi, there was no significant mortality after 48 h, indicating the importance of immune responses during the first two days after bacterial inoculation. We used live imaging of transparent zebrafish embryos to study the nature of the host–pathogen interaction during the first hours of infection. Notably, this method revealed clear co-localization and phagocytosis of pneumococci by myeloid cells. The increased production of pro-inflammatory cytokines also indicated that pneumococci were recognized by immune cells.

An important advantage of the zebrafish model is its easy genetic manipulation with Morpholino injections. Here we used this technique to transiently knockdown some of the essential genes regulating innate immune functions. The ablation of myeloid cells by knocking down the transcription factor Pu.1 led to a severely compromised zebrafish immune response against pneumococci. This indicates that the immune defense at this developmental stage is strongly dependent on myeloid cells i.e. macrophages and neutrophils. To test the role of phagocytosis more directly, morpholinos targeting an essential gene for phagocytosis, WASP, were used. In the zebrafish genome, the gene encoding WASP is duplicated, and thus both *WASP* genes (*WASP1, WASP2*) were blocked. The WASP morphants showed decreased survival after pneumococcal challenge suggesting a central role for myeloid cells and their phagocytic activity in resistance against pneumococci. This finding is in line with the results that WASP −/−mice are more susceptible to *S. pneumoniae* infection (Andreansky et al., 2005). Furthermore, WAS patients often suffer from pneumococcal diseases (Sullivan et al., 1994).

Earlier studies have shown that the zebrafish model can be used to elucidate the virulence factors of *Escherichia coli* (Wiles et al., 2009) and *Pseudomonas aeruginosa* (Brannon et al., 2009). Importantly, our data demonstrated significant differences in the survival of attenuated mutant derivatives compared to wild type pneumococci. Furthermore, unencapsulated pneumococci were avirulent in this model, which highlights the antiphagocytic properties of the capsule. Also injections of other mutant strains lacking pneumolysin secretion, autolysin and pneumococcal pili showed higher survival rates compared to wild type pneumococci. All of these virulence factors have been shown to hamper innate immune responses, and a resulting reduction in pathogenicity has been reported in mammalian models (Barocchi et al., 2006; Berry et al., 1989a,b; Nelson et al., 2007b). Although, the ecological niche for zebrafish is very different from the human host, our findings show that zebrafish embryos may be used to study pneumococcal virulence factors. Moreover, the model could be ideal for the functional analyses of pneumococcal virulence factors and for large-scale mutagenesis screens for new virulence factors in invasive diseases. This approach has been used for screening new virulence genes of *S. iniae* in adult zebrafishes (Miller and Neely, 2005).

5. Conclusion

In conclusion, we report the use of zebrafish embryos for studying the pathogenesis of pneumococcal infections in a new vertebrate host. We have evaluated some of the key factors involved in the host's innate immune response against *S. pneumoniae*. Furthermore, we have shown the importance of selected pneumococcal virulence determinants in this system. This model appears to be a powerful tool for studying this important human pathogen. Available genetic manipulation techniques and easy live imaging make zebrafish very suitable for large-scale screening and for the delicate modeling of pathogen–host interactions.

Conflicts of interest

None declared.

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PUBLICATION II

Adult Zebrafish Model of Bacterial Meningitis in *Streptococcus agalactiae* Infection

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Adult zebrafish model of bacterial meningitis in *Streptococcus agalactiae* infection

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ABSTRACT

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is the major cause of severe bacterial disease and meningitis in newborns. The zebrafish (*Danio rerio*) has recently emerged as a valuable and powerful vertebrate model for the study of human streptococcal infections. In the present study we demonstrate that adult zebrafish are susceptible to GBS infection through the intraperitoneal and intramuscular routes of infection. Following intraperitoneal challenge with GBS, zebrafish developed a fulminant infection 24–48 h post-injection, with signs of pathogenesis including severe inflammation at the injection site and meningoencephalitis. Quantification of blood and brain bacterial load confirmed that GBS is capable of replicating in the zebrafish bloodstream and penetrating the blood–brain barrier, resulting in the induction of host inflammatory immune responses in the brain. Additionally, we show that GBS mutants previously described as avirulent in the mice model, have an impaired ability to cause meningitis in this new *in vivo* model. Taken together, our data demonstrates that adult zebrafish may be used as a bacterial meningitis model as a means for deciphering the pathogenesis and development of invasive GBS disease.
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1. Introduction

Streptococcus agalactiae, or Group B *Streptococcus* (GBS), is the predominant cause of bacterial meningitis and sepsis in newborn infants. This gram positive encapsulated bacterium is also responsible for invasive disease in adults; particularly in the elderly and in individuals with underlying disease (Skoff et al., 2009). GBS is an opportunistic pathogen which asymptomatically colonizes the lower gastro-intestinal and genito-urinary tracts of approximately 10–30% of the healthy adult population (Johri et al., 2006; Melin, 2011). Neonatal GBS disease occurs in approximately 0.2–4 cases per 1000 live births, and is considered a global health burden in fetal medicine (Edmond et al., 2012). Newborns become infected intrapartum or during delivery from colonized mothers, and despite the use of intrapartum antimicrobial prophylaxis (IAP) to prevent its transmission, GBS remains the leading cause of neonatal bacterial meningitis in Europe, Asia and the United States (Chang et al., 2011; Edmond et al., 2012). Surviving newborns frequently develop life-long neurological sequelae as a consequence of GBS meningitis, and can suffer from seizures, hearing impairments and cognitive defects (Chang et al., 2011; Senn et al., 2011). In these instances, it is likely that brain damage is a result of blood-borne GBS disrupting and penetrating the blood–brain barrier (BBB). This barrier is responsible for the protection of the central nervous system (CNS) from invading microbial pathogens, and is formed by a single layer of specialized brain microvascular endothelial cells (BMECs) (for a review, see Kim, 2008). All ten known GBS serotypes can infiltrate the CNS and cause meningitis; however serotype III, in particular, is responsible for more than 80% of all neonatal meningitis cases (Doran et al., 2005; Ippolito et al., 2010; Melin, 2011; Tazi et al., 2010).

In order to cause meningitis, GBS must infect BMECs as a means of inducing a host inflammatory response and disrupting the BBB, thus permitting its entry into the CNS. *In vitro* and *in vivo* studies have previously confirmed that both GBS virulence factors and host immune responses contribute significantly to the development of bacterial meningitis. Several animal studies have demonstrated that high-levels of bacteria in the blood correlate with the ability of GBS to breach the BBB and invade the CNS (Doran et al., 2005; Ferrieri et al., 1980). *In vitro* investigations have also established that GBS virulence factors contribute to the adherence and invasion of human BMECs (HBMECs), resulting in the activation of acute inflammatory responses which inevitably compromise the

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integrity of the BBB (Nizet et al., 1997; Tenenbaum et al., 2007). Murine models further support these findings and have been used to identify specific GBS virulence factors. The GBS polysaccharide capsule and β-hemolysin/cytolysin are the best characterized virulence factors (for a review, see Maisey et al., 2008; Nizet, 2002). The capsule protects the bacterium from killing by complement-dependent phagocytosis, whereas the β-hemolysin/cytolysin (β-h/c) activates specific signaling pathways in HBMECs resulting in IL-8 release, neutrophil recruitment and better access to the central nervous system (CNS) (Doran et al., 2003). The development of new *in vitro* and *in vivo* models are imperative for better understanding of the molecular mechanisms involved in CNS invasion by GBS.

No commercial vaccine is currently available for GBS disease, although conjugate vaccine preparations have demonstrated promising results following Phase I and II clinical trials in the USA (Baker et al., 2003; Bottomley et al., 2012). Of note, major concerns and ethical issues associated with maternal vaccination may impede Phase III evaluations of candidate GBS vaccines. On the other hand, the increased use of IAP is associated with the risk of emerging antibiotic-resistant strains, and rare GBS isolates with decreased susceptibility to penicillin have recently been reported in the USA and Japan (Dahesh et al., 2008). Therefore new prophylactic and therapeutic strategies are strongly needed.

Over the last decade, the zebrafish (*Danio rerio*) has emerged as a valuable vertebrate model for the study of infectious diseases (van der Sar et al., 2004). The zebrafish, a teleost, shares advantageous qualities with both invertebrate and mammalian model organisms. Like invertebrate models, zebrafish have a short generation time, give rise to large numbers of progeny and are amenable to large-scale genetic screens (Meeker and Trede, 2008; Sullivan and Kim, 2008). Adult zebrafish possess both innate and adaptive immune systems, surpassing classic invertebrate models such as *Drosophila melanogaster* and *Caenorhabditis elegans* who lack functional components of adaptive immunity (van der Sar et al., 2004). Compared to these invertebrate model systems, zebrafish are evolutionary closer to mammals and share many orthologous genes with higher vertebrates including mice and humans (Yoder et al., 2002). Nearly all components of the human immune system have been identified in zebrafish, including myeloid and lymphoid cell lineages (Rombout et al., 2011; Sullivan and Kim, 2008; Yoder et al., 2002), pathogen recognition receptors (Jault et al., 2004), and cytokines and chemokines (Peatman and Liu, 2007; Roca et al., 2008). Sequencing of the zebrafish genome has identified the conservation and homology between human and zebrafish, thus allowing effective modeling of human diseases (Allen and Neely, 2010).

Multiple streptococcal infection models have already been established in zebrafish, including *Streptococcus pyogenes* and *Streptococcus pneumoniae* (Neely et al., 2002; Rounioja et al., 2012), and also more recently *Streptococcus agalactiae* (GBS) (Hanson et al., 2012; Hsieh et al., 2010; Pan et al., 2011). In the present study, we show for the first time that zebrafish may be used as a bacterial meningitis model in GBS infection. We demonstrate that zebrafish are susceptible to GBS infection following intraperitoneal (IP) and intramuscular (IM) challenge. We found that IP infection causes a fulminant infection, with signs of pathogenesis including severe inflammation at the injection site and meningoencephalitis. Furthermore, we show that GBS can successfully replicate within the bloodstream of the zebrafish and disseminate across the BBB, resulting in the infiltration of the CNS and induction of host inflammatory immune responses in the brain. We also found that infection with mutant GBS strains resulted in decreased mortality and bacterial BBB dissemination in our *in vivo* meningitis model. These results constitute a proof of concept study for the use of *Danio rerio* in understanding GBS pathogenesis.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Streptococcus agalactiae (GBS) FIM314 used throughout the majority of this study was isolated from a septic neonatal patient, and it belongs to capsular serotype III, ST-17. Other GBS strains included the hypervirulent COH1 (serotype III, ST-17), wild-type NEM316 (serotype III, ST-23), and the isogenic NEM316 mutants deficient in β-hemolysin (Δ*cylE*) (Forquin et al., 2007), the polysaccharide capsule (Δ*cpsD*) (Poyart et al., 2001), and the major two-component signal transduction system CovS/CovR (Δ*covSR*) (Lamy et al., 2004).

All GBS strains were grown overnight on 5% lamb blood agar plates (37 °C and 5% CO_2). Cells were suspended in 5 ml Todd-Hewitt broth (Becton, Dickinson and Company, BD) supplemented with 0.5% Todd-Hewitt yeast extract (THY), and incubated at 37 °C to allow cells to reach mid-log phase of growth. Bacteria were then harvested when they reached an OD620 of 0.4 (corresponding to 10^8 colony forming units cfu/ml) by centrifugation (7000 rpm, 10 min). Cell pellets were washed and re-suspended in 0.2 M KCl to obtain the desired concentrations for the infection doses. The final bacterial concentrations were confirmed by preparing eight ten-fold serial dilutions and plating them onto 5% lamb blood agar plates (overnight, 37 °C, 5% CO_2).

2.2. Zebrafish

Wild-type AB adult zebrafish (6–9 months old) were used throughout this study. Infected zebrafish were transferred to an isolated PP module stand-alone unit with a separate flow-through system. Care and maintenance of zebrafish used in this study followed established protocols (Nusslein-Volhard and Dahm, 2002), and experiments were approved by the Animal Experiment Board in Finland. All animal studies were conducted in accordance with the European Union regulations for animal experimentation.

2.3. Infection of adult zebrafish

Groups of 15 fish were used for survival assays. Adult zebrafish were first anesthetized with buffered 0.02% tricaine (Sigma–Aldrich). For IP injection, an anesthetized fish was placed supine (i.e. abdomen facing upwards) and supported by a moistened foam bed to ensure it remained in an upright position. A U-100 insulin syringe with a 12 mm needle was used to administer 5 μl of bacterial suspension into the fish (Supplementary Fig. 1). The needle was inserted into the midline of the abdomen posterior to the pectoral fins, as described by Phelps et al. (2009). No more than the tip of the needle was inserted into the abdomen of each fish, as a means of preventing damage of internal organs. For the IM injection, an anesthetized fish was placed inside a moistened foam bed, as above, and positioned prostrate (i.e. abdomen facing downwards). A micromanipulator was used to position the microcapillary needle anterior to the dorsal fin, at a 45° angle relative to the spine (Neely et al., 2002; Phelps et al., 2009) (Supplementary Fig. 1). Five microliters of the bacterial suspension was delivered into the dorsal muscle of the fish using a PV830 Pneumatic PicoPump microinjector (World Precision Instruments). To visualize the possible leakage of solution during injections, sterile-filtered phenol red (3 mg/ml) was added to bacterial suspensions before injections (1:10 ratio).

In the survival assays, strictly defined non-terminal humane endpoint criteria were followed and approved by the National Animal Experiment Board. Fish seen as displaying aberrant swimming behaviors (i.e. spiral-like swimming movements) or observed to be

suffering were euthanized, and time of death was recorded. Fish were monitored and mortalities recorded three times a day, over a seven-day period, in order to obtain survival rates.

2.4. Collection of blood samples

Following IP infection, 5 fish were randomly sampled and anesthetized at the specified time points. The fish's neckline was cut using a sterile scalpel blade and a 5 μl blood sample collected. Blood samples were transferred to an eppendorf tube containing 45 μl KCl buffer on ice to prevent the blood from coagulating. Eight ten-fold serial dilutions of each recovered blood sample were prepared using 0.2 M KCl and plated onto selective 5% lamb blood agar plates (erythromycin, 1 μg/ml) and incubated overnight (37 °C and 5% CO_2) for enumeration of bacterial colonies.

2.5. Isolation of the brain

Fish were collected at the specified time points and euthanized with tricaine. Fish were sprayed with 95% EtOH before being placed on a sterile dissecting board and pinned dorsal side up. A sterilized scalpel blade was used to remove the skull plate and expose the brain cavity (Phelps et al., 2009). Whole brain tissue was removed using sterile forceps and transferred to an eppendorf tube containing 200 μl sterile phosphate buffered saline (PBS) solution. Eight ten-fold serial dilutions of brain samples were prepared using 0.2 M KCl and plated onto selective 5% lamb blood agar plates, as before. After overnight incubation, the bacterial colonies were counted and the brain bacterial loads were determined.

2.6. Real-time qRT-PCR analysis

Fish were first injected IP with either 10^5 cfu FIM314 or 0.2 M KCl, and five fish were randomly sampled from each group at 2, 6, 12, 24 and 48 h post-infection. Fish were euthanized with tricaine and their brains recovered, as described previously, for qRT-PCR analysis. Total RNA extraction was performed using the TRIsure RNA Purification Kit (Bioline) according to the manufacturer's instructions. Real-time qRT-PCR for mRNA expression levels of IL-1β, IL-6 and β-actin were performed using the iScript™ One Step RT-PCR Kit with SYBR® Green (Bio-Rad) and the ABI7000 instrument (Applied Biosystems) according to the manufacturer's instructions. Reactions were carried out in triplicate, and relative expression levels were calculated using the standard curve method (Larionov et al., 2005). Expression levels of IL-1β and IL-6 were normalized to the corresponding β-actin values and non-treated controls. Results were analyzed with the ABI 7000 System SDS software, version 1.2.3. The primers used in this assay are listed in Table 1. IL-6 primers were obtained from a study carried out by Varela et al. (2012), and IL-1β and β-actin primers from Pressley et al. (2005).

2.7. Statistical analyses

Each experiment was repeated three times. The 50% lethal doses (LD_{50}) for IP and IM infection routes were determined by performing three separate survival experiments and infecting groups of 15 fish with concentrations of GBS, ranging between 10^2 and 10^6 cfu. For each infection route, the survival data was collated and the dose that killed 50% of a test population was considered as the LD_{50}. Survival data were tested using the Kaplan–Meier survival analysis, and qRT-PCR data analyzed using one-way ANOVA. Statistical analysis of blood and brain cfu was performed using an unpaired two-tailed Student's *t*-test, and Pearson's correlation coefficients were calculated to evaluate the relationship between blood and brain bacterial loads. All data were analyzed using the GraphPad Prism® 5.0 software. Values of $P \leqslant 0.05$ were accepted as statistically significant.

Table 1
Primer sequences used for real-time qRT-PCR.

Target gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
β-actin	ATG GAT GAG GAA ATC GCT G	ATG CCA ACC ATC CCC TG
IL-1β	TGG ACT TCG CAG CAC AAA ATG	GTT CAC TTC ACG CTC TTG GAT G
IL-6	TCA ACT TCT CCA GCG TGA TG	TCT TTC CCT CTT TTC CTC CTG

3. Results

3.1. GBS infection in adult zebrafish

We first sought to determine whether the FIM314 GBS strain causes significant disease through the intraperitoneal (IP) and intramuscular (IM) routes of infection. In order to investigate the virulence capacity of FIM314, groups of 15 adult fish were challenged with various doses of FIM314 ranging from 10^2 to 10^6 cfu. Survival rates were recorded daily and followed over a period of 7 days following infection. Regardless of the injection route, mortality was dose-dependent following infection with FIM314 (Fig. 1A and B). Importantly, the LD_{50} for the IP infection route (10^2 cfu) was relatively lower than that of the IM route (10^4 cfu). Time of death also differed between these two routes, with IP injections causing mortalities from 24 to 96 h post-injection (hpi) and IM injections from 48 to 120 hpi. Infection with 10^6 cfu resulted in the death of all zebrafish ($n = 15$) for both IP and IM routes, and was associated with the most rapid progression of disease. Control fish, which were injected IP or IM with sterile KCl buffer, exhibited no abnormal behaviors and survived throughout these experiments.

These survival assays revealed that the zebrafish immune system is capable of resolving GBS infection at lower doses. To validate these findings and examine the expansion of bacteria in the bloodstream, fish were injected IP with 10^2 cfu FIM314 and blood samples were recovered from fish at the specified time points following infection (Fig. 1C). Both healthy and sick fish were included in this assay to quantify the level of bacteremia. Fig. 1C shows that FIM314 is capable of replicating within the zebrafish bloodstream post-infection, and that survival is associated with the activation of bacterial clearance mechanisms. Some fish were capable of resolving the infection better than others, and dying fish had higher levels of bacteremia compared to those evidently coping with the infection. However, no fish appeared sick or dying 96 h following infection, and almost all fish had completely cleared FIM314 from the bloodstream 120 hpi. Taken together, these results demonstrate that GBS can successfully replicate and establish infection in zebrafish, and that the zebrafish immune system is capable of resolving GBS infection following injection of low bacterial doses.

3.2. Hematogenous dissemination of GBS into the brain

As IP injection results in an immediate spread of GBS to the bloodstream (Supplementary Fig. 2) and causes an overwhelming systemic infection similar to what is observed during human neonatal GBS sepsis, we used this injection route for the rest of the study. Fish infected with 10^5 and 10^6 cfu via the IP route were examined 48 hpi for signs of systemic infection. All fish suffered from severe trauma at the injection site (Fig. 2F). Interestingly, fish exhibited visible swollen brain cavities and cerebral edema (Fig. 2C–E), suggesting high bacterial loads in the brain. These fish

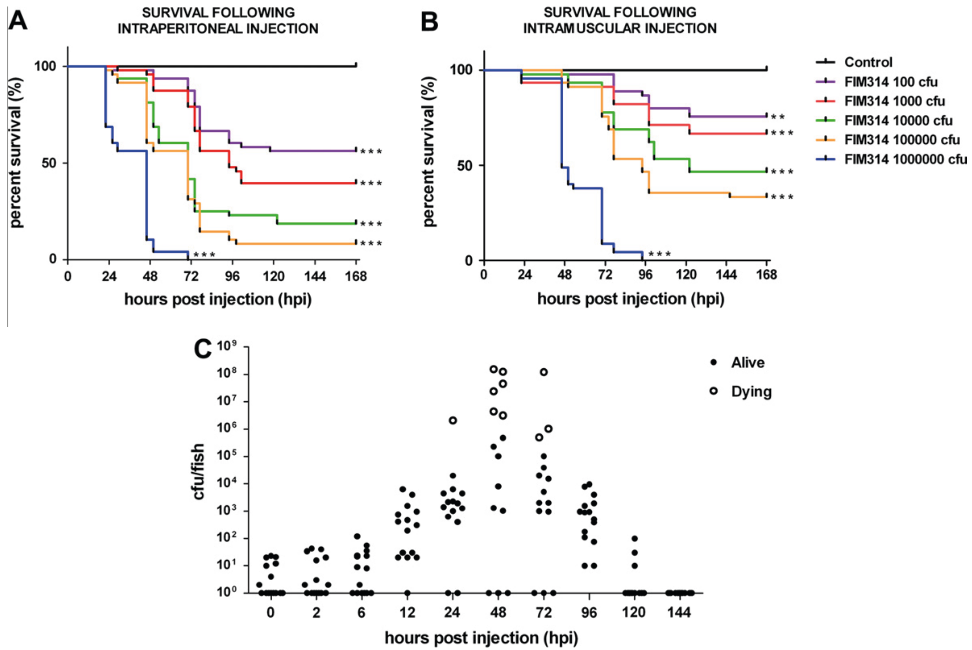

Fig. 1. Kaplan–Meier survival curves of adult zebrafish following intraperitoneal (IP) (A) and intramuscular (IM) (B) injection with the indicated doses of FIM314. Data comprise of three replicate experiments ($n = 15$ per group, per experiment). Control fish were injected either IP or IM 0.2 M sterile KCl buffer. The asterix (*) depicts a statistically significant difference compared to the control group, where $^{**}P \leqslant 0.005$, $^{***}P \leqslant 0.0005$. (C) Immune clearance of GBS infection following IP challenge with 10^2 cfu FIM314. Each circle represents one blood sample taken from an individual zebrafish at each indicated time point. Both surviving and dying fish were analyzed in this clearance assay. Graph represents collated data from three replicate experiments ($n = 5$ per time point, per experiment).

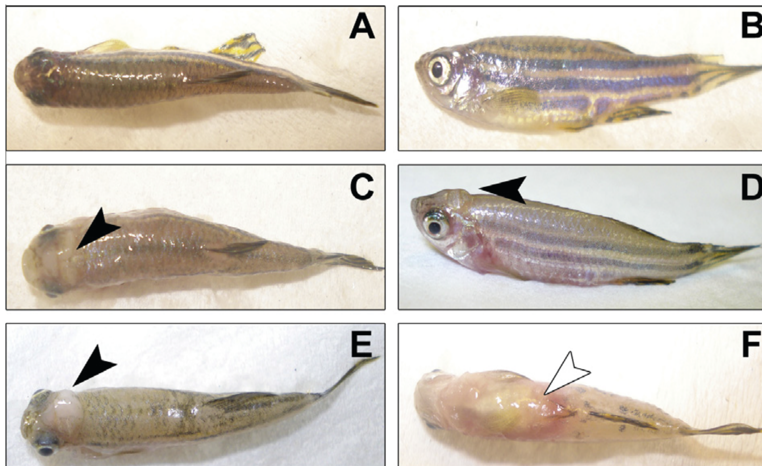

Fig. 2. Gross pathology of adult zebrafish infected IP with GBS. Following IP injection with 10^5 cfu (C and D) and 10^6 cfu (E and F) FIM314, fish were examined 48 hpi for signs of visible GBS disease. Black arrowheads indicate visible cerebral edema and the white arrowhead indicates severe inflammation at the injection site. Control fish were injected IP with sterile 0.2 M KCl (A and B).

also displayed abnormal, spiral-like swimming patterns at the time of death. Control fish injected IP with sterile KCl buffer displayed no such behaviors and showed no signs of disease (Fig. 2A and B). These observations led us to hypothesize that GBS could penetrate the BBB and cause meningitis in adult zebrafish.

To investigate whether GBS are capable of infiltrating the brain and thus causing the observed cerebral edema, we next quantified the bacterial loads in blood and whole brain tissue samples. Fish were collected at the specified time points following IP challenge with 10^5 cfu FIM314. Fig. 3A shows a direct correlation between

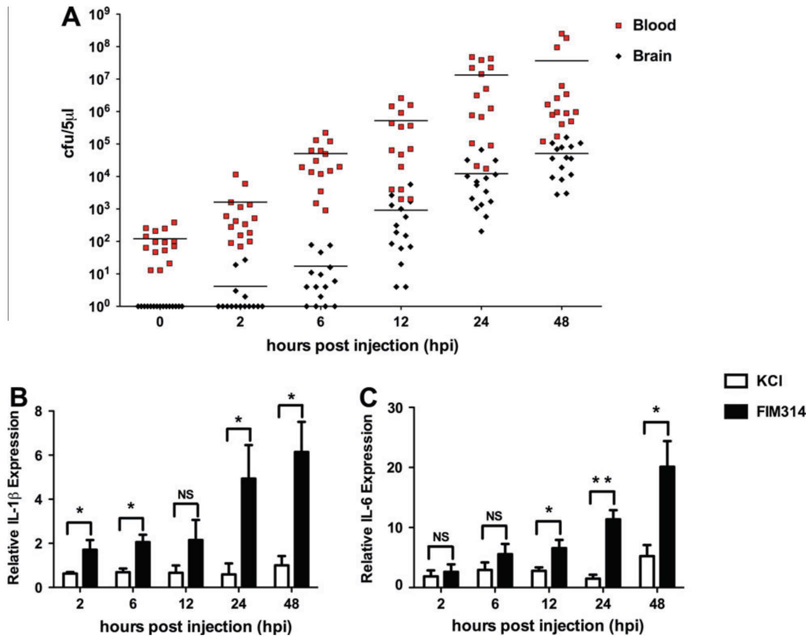

Fig. 3. Hematogenous dissemination of GBS into the CNS induces an inflammatory response in adult zebrafish brain. (A) GBS dissemination from the bloodstream into the brain following IP challenge with 10^5 cfu FIM314. Each symbol represents one blood and brain sample taken from individual zebrafish at each indicated time point. Graph shows collated data from three replicate experiments ($n = 5$ per time point, per experiment). Bars represent mean bacterial cfu. Real time qRT-PCR analysis of IL-1β (B) and IL-6 (C) in zebrafish brain following IP injection with 10^5 cfu FIM314. RNA was extracted from adult zebrafish brains sampled at the specified time points. Data shown are the mean ± standard error of the mean of three replicate experiments. $^*P \leqslant 0.05$, $^{**}P \leqslant 0.005$; NS = not significant compared to FIM314.

the level of bacteremia and the bacterial load in the brain after infection. GBS was present in the bloodstream almost immediately after injection; however bacteria were not present in the brain until 6 hpi. GBS colonies were detected in all brain samples recovered 12 hpi, and mean bacterial cfu increased from 12 to 48 hpi. No GBS colonies were present in blood and brain samples collected from KCl-injected fish (data not shown).

To evaluate the host inflammatory response to GBS infection in the brain, we used quantitative RT-PCR to measure the production of pro-inflammatory cytokines IL-1β and IL-6 following IP injection with 10^5 cfu FIM314. Fig. 3B and C show an increase in both cytokines 24 hpi, suggesting that the zebrafish brain promotes the production of pro-inflammatory cytokines in response to CNS invasion. These results indicate that GBS induces an innate inflammatory response following CNS invasion, in agreement with previous work using mouse models of GBS meningitis (Banerjee et al., 2011; Doran et al., 2005).

3.3. GBS virulence factors contribute to the pathogenesis of meningitis in zebrafish

The zebrafish temperature (28.5 °C) is not considered optimal for human-adapted pathogens such as GBS; however all GBS strains used in this study grow at a comparable rate in THY medium at 28.5 and 37 °C (Supplementary Fig. 3). Earlier rodent studies have identified specific virulence factors important for GBS meningitis (for a review, see Maisey et al., 2008; Tazi et al., 2012). Thus, we next employed our GBS-meningitis model to determine whether mutant GBS strains lacking particular virulence factors were attenuated in this *in vivo* model system. Fish were infected IP with 10^6 cfu of the non-hemolytic mutant NEM316Δ*cylE*, the non-capsulated mutant NEM316Δ*cpsD* and the NEM316Δ*covSR* mutant lacking the CovS/CovR regulatory system (Fig. 4). Survival rates were followed over a period of 7 days, as before, following infection with mutant GBS strains. It has been previously reported that GBS mutants lacking the *cylE* gene encoding for β-hemolysin show decreased virulence in mice and have an impaired ability to penetrate the BBB (Doran et al., 2003; Liu et al., 2004). Following infection with 10^6 cfu of NEM316Δ*cylE*, zebrafish showed significant higher rates of survival (82%) compared to those infected with WT NEM316 (65%) ($P \leqslant 0.05$). Moreover, NEM316Δ*cpsD* appeared avirulent in adult zebrafish, exhibiting higher survival rates (84%) compared to WT NEM316 ($P \leqslant 0.05$). The polysaccharide capsule is another virulence factor which has been implicated in the pathogenesis of GBS, as it prevents the binding of opsonizing antibodies thus promoting GBS survival in the bloodstream (Rubens et al., 1987). Correspondingly, fish infected IP with the highly encapsulated COH1 strain showed enhanced virulence, resulting in the

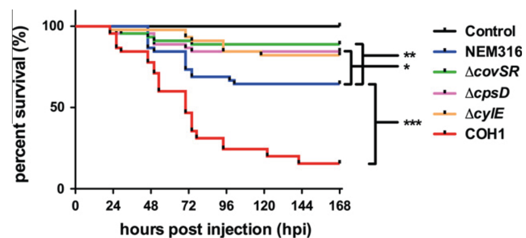

Fig. 4. Kaplan–Meier survival curve of adult zebrafish following IP infection with 10^6 cfu wild-type NEM316, mutant NEM316 strains and hypervirulent GBS strain COH1. Graph represents data from three replicate experiments ($n = 15$ per group, per experiment). $^*P \leqslant 0.05$, $^{**}P \leqslant 0.005$, $^{***}P \leqslant 0.0005$ compared to wild-type NEM316.

death of 85% of fish ($P \leqslant 0.0005$). The CovS/CovR system (also known as the CsrRS system) has been proven to regulate the expression of important GBS virulence factors, including β-hemolysin and the polysaccharide capsule (Jiang et al., 2005; Lamy et al., 2004), and NEM316Δ*covSR* also showed significantly higher survival rates (89%) in zebrafish than WT NEM316 ($P \leqslant 0.005$).

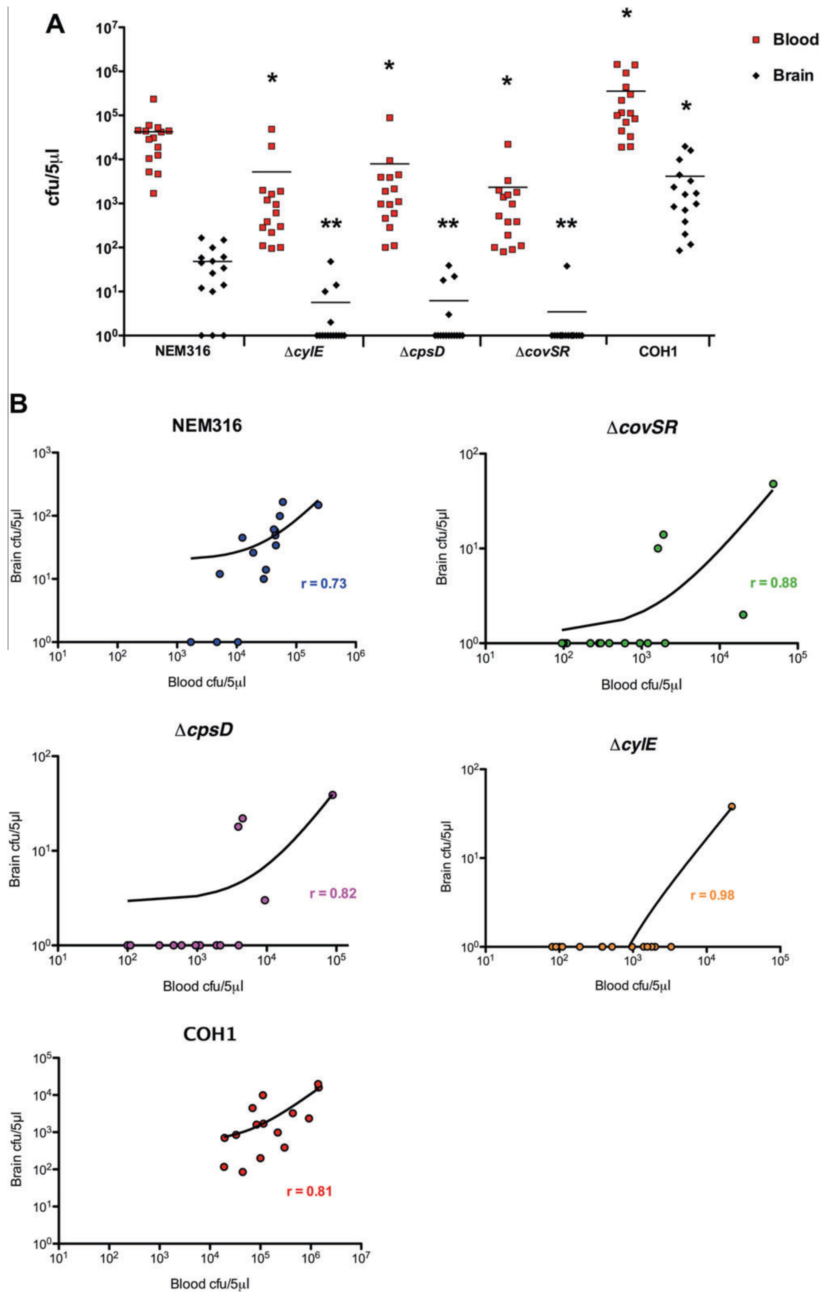

Fig. 5. Attenuation of mutant GBS strains in zebrafish meningitis model. (A) Bacterial load in blood and brain 24 h post IP injection with 10^6 cfu wild-type NEM316, NEM316-derived mutant strains and hypervirulent GBS strain COH1. Each symbol represents one blood and brain sample recovered from individual zebrafish 24 hpi. Bars represent mean bacterial cfu. Graph shows collated data from three individual experiments ($n = 5$ per time point, per experiment). $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.005$ compared to wild-type NEM316. (B) Data from figure 5A represented as scatter plots, illustrating the direct correlation between the level of bacteremia and brain bacterial load following IP infection with 10^6 cfu of each strain. Pearson's correlation coefficients (r) were calculated to evaluate the relationship between blood and brain bacterial loads.

To investigate whether β-hemolysin, the polysaccharide capsule and the CovS/CovR regulatory system were essential for GBS to cause meningitis in zebrafish, blood and brain samples were recovered from fish 24 h following IP challenge with 10^6 cfu (Fig. 5A). The majority of brain samples recovered from fish infected with mutant GBS strains were clear of bacteria (80%), indicating that these virulence factors are important for BBB penetration. These fish also had significantly lower mean levels of bacteremia ($\leqslant 8 \times 10^3$ cfu/5 μl) compared to those infected with WT NEM316 (4.2×10^4 cfu/5 μl) ($P \leqslant 0.05$). Conversely, COH1-infected fish exhibited significantly higher mean bacterial loads in the blood (3.5×10^5 cfu/5 μl) and brain (4.2×10^3 cfu/5 μl) compared with WT NEM316-infected fish ($P \leqslant 0.05$). As shown in Fig. 5B, a direct correlation exists between the level of bacteremia and the bacterial load in the brain after infection with WT and mutant NEM316 strains. In summary, our results prove that adult zebrafish may be used as an *in vivo* model to identify and further evaluate the role of GBS virulence factors in the pathogenesis of meningitis.

4. Discussion

The zebrafish has recently emerged as a valuable infection model for human streptococcal diseases. Zoonotic streptococcal pathogens such as *Streptococcus iniae* and *Streptococcus suis* have been studied previously in adult zebrafish (Neely et al., 2002; Wu et al., 2008), as well as important human pathogens *Streptococcus pyogenes* and *Streptococcus pneumoniae* (Miller and Neely, 2004; Rounioja et al., 2012). In the present study, we have established an adult zebrafish model of GBS meningitis, proving that GBS successfully causes systemic disease and infiltrates the CNS by disseminating from the bloodstream and penetrating the BBB.

Adult zebrafish are susceptible to infection by FIM314 through the IP and IM injection routes. The low LD_{50} of 10^2 cfu following IP injection highlights the lethality of GBS and its ability to induce a fulminant infection in zebrafish, resembling infection in human neonates. Notably, the visible cerebral edema was observed more frequently, and earlier (48 hpi), in those fish which were injected IP with FIM314 than by the IM route. As IP injections give bacteria direct access to the vasculature, GBS can establish an almost immediate systemic infection and invade the CNS. IM injections resulted in localized infections, allowing for the activation of the zebrafish's innate immune defense mechanisms involved in the resolution of the infection. As our results revealed that injection into the peritoneal cavity resulted in rapid GBS disease, the IP injection route was used throughout our study to determine whether GBS disseminates to the CNS hematogenously in zebrafish, thus causing meningitis. We note that the IP delivery route is not physiologic for zebrafish; however it is a well-established infection route for this model organism and has been successfully implemented in several previous zebrafish infection studies (Miller and Neely, 2004; Neely et al., 2002; Phelan et al., 2005; Pressley et al., 2005; Prouty et al., 2003).

In vivo models have previously reported that the ability of GBS to invade the CNS is associated with the level and duration of bacteremia (Doran et al., 2005; Ferrieri et al., 1980). In the present study, we demonstrate that a direct correlation exists between the level of bacteremia and brain bacterial load following GBS infection in zebrafish. Recent murine studies have also confirmed that GBS infection promotes pro-inflammatory cytokine expression in the brain endothelium; in particular IL-1β and IL-6 have been shown to be produced early in CNS infection (Banerjee et al., 2011; Barichello et al., 2011). Quantification of bacterial loads in the brain confirmed high levels of GBS present 24 h post-infection, coinciding with the qRT-PCR results that revealed an upregulation of IL-1β and IL-6 production in the brain 24 h post-injection. IL-1β is of particular importance in the pathogenesis of bacterial meningitis as it promotes the production of other pro-inflammatory cytokines (including IL-6) and promotes trafficking of peripheral leukocytes to the brain, resulting in increased inflammation at the BBB and thus compromising its integrity (Leib and Täuber, 1999). Overall our findings provide evidence that an innate inflammatory response is initiated in the brain in response to bacterial infiltration of the CNS 24 h post-infection with GBS; in agreement with previously reported results from meningitis mouse models.

We report that β-hemolysin and the polysaccharide capsule have important roles in the development of meningitis in zebrafish. Infection with mutant GBS strains deficient in these virulence factors are attenuated in our zebrafish meningitis model, resulting in significantly higher survival rates and reduced CNS infiltration compared to the wild-type NEM316 strain. β-hemolysin and the polysaccharide capsule (also known as the CPS) have been recognized as important virulence factors which impact the pathogenesis of GBS meningitis. β-hemolysin is a pore-forming cytolysin proven to cause damage to a wide array of host cell types, including BMECs which protect the CNS from bacterial invasion (Doran et al., 2003, 2005; Nizet et al., 1997). The sialylation of the polysaccharide capsule allows GBS to mimic host cell surfaces and evade immune recognition, thus prolonging GBS survival in the bloodstream (Chaffin et al., 2005; Doran and Nizet, 2004). Hanson et al. (2012) demonstrated that infection with a mutant GBS strain lacking the CpsA protein (responsible for capsule synthesis) is attenuated in zebrafish, highlighting the importance of the polysaccharide capsule in the pathogenesis of GBS disease.

The two-component CovS/CovR system has been shown to directly regulate the expression of over one hundred of GBS genes (Lamy et al., 2004; Jiang et al., 2005). We report that the CovS/CovR-deficient strain is also attenuated in our zebrafish meningitis model, showing higher survival rates and poor CNS infiltration compared with the wild-type GBS strain. Our results are in agreement with Lamy et al. (2004), who showed that the CovS/CovR system is critical for GBS virulence in the neonate rat model, and reported a 3 log increase of the LD_{50} of the Δ*covSR* strain compared to the wild-type strain. Conversely, Lembo et al. (2010) demonstrated that infection with a CovR-deficient GBS strain promoted inflammatory cytokine production and increased BBB penetration in a murine model of GBS meningitis. Thus, our results suggest that both the CovS and CovR components are important for BBB penetration and CNS invasion. Overall our results with these mutant GBS strains are in line with murine models of GBS meningitis and what is known in human GBS disease; validating our research model despite the fact that GBS is not natural pathogen of zebrafish. As such, this zebrafish meningitis model may be employed in the future as a means of providing further mechanistic insight into the role of GBS virulence factors in the pathogenesis of meningitis.

5. Conclusion

In this study, we report the use of the adult zebrafish as a model for GBS-induced meningitis. Our results coincide with those previously reported in murine studies of GBS meningitis, highlighting that the zebrafish is another valuable *in vivo* model for studying the pathogenesis of GBS meningitis. This zebrafish model is highly suitable for large-scale genetic screens which could identify and evaluate novel GBS virulence factors and vaccine candidates. We hope that this new model will provide tools to decipher the molecular mechanisms exploited by GBS to invade the CNS, hence influencing the progression and outcome of GBS disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2012.07.007.

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Adult zebrafish model for pneumococcal pathogenesis

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ABSTRACT

Streptococcus pneumoniae (pneumococcus) is a leading cause of community acquired pneumonia, septicemia, and meningitis. Due to incomplete understanding of the host and bacterial factors contributing to these diseases optimal treatment and prevention methods are lacking. In the present study we examined whether the adult zebrafish (*Danio rerio*) can be used to investigate the pathophysiology of pneumococcal diseases. Here we show that both intraperitoneal and intramuscular injections of the pneumococcal strain TIGR4 cause a fulminant, dose-dependent infection in adult zebrafish, while isogenic mutant bacteria lacking the polysaccharide capsule, autolysin, or pneumolysin are attenuated in the model. Infection through the intraperitoneal route is characterized by rapid expansion of pneumococci in the bloodstream, followed by penetration of the blood–brain barrier and progression to meningitis. Using Rag1 mutant zebrafish, which are devoid of somatic recombination and thus lack adaptive immune responses, we show that clearance of pneumococci in adult zebrafish depends mainly on innate immune responses. In conclusion, this study provides evidence that the adult zebrafish can be used as a model for a pneumococcal infection, and that it can be used to study both host and bacterial factors involved in the pathogenesis. However, our results do not support the use of the zebrafish in studies on the role of adaptive immunity in pneumococcal disease or in the development of new pneumococcal vaccines.
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1. Introduction

Despite significant advances in the development of vaccination strategies against *Streptococcus pneumoniae* (pneumococcus), the bacterium is still a major human pathogen causing over one million deaths every year, a majority among children (Lynch and Zhanel, 2010). Pneumococci are common colonizers of the human upper respiratory tract and can persist there as asymptomatic, harmless inhabitants (van der Poll and Opal, 2009). In many cases, however, pneumococci spread to the lower respiratory tract or further into the bloodstream, and cause a variety of diseases (van der Poll and Opal, 2009). These diseases range from relatively mild but very common infections, such as sinusitis or otitis media to life-threatening diseases such as pneumonia, sepsis, and meningitis (Gladstone et al., 2011).

S. pneumoniae is the leading cause of community acquired pneumonia worldwide (Chiavolini et al., 2008; Said et al., 2013). It has been estimated to be responsible for up to 5 million cases of pneumonia annually, with around 10% of all cases leading to death (Feldman and Anderson, 2011). Pneumococci are also one of the most important causative agents of bacterial meningitis (Brouwer et al., 2010; Randle et al., 2011). Pneumococcal meningitis is associated with a lethality of as high as 37% (Lynch and Zhanel, 2010; Mook-Kanamori et al., 2011). In addition, up to 52% of surviving patients suffer from neurological sequelae such as hearing loss, cognitive impairment, and seizures (Mook-Kanamori et al., 2011; Randle et al., 2011). Furthermore, recurrent ear infections caused by pneumococci are also the most common reason for doctors' consultations and the use of antibiotics (Boonacker et al., 2011).

Pneumococci express a range of virulence factors, such as a polysaccharide capsule, pneumolysin, autolysin, and a pilus structure, which contribute to the pathogenesis and the progression of the infection (Jedrzejas, 2001; Kadioglu et al., 2008; Orrskog et al., 2012). The polysaccharide capsule is commonly accepted as the most important virulence factor of the bacterium (Mitchell and Mitchell, 2010; Vernatter and Pirofski, 2013). The capsule is highly antiphagocytic, and un-encapsulated pneumococcal mutants are avirulent in several animal models (Nelson et al., 2007;

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Watson and Musher, 1990). The polysaccharide composition of the pneumococcal capsule is also the basis for the division of pneumococci into the so far recognized 93 serotypes (Gladstone et al., 2011). Several epidemiological studies have shown that pneumococcal capsular serotypes differ in their frequency and geographical occurrence, as well as in their tendency to cause infections in humans (Brueggemann et al., 2003; Sandgren et al., 2004; Sjöstrom et al., 2006). These studies have also shown that some pneumococcal serotypes, more often than are others, are associated with severe invasive diseases in humans. Serotypes with a high potential to cause invasive disease are for example types 1, 4 and 7F (Sandgren et al., 2005; Sjöström et al., 2006). Types 6B and 14 have a medium invasive potential, and serotype 19F a low invasive potential (Sandgren et al., 2005; Sjöström et al., 2006). However, when the different strains caused an invasive disease, serotypes belonging to the group with a high invasive potential (i.e., types 1, 4 and 7F) had a lower mortality rate than did serotypes with a lower invasive potential such as type 19F (Sjöström et al., 2006). Pneumococci of the same serotype can also vary in their pathogenicity, indicating that clonal properties other than the capsular structure also affect the ability of the bacterium to cause an invasive disease (Brueggemann et al., 2003; Sandgren et al., 2005). Although the serotype and clone specific properties are evident, the factors contributing to these differences are still poorly understood.

Two vaccine formulations against pneumococcal diseases have been developed and are in use globally: a 23-valent polysaccharide vaccine and a conjugated vaccine (Gladstone et al., 2011). These vaccines are serotype specific polysaccharide-based formulations directed against the most prevalent pneumococcal serotypes found to cause an invasive disease (Gladstone et al., 2011). However, the complex nature and structural variability of pneumococci pose challenges for the prevention and treatment of the diseases associated with this pathogen, and despite the positive impact of current vaccines pneumococci remain to be eradicated. In addition, increasing antibiotic resistance among clinical strains further complicates the control of pneumococcal spread (Lynch and Zhanel, 2010; Song et al., 2012). In order to overcome these challenges, the best possible understanding of the interactions occurring between the host and the pneumococci during the infection is required. These details are best revealed by studying animal models where both pathogenic and host specific determinants of infection can be investigated. Several rodent models have proved their feasibility and reliability in infection studies, but methodical difficulties in addition to ethical issues concerning these models complicate their use in this field (van der Sar et al., 2004). The zebrafish, *Danio rerio*, provides an attractive alternative non-mammalian vertebrate model for the study of infection biology and immunology (Lohi et al., 2013). In addition to being more ethical, zebrafish are cost-effective and easy to manipulate genetically. Importantly, the adult fish has fully developed innate and adaptive immune systems. These factors make the zebrafish an ideal infection model suitable also for large scale genetic screens (Phelps and Neely, 2005; van der Sar et al., 2004). So far, the zebrafish has been successfully used to model host-pathogen interaction in infectious diseases caused by various pathogens, including both zoonotic (e.g., *Mycobacterium marinum*) and human specific pathogens (e.g., *Salmonella typhimurium*, *Staphylococcus aureus*) (Parikka et al., 2012; Prajsnar et al., 2008; Prouty et al., 2003; van der Sar et al., 2003). These pathogens also include several streptococcal species, such as *Streptococcus iniae* and *Streptococcus agalactiae* (Neely et al., 2002; Patterson et al., 2012).

Here we introduce a new non-mammalian model to be used in the study of pathophysiological mechanisms of pneumococcal infection. We have previously shown that pneumococci can cause a fulminant infection in zebrafish embryos and that surviving embryos can combat the infection using innate immunity mechanisms (Rounioja et al., 2012). Here we show that a pneumococcal infection can also be established in adult zebrafish, which provides a more diverse model for the study of pneumococcal infection.

2. Materials and methods

2.1. Bacterial strains

The wild type pneumococcal strain TIGR4 (T4) used throughout these studies was originally isolated from a Norwegian patient suffering from a systemic pneumococcal infection (Aaberge et al., 1995). It belongs to capsular serotype 4 and a highly invasive clone ST205 and has previously been used in a zebrafish embryo model (Rounioja et al., 2012). The other pneumococcal strains used were four clinical isolates belonging to serotypes with different invasive disease potentials (serotype 1 (ST306), serotype 14 (ST124), serotype 6B (ST138) and serotype 19F (ST162)) as well as four T4 isogenic mutants deficient in either the polysaccharide capsule (T4R), autolysin (T4Δ*lytA*), pneumolysin (T4Δ*ply*), or the pilus-like structure (T4Δ*rlr*).

All the pneumococcal strains used were grown overnight on blood agar plates at 37 °C and 5.0% CO_2. Bacterial cells were suspended in 5 ml of Todd Hewitt broth (Becton, Dickinson and Company, New Jersey, USA) supplemented with 0.5% Todd-Hewitt yeast extract (Becton, Dickinson and Company) until they reached an OD_{620} of 0.1. Bacteria were grown in the media until they reached an OD_{620} of 0.4, after which the bacterial cells were harvested by centrifugation (4000 rpm, 10 min) and re-suspended in sterile 0.2 M KCl to obtain the desired concentrations. To be able to visualize the injection, 10% filtered phenol red (3 mg/ml; Sigma–Aldrich, St. Louis, Missouri, USA) was added to the injection solution. The exact bacterial numbers in inoculates were confirmed by quantitative plating before and after the experiment.

2.2. Zebrafish and maintenance

Wild type AB zebrafish of 5–8 months of age were used in the majority of the experiments. In addition, the mutant fish line ($Rag1^{hu1999}$) (from ZIRC), which lack active lymphocytes was used to assess the role of the adaptive immunity. All the animal studies were conducted in accordance with the regulations of the Animal Experiment Board in Finland and the fish were maintained according to standard protocols (Nusslein-Volhard and Dahm, 2002). Following infection fish were kept in an isolated stand-alone unit with a separate flow-through system.

2.3. Infection of adult zebrafish by intraperitoneal and intramuscular injection

Generally, fish were randomly divided into groups of 15–20 fish, each group receiving a different concentration of bacterial inoculation. Prior to injection the fish were anesthetized in water containing 0.02% 3-aminobenzoic acid ethyl ester (pH 7.0) (Sigma–Aldrich) and gently laid on a moistened foam bed ventral side up. An Omnican 100 30G insulin needle (Braun, Melsungen, Germany) containing 5 μl of the desired injection suspension was held parallel to the fish and inserted into the midline of the abdomen, between the pectoral fins and the entire content of the needle was carefully injected into the fish peritoneum. Similarly, the fish in the control group received 5 μl 0.2 M KCl. If any leakage of the injection solution was observed at the injection site, the fish was euthanized. Successfully infected fish were transferred to a fresh water tank and their recovery was verified before moving them

into the water unit. Any fish observed to be bleeding or otherwise suffering was immediately euthanized.

For the intramuscular (i.m.) injection, anesthetized fish were placed on a moistened foam bed dorsal side up. The i.m. injection was carried out using a micromanipulator and the PV830 Pneumatic PicoPump microinjector (World Precision instruments, Sarasota, Florida, USA). A microcapillary needle filled with 5 μl of bacterial suspension was positioned anterior to the dorsal fin at a 45° angle in relation to the back of the fish and the suspension was delivered into the dorsal muscle. As above, successfully infected fish were transferred to the fresh water tank to recover.

To obtain survival rates, the mortality of the infected fish was followed twice a day during a 7-day period. Dead fish were removed and the fish likely to be suffering were euthanized and the time of death was recorded. Strict humane endpoint criteria were followed during the survival assays. Aberrant swimming and behavior, increased rate of respiration, and lack of movement, for example, were considered as criteria for euthanasia.

2.4. Collection of blood samples

Blood samples were taken from five randomly chosen infected fish at given time points (0, 2, 6, 12, 24, 48, 72 and 96 h post infection, hpi). The fish were briefly anesthetized as described above (Section 2.3) and laid on a sterile plastic surface. Blood samples were taken by cutting the fish neckline with a scalpel blade and collecting 5 μl of blood which we estimate to represent on average one fourth of the total volume of adult zebrafish blood. Blood samples were transferred to Eppendorf tubes containing 45 μl 0.2 M KCl and mixed carefully to prevent coagulation. Each blood sample was serially diluted and the dilutions were plated onto selective lamb blood agar plates containing 1 μg/ml erythromycin. After an overnight incubation at 37 °C and 5.0% CO_2 the bacterial colonies were counted to determine the bacterial numbers in each blood sample.

2.5. Isolation of the brain

Five infected fish from each group were randomly collected and prepared for the isolation of the brain. The fish were euthanized as above and placed on a dissecting board dorsal side up. Fish were tacked to the board by pinning and the surface of the fish was sterilized with 70% ethanol in order to prevent the contamination of the brain. A sterile scalpel blade and forceps were used to remove the skull plate and expose the brain. The exposed brain tissue was then completely removed using sterile equipment and transferred to an Eppendorf tube containing 200 μl of phosphate buffered saline (PBS) solution. Special care was taken to prevent contamination of the brain tissue. The brain tissue in PBS was then homogenized using a sterile pestle, after which the homogenate was serially diluted in PBS. The dilutions were plated onto selective blood agar plates and incubated overnight (37 °C and 5.0% CO_2). After incubation, the colony forming units were counted and the bacterial load in each brain homogenate was determined.

2.6. Immunization

20–30 randomly chosen fish were immunized with a heat-killed T4, a non-lethal dose of live T4, or a live un-encapsulated mutant of T4 (T4R). To prepare the killed bacteria for immunization, bacteria were first grown in suspension as described in Section 2.1. Thereafter, the bacterial cells were harvested by centrifugation (4000 rpm, 10 min) and resuspended in 500 μl 0.2 M KCl. The bacterial suspension was then autoclaved to heat-kill the bacteria. These autoclaved immunization stocks were then either used for immunization or stored at −20 °C to be used later. Both i.p. and i.m. injection routes (described in Section 2.3) were tested for immunization. After immunization the humoral immune response was allowed to develop for 2 or 3 weeks after which the immunized individuals were infected with a semilethal dose (2.5×10^6 cfu) of pneumococcus either through the i.p. or the i.m. route. The infection dose was verified as above. Two negative control groups received either a KCl immunization followed by infection or infection without immunization. The mortality of the infected fish was monitored for approximately 14 days.

2.7. Statistical analyses

Survival rates in the experiments were analyzed with the Graph Pad Prism 5.02 software. The statistical significance in these assays was measured using the Mantel-Cox log-rank test. p values of <0.05 were considered significant.

3. Results

3.1. The adult zebrafish can be used as a disease model for a systemic pneumococcal infection

We first determined if pneumococci can cause a systemic infection in adult zebrafish using the pneumococcal strain T4. Since pneumococci are not natural fish pathogens, and their optimal growth temperature (+37 °C) differs from the optimal temperature for zebrafish (+28 °C), the ability of the bacteria to grow and replicate in this environment was verified. To this end, the growth of T4, T4 mutants and the four clinical strains used in this study was tested in conditions resembling the host environment (at 28 °C in poor medium). All the strains were able to replicate under these conditions. Most importantly, there were no significant differences between the growth rates of the different pneumococcal strains (data not shown).

To investigate pneumococcal virulence in adult zebrafish, we infected fish ($n = 15$/group) with wild type T4 via either intraperitoneal (i.p.) or intramuscular (i.m.) injection using three bacterial doses ranging from 1×10^6 to 5×10^6 cfu. The survival and symptoms of the infected fish were then recorded twice a day for 7 days. The infected fish showed signs of severe infection including edema at the site of injection, abnormal spiral-like swimming, increased respiration, and ultimately, death. As is shown in Fig. 1 both the i.p. and i.m. injections lead to a dose-dependent mortality. For the i.p. infection the combined mean survival rate with 1×10^6 cfu was 87%, with 2.5×10^6 cfu it was 44% and with 5×10^6 cfu the survival was 7% ($p < 0.0001$) (Fig. 1A). The mortality rate during the infection was also dependent on the bacterial dose and the majority of the fish infected with the highest dose died during the first 24 h, while in the other groups the highest mortality rate was reached during the first 48 and 72 hpi (Fig. 1A).

Noteworthy, infecting the zebrafish i.m. resulted in a slower disease progression and a higher survival than did the i.p. infection route (Fig. 1B). The highest mortality rates were reached by 48 h for an inoculation dose of 5×10^6 cfu, by 72 h for 2.5×10^6 cfu and by 96 h for 1×10^6 cfu and the mean survival percentages were 12%, 66% and 93%, respectively. Visible signs of local infection could also be detected after the i.m. infection; most of the infected fish had a hypopigmented lesion and notable tissue necrosis at the dorsal muscle. The lesions were visible in both dead and surviving individuals. With both injection routes, the control fish injected with KCl had no visible signs of the disease and showed 100% survival. In conclusion, both infection routes can cause a lethal, dose-dependent infection in adult fish. With moderate doses, some individuals are able to resist the infection and survive.

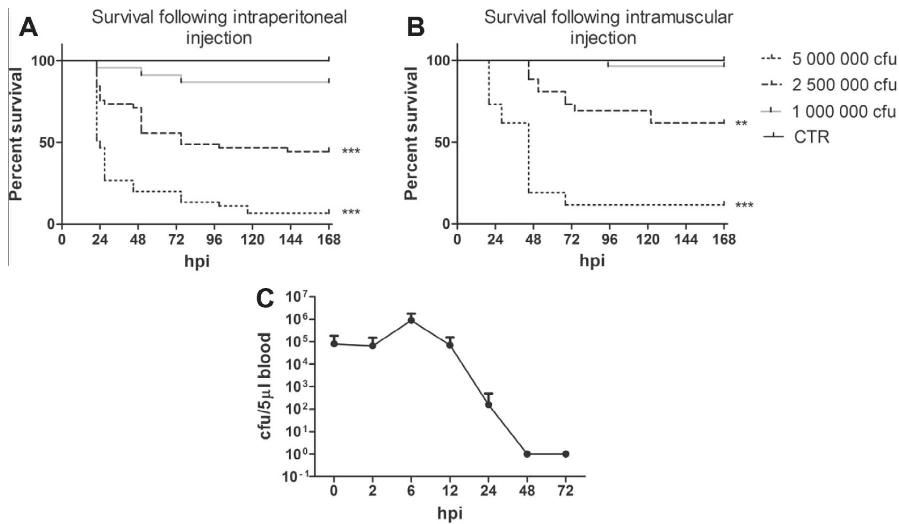

Fig. 1. *Streptococcus pneumoniae* infection in adult zebrafish. The survival of adult zebrafish after intraperitoneal (A) and intramuscular (B) injection of wild type (T4) pneumococcus is dose-dependent. The figures depict the survival of adult zebrafish infected either i.p. or i.m. with the three indicated doses. Control fish received sterile 0.2 M KCl. The asterisk (*) depicts the statistically significant difference compared to the control group, where ** $p < 0.001$ and *** $p < 0.0001$. (C) Bacterial burden in adult zebrafish during pneumococcal infection. The bacterial burden was measured at the indicated time points in adult zebrafish infected i.p. with 2.5×10^6 cfu of T4. The figure represents the mean (+SD) bacterial burden in 10 individuals measured from blood samples taken at specific time points. Cfu = colony forming units. Hpi = hours post infection.

Next we examined bacteremia levels and the ability of the fish to clear injected bacteria. The level of bacteremia was measured at different time points after an i.p. injection of 2.5×10^6 cfu of T4. Some individuals died during the 96 h period in which the samples were taken and the bacteremia levels were only measured from live fish. As is shown in Fig. 1C, pneumococci are present in the blood immediately after injection (0 h time point), as expected, since the fish peritoneal cavity is directly connected to the vasculature (Neely et al., 2002). During the first hours the bacterial burden in the blood increases and reaches its highest level 6 h pi. The observed increase in the bacteremia level is likely due to the bacterial replication occurring in the blood but it may also be, in part, due to the transfer of injected bacteria from the peritoneal cavity. During the early time points the mortality rate is the highest and most of the fish unable to resolve the infection die. Thereafter, the bacterial burden starts to decrease in the surviving individuals, and 48 h pi most of the surviving fish are free of pneumococci. However, in some individuals the bacterial burden remains high up to 96 h pi (data not shown). These findings indicate that a pneumococcal infection in adult zebrafish is associated with rapid bacterial growth in the blood, and if unrestricted, the bacterial growth leads to the death of the fish. However, some individuals are able to resolve the pneumococcal infection leading to the clearance of the bacteria in the blood and survival.

3.2. Mutants in important pneumococcal virulence factors are attenuated in the adult zebrafish model

We next assessed the role of important pneumococcal virulence factors in the adult zebrafish model using pneumococcal mutants lacking known virulence factors that have been shown to be attenuated in murine models as well as in the zebrafish embryo model (Berry et al., 1989a,b; Nelson et al., 2007; Rounioja et al., 2012; Watson and Musher, 1990). We challenged a group of 15–20 fish with 2.5×10^6 cfu of the following four isogenic mutants in the T4 background: non-encapsulated (T4R), pneumolysin-deficient (T4Δ*ply*), autolysin-deficient (T4Δ*lyt*) and pilus-deficient (T4Δ*rlr*) mutant.

The pneumococcal polysaccharide capsule is essential for every step of the pneumococcal disease progression, playing a role in colonization, adherence and in invasion and it enables the evasion of bacteria from the host immune response (Mook-Kanamori et al., 2011; Zancolli et al., 2011). Using the un-encapsulated T4R strain we show that the capsular knockout is attenuated in our adult zebrafish model with significantly higher survival rates (87%) compared to the wild type T4 (38%) ($p < 0.05$) (Fig. 2). Pneumolysin is a pore-forming toxin that is expressed by virtually all clinical strains of pneumococci (Kadioglu et al., 2008; Mitchell and Mitchell, 2010). This cytosolic protein is released when the bacteria undergo autolysis and when released, its two main functions are to cause host cell lysis and activate the complement system (Mitchell

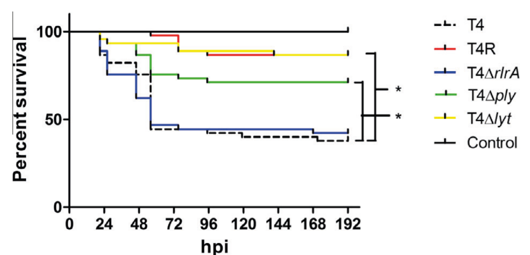

Fig. 2. Survival of adult zebrafish after i.p. infection with T4 mutants. The Non-encapsulated mutant (T4R), the pneumolysin deficient mutant (T4Δ*ply*) and the autolysin deficient mutant (T4Δ*lyt*) of T4 have an attenuated virulence in adult zebrafish compared to the wild type T4. The data comprise the combined results from three individual experiments ($n = 15$) where the fish were infected with 2.5×10^6 cfu of the desired pneumococcal strain. The asterix (*) depicts the statistically significant difference compared to the wild type T4, where * $p < 0.05$. Control fish were injected with 0.2 M KCl. Hpi = hours post infection. T4Δ*rlr* = T4 pilus mutant.

and Mitchell, 2010). The pneumolysin-deficient mutant of T4 (T4Δ*ply*) also showed an attenuated virulence resulting in a mean survival rate of 71% (Fig. 2). Yet another virulence factor is the pneumococcal autolysin LytA (Jedrzejas, 2001; Kadioglu et al., 2008). Its contribution to bacterial virulence is thought to be mediated, at least in part, by pneumolysin, as it functions by causing the autolysis of pneumococcal cells, which leads to the release of pneumolysin (Kadioglu et al., 2008). In the i.p. infection of adult zebrafish, the virulence of the autolysin-deficient mutant (T4Δ*lytA*) was clearly low as the survival rate of the inoculated fish was significantly higher (87%) compared to the fish inoculated with wild type bacteria ($p < 0.05$) (Fig. 2). Finally, we investigated the role of the pneumococcal pilus-like structure in the virulence of pneumococcus in adult zebrafish. The pilus structure is a relatively recently discovered virulence factor which mediates the adhesion of pneumococci to epithelial cells, and therefore, it has been suggested to contribute to early stages of the infection (Barocchi et al., 2006; Orrskog et al., 2012). In our model, the pilus structure seems to have no role in the pneumococcal disease as the infection of zebrafish with a pilus-deficient mutant of pneumococcus (T4Δ*rlr*) resulted in a survival rate similar to an infection with wild type bacteria (42%).

3.3. Invasive disease potentials of pneumococcal clinical isolates

Serotype- and clone-specific properties have been shown to contribute to the ability of pneumococci to cause an invasive disease in humans. To study the potential impact of serotypes and clonal types we challenged adult zebrafish i.p. with 2.5×10^6 cfu of clinical isolates belonging to serotypes with different invasive disease potential in man and in mice, serotypes 1, 6B, 14 and 19F (Sandgren et al., 2004, 2005; Sjöström et al., 2006). As is shown in Fig. 3, these isolates have varying virulence capacities in adult zebrafish; the mean survival percentages associated with the strains differed from 4% for type 19F to 93% for type 6B. The strains of serotypes 1, 4 and 14 caused an infection with moderate lethality, with mean survival percentages of 19%, 49% and 33%, respectively. These results indicate that the zebrafish model can distinguish between the virulence differences of pneumococcal isolates and can potentially be used in the characterization of the virulence potentials of pneumococcal strains.

3.4. The adult zebrafish model can be used to study pneumococcal meningitis

Adult zebrafish infected with pneumococci show swollen brain cavities and visible cerebral edema, apparent signs of inflammation of the brain (Fig. 4). Therefore, we next examined whether pneumococci can cross the blood–brain barrier and consequently cause meningitis in adult zebrafish. We quantified the bacterial loads in the whole brain tissue of adult zebrafish during a pneumococcal infection after an i.p. challenge with 2.5×10^6 cfu of T4. Blood samples were collected and the brain tissue removed at specific time points. As is shown in Fig. 5, substantial amounts of bacteria are present in the zebrafish blood and brain already 2 h pi. Thereafter, the bacterial load keeps increasing until 12–24 h pi. Noteworthy, bacterial loads in the brain correlated with the bacterial loads in the bloodstream (Fig. 5). These results indicate that pneumococci are able to cross the blood–brain barrier and cause meningitis in adult zebrafish.

Furthermore, we examined whether the pneumococcal virulence factors tested above contribute to meningitis in this model and determined the bacterial burden 6 h pi in the blood and brain of adult zebrafish infected with the four T4 mutant strains. Infection with the capsular mutant T4R and the autolysin mutant T4Δ*lytA* resulted in the lowest number of bacteria in the blood and brain compared to the wild type T4, but only the difference between T4R and wild type bacteria was statistically significant ($p < 0.05$) (Fig. 6A and B). Also, we challenged the fish with the four clinical isolates to study meningitis. The bacterial numbers in the blood and brain of the infected fish were measured as above 6 h pi. Although these isolates had different levels of virulence in adult zebrafish (Fig. 3), they cause equivalent levels of bacteremia and meningitis (Fig. 6A and B).

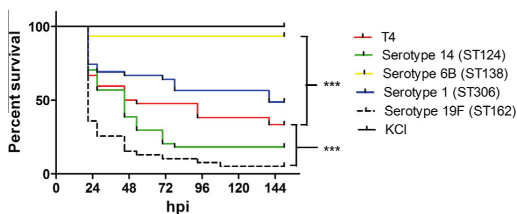

Fig. 3. The virulence potential of invasive pneumococcal isolates in zebrafish. Survival of adult zebrafish after an i.p. infection with serotypes 4 (T4), 14, 6B, 1 and 19F. The figure combines the results of three independent experiments ($n = 20$) where zebrafish were infected with 2.5×10^6 cfu of certain serotype. The asterisk (*) depicts the statistically significant difference compared to the T4 strain (***$p < 0.0001$). Control fish were injected with 0.2 M KCl. Hpi = hours post infection.

Fig. 4. Gross pathology of adult zebrafish after i.p. infection with T4. (A) Control fish injected with 0.2 M sterile KCl. (B) Zebrafish injected i.p. with 2.5×10^6 cfu of T4. The black arrow indicates visible cerebral edema.

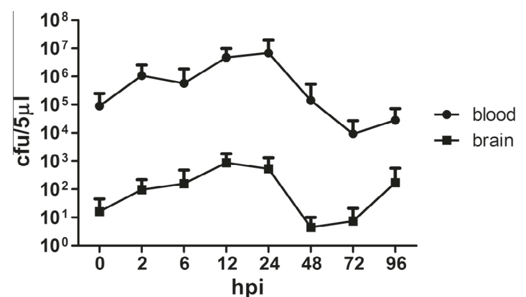

Fig. 5. Bacterial loads in the blood and brain of adult zebrafish after an i.p. infection with pneumococcus. The bacterial loads were measured at specific time points from fish infected with 2.5×10^6 cfu of T4. The figure represents the mean (+SD) bacterial burden in 5 μl of blood and 5 μl of a brain homogenate measured from 10 individuals. Hpi = hours post infection. Cfu = colony forming units.

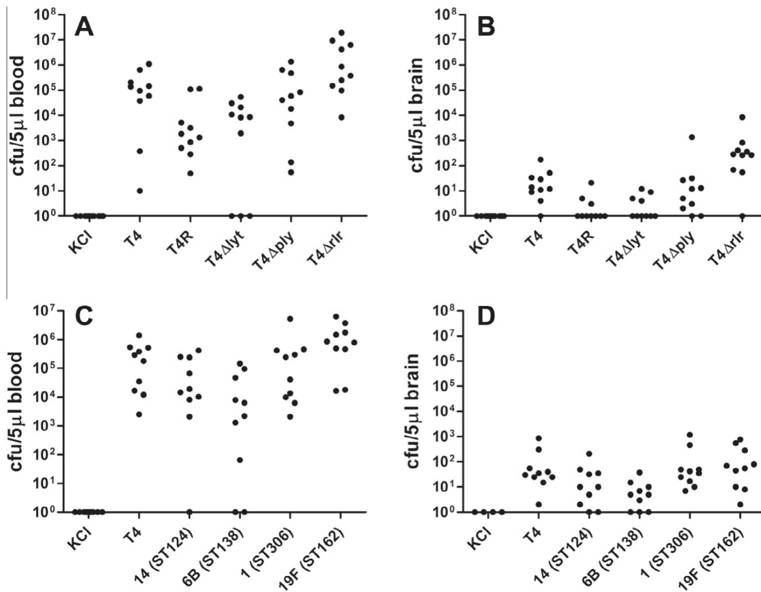

Fig. 6. Levels of bacteremia and meningitis in the adult zebrafish infected with T4 mutants and clinical strains of different serotypes. The bacterial loads were measured at 6 hpi from 5 µl of blood and 5 µl of a brain homogenate from adult fish infected i.p. with 2.5×10^6 cfu of the given strain. (A+B) Bacterial loads 6 hpi in the blood (A) and the brain (B) of adult fish infected with T4, the un-encapsulated mutant (T4R), the autolysin deficient mutant (T4Δ*lyt*), the pneumolysin deficient mutant (T4Δ*ply*), or the pilus mutant (T4Δ*rlr*). (C+D) Bacterial loads at 6 hpi in the blood (C) and the brain (D) of adult fish infected with serotypes 4 (T4), 14, 6B, 1 and 19F. Control fish were injected with 0.2 M KCl. Hpi = hours post infection. Cfu = colony forming units.

3.5. The adult zebrafish model is not an appropriate model for vaccine studies

The survival assays indicated a rapid disease progression in adult zebrafish after an i.p. infection with wild type T4. This suggests that the acute infection might be mainly resolved by components of the innate immunity. In order to test the role of the adaptive immunity in the acute infection, we used the transgenic fish line, Rag1^{hu1999}, which lacks the ability to undergo somatic rearrangements, and therefore, active lymphocytes. The Rag1 mutants were infected i.p. with 2.5×10^6 cfu of T4 and assayed for survival. When compared to wild type fish, no significant difference in the survival was observed (Fig. 7A). This result verifies our previous suggestion, that the infection kinetics of acute pneumococcal infection is too fast to activate the adaptive arm of immunity and that the adult zebrafish resist the acute pneumococcal infection primarily through mechanisms of their innate immunity.

To test whether immunization can confer protection against a pneumococcal infection in zebrafish, groups of 20–30 fish were infected with non-lethal doses of live T4, heat-killed T4, or live T4R pneumococci prior to infection. Similarly, we also tested the protective effect of two commercially available pneumococcal vaccines, the 13-valent conjugate vaccine Prevenar 13® (Pfizer) and the 23-valent polysaccharide vaccine Pneumovax 23® (Merck). Both the i.p. and i.m. injection routes were tested for immunization and the following infection was introduced through the same route 2 or 3 weeks later. Previously, it was shown that a teleost fish can develop a protective antibody response in 3 weeks after sub-lethal infection (Castro et al., 2013). As is shown in Fig. 7B, the pre-infection with T4, killed T4 or T4R did not significantly reduce the mortality of T4 infected adult fish compared to the mock immunized or unimmunized fish. Similarly, the human pneumococcal vaccines were not able to confer protection against a pneumococcal infection in the zebrafish model (data not shown). In light of these results, we conclude that immunization does not confer protection against pneumococci in this model.

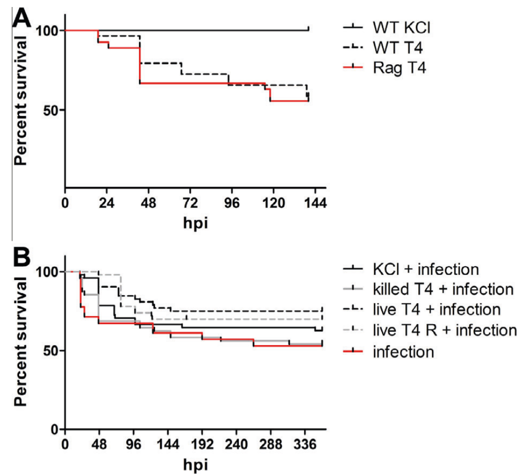

Fig. 7. The role of the adaptive immunity in a pneumococcal infection in adult zebrafish. (A) Pneumococcal infection in zebrafish Rag1 mutants. The Rag1 and wild type fish were infected with 2.5×10^6 cfu of pneumococcus, after which the mortality was recorded. No difference in the survival of these two groups can be seen ($p = 0.700$). WT+KCl = wild type zebrafish injected with KCl; WT+T4 = wild type zebrafish infected with T4; Rag+T4 = Rag1 mutant fish infected with T4. (B) Immunization assays in adult zebrafish. Groups of 20–30 adult fish were immunized with live T4, live T4R, or killed T4 by an i.m. injection. Control fish received either KCl immunization or no immunization. Immunized and control fish were infected i.m. 2–3 weeks after immunization with 2.5×10^6 cfu of T4. Immunization assays were repeated at least 3 times in each case, and the figure combines the results of three separate experiments ($n = 20–30$). Hpi = hours post infection.

4. Discussion

Zebrafish are cost effective, easy to handle, and genetically manipulate. These features make them, a very feasible tool for clarifying the mechanisms of disease pathogenesis, for testing novel drugs, and for investigating the role of host specific and pathogenic factors in a variety of infections. Zebrafish have been previously used to model infections caused by important β-hemolytic streptococcal pathogens such as zoonotic *S. iniae* (Neely et al., 2002) and *Streptococcus suis* (Wu et al., 2008) as well as the human specific strains *S. agalactiae* (Patterson et al., 2012) and *Streptococcus pyogenes* (Neely et al., 2002). Furthermore, we have previously established a zebrafish embryo model for pneumococcal pathogenesis (Rounioja et al., 2012). In the present study we introduce an adult zebrafish model for a systemic pneumococcal infection and for pneumococcal meningitis.

Our results show that the wild type pneumococcus strain T4 causes a fulminant infection in adult zebrafish when introduced through either an i.p. or an i.m infection route. Regardless of the injection site, the infection is dose-dependent and leads to death within 2–3 days after injection. With both injection routes the lethal dose of pneumococcus that kills 50% of infected fish (OD_{50}) is approximately 2.5×10^6 cfu. When compared to other streptococci-zebrafish models, for example *S. iniae* (LD_{50} of 5×10^3 cfu for i.p. and i.m.), *S. pyogenes* (LD_{50} of 1×10^3 cfu for i.p. and 5×10^4 cfu for i.m.) and *S. agalactiae* (LD_{50} of 1×10^2 cfu for i.p. and 1×10^4 cfu for i.m.) pneumococcus seems to be less virulent in adult zebrafish and a bigger dose of bacterial inoculate is needed to cause a notable infection (Neely et al., 2002; Patterson et al., 2012). The i.p. infection of adult zebrafish leads to a systemic pneumococcal infection associated with high levels of bacteremia and therefore, this infection route was used throughout the study. Rapid bacterial expansion in the blood demonstrates the ability of pneumococcus to replicate in zebrafish and cause an acute infection. If unrestricted, pneumococcal expansion leads to the death of the fish. Simultaneously, we noticed that some zebrafish were able to resist the bacterial expansion leading to the complete clearance of the bacteria within 4 days. Because of such a short time period, we hypothesize that in lethal and sublethal infections in adult zebrafish the pneumococcus is primarily confronted by components of the innate immunity and that the clearance of the pneumococcus is mainly dependent on these factors. In our previous study the innate immunity mechanisms associated with the clearance of pneumococcus were investigated using the zebrafish embryo model for pneumococcal pathogenesis (Rounioja et al., 2012). In that study we reported that pneumococcus activates the common actors of the innate immunity, including phagocytosis and the production of pro-inflammatory cytokines as a response to infection in zebrafish embryos. Although the clearance mechanisms were not investigated more closely in the present study, we suggest that the above listed innate responses are also important for resisting pneumococcus in the adult zebrafish model.

In addition to their innate immunity, adult zebrafish possess a fully developed adaptive immunity including both cellular and humoral responses. An important role of the adaptive immunity in the protection of zebrafish from a *M. marinum* infection has been reported (Meijer et al., 2005; Swaim et al., 2006). Importantly, a *M. marinum* infection in adult zebrafish can last months, long enough for the activation of the adaptive immunity. In contrast, a pneumococcal infection leads to death within a few days, a time period probably too short for engaging the adaptive arm of immunity. In order to verify this suggestion, we tested whether adult fish from the transgenic line $Rag1^{hu1999}$, which lack active lymphocytes, are hypersusceptible to pneumococcal infection. No differences between the survival of Rag1 mutants and wild type fish were seen, indicating that the adaptive immunity has a limited role in an acute pneumococcal infection in adult zebrafish.

Although there is limited information on the zebrafish in vaccination studies, its ability to induce an immunological memory upon immunization has been verified (Lam et al., 2004). Experimental evidence for the effectiveness of immunization in zebrafish also exists, since pre-infections of adult zebrafish with an attenuated *M. marinum* mutant as well as an attenuated viral hemorrhagic septicemia virus have been shown to protect zebrafish from future infection (Cui et al., 2010; Novoa et al., 2006). However, we discovered that a pre-infection with inactivated T4, live T4 or T4R does not seem to confer protection against future pneumococcal infection indicating that pneumococcus does not induce a protective immunological memory in our setting.

The adult zebrafish provides an attractive model for the study of specific host-pathogen interactions associated with a pneumococcal infection. Previously, adult zebrafish have been successfully used in the search for novel virulence factors of *S. iniae* and *S. pyogenes* (Kizy and Neely, 2009; Miller and Neely, 2005). The model proved to be particularly feasible and practical in large-scale screens where previously unknown virulence determinants of these streptococci were revealed. In the present study, we demonstrate that the adult zebrafish model for a pneumococcal infection could also be reliably used in the search for novel virulence factors of the pneumococcus; our result show a significant attenuation in virulence of T4 mutants lacking certain virulence factor compared to the wild type T4. Most importantly, the un-encapsulated mutant of T4, which has previously been shown to be avirulent (Nelson et al., 2007; Watson and Musher, 1990), is almost completely attenuated in adult zebrafish. I.p. injections of the pneumolysin-deficient and the autolysin-deficient mutants of T4 also resulted in significantly higher survival of the adult zebrafish compared to those infected with wild type T4. Previously, the important roles of all these virulence factors in the pathogenesis of pneumococcus as well as the attenuated virulence of the corresponding mutants have been shown in rodent models of a pneumococcal infection (Berry et al., 1989a,b). Therefore, our results demonstrate that also pneumococcal virulence factors can be successfully studied in the adult zebrafish model.

An epidemiological study by Sjöström et al. (2006) reported a correlation between disease severity and pneumococcal capsular serotype. Serotypes 1, 4 and 7F have the highest invasive disease potential in humans but rarely cause deaths, while other serotypes such as 6B, 14 and 19F are more often associated with lethal infections. Previously, a mouse model for a pneumococcal infection was used to investigate the varying virulence capacities of pneumococcal serotypes and clonal types (Sandgren et al., 2005). In the present study we used the adult zebrafish model to compare the virulence of five invasive pneumococcal serotypes 4, 14, 6B, 1 and 19F. Importantly, clear differences in the ability of these isolates to cause a lethal infection were detected; serotypes 19F and 14 were the most virulent while an infection with 6B resulted in almost 100% survival. Interestingly, the virulence of these strains seems to differ from the virulence observed in mice, where i.p. and intranasal injections of serotypes 4 and 6B caused the highest lethality and level of bacteremia while 19F and 14 caused a more moderate infection (Sandgren et al., 2005). The opposite findings in mice and zebrafish can in part be explained by the varying host conditions (e.g., temperature) between these two model organisms. Furthermore, pneumococcal strains are highly specialized in infecting humans, where the specific host-pathogen interactions required for the infection occur. Although no model is perfect for the investigation of human isolates, we strongly suggest that the zebrafish model can be employed for assessing the disease potential of different pneumococcal serotypes and clonal types. Most

importantly, this model could help to reveal the detailed host-pathogen interactions associated with the different disease capacities.

Pneumococcus is one of the most common causative agents of bacterial meningitis and is associated with high mortality and severe neurological sequelae in surviving patients (Chiavolini et al., 2008). In humans the development of meningitis is usually preceded by pneumococcal bacteremia followed by the translocation of the bacteria across the blood–brain barrier (Mook-Kanamori et al., 2011). In the present study we demonstrate that a pneumococcal bacteremia caused by an i.p. injection of T4 into adult zebrafish results in the invasion of the brain and develops into meningitis. The development of meningitis in zebrafish mimics the common route of infection in humans and is associated with visible signs of meningeal inflammation, including cerebral edema and swollen brain cavities. Noteworthy, the severity of meningitis in adult zebrafish correlates with the level of the preceding bacteremia. We also evaluated the ability of pneumococcal mutants to invade the brain and cause meningitis in this model. In particular, the contribution of pneumolysin and autolysin in the onset and progression of meningitis has been frequently studied in several animal models (Friedland et al., 1995; Hirst et al., 2008; Wellmer et al., 2002). However, results have been controversial, as Friedland et al. reported no role for pneumolysin in the pathogenesis of meningitis in rabbits, while Hirst et al. and Wellmer et al. showed that the lack of pneumolysin and autolysin results in significantly decreased signs of meningitis in mice and rats compared to the wild type pneumococcus (Friedland et al., 1995; Hirst et al., 2008; Wellmer et al., 2002). In agreement with Hirst et al. we found a slight, although not significant, decrease in the bacterial numbers in the brain of autolysin-mutant infected fish compared to fish infected with wild type bacteria (Hirst et al., 2008). In addition, an infection with un-encapsulated mutants also resulted in a lower amount of bacteria in the brain compared to wild type. Importantly, in each case the lower levels of T4 mutants in the brain were accompanied by a decreased bacterial load in the blood. This suggests that the decreased ability of pneumococcal mutants to cause meningitis in adult zebrafish is mostly due to their more effective clearance by innate immunity mechanisms in the blood, instead of an impaired ability to invade the brain tissue of the host.

5. Conclusions

The new vertebrate model introduced here shows potential for use in future pneumococcal studies where both pathogenic and host specific factors are investigated. We also report that pneumococcal meningitis can be established in adult zebrafish, which provides a valuable new tool for the forthcoming investigation of pathophysiological mechanisms of bacterial meningitis and for testing the efficacy of novel drugs against the disease. Furthermore, adult zebrafish could provide a practical and more ethical model to be used in large-scale mutagenesis screens where novel virulence determinants of pneumococcus are investigated. Finally, we report that the adaptive immunity has a limited role in an acute pneumococcal infection in adult zebrafish, and that the bacterium does not induce a protective immunological memory in the fish, indicating that this model cannot be used in the search for new vaccine candidates against pneumococcus.

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PUBLICATION IV

Characterization of the Innate Immune Response to *Streptococcus pneumoniae* Infection in Zebrafish

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Submitted manuscript