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Role of PspC interaction with human polymeric immunoglobulin receptor and Factor H in *Streptococcus pneumoniae* infections and host cell induced signalling

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Greifswald, November 2008

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Contents

1. Zusammenfassung

Streptococcus pneumoniae ist ein Gram-positives Bakterium und ein Kommensale des humanen Nasenrachenraums. Pneumokokken sind andererseits auch die Verursacher schwerer lokaler Infektionen wie der Otitis media, Sinusitis und von lebensbedrohenden invasiven Erkrankungen. So sind Pneumokokken die wichtigsten Erreger einer ambulant erworbenen Pneumonie und sie sind häufige Verursacher von Septikämien und bakteriellen Meningitiden. Die initiale Phase der Pathogenese ist verbunden mit der Besiedelung der mukosalen Epithelzellen des Rachenraumes. Diese Kolonisierung erleichtert die Aufnahme der Bakterien in die Zelle bzw. deren Dissemination in submukosale Bereiche und den Blutstrom. Die Konversion des Kommensalen zu einem invasiven Mikroorganismus ist assoziiert mit der Anpassung des Krankheitserregers an die verschiedenen Wirtsnischen und wird auf der Wirtsseite durch die Zerstörung der transepithelialen Barriere begleitet. Die Anpassung des Erregers ist vermutlich ein in hohem Grade regulierter Prozess.

Die Oberfläche von *Streptococcus pneumoniae* ist mit Proteinen bedeckt, die kovalent oder nicht kovalent mit der Zellwand verknüpft sind. Eine einzigartige Gruppe von Oberflächenproteinen in der Zellwand der Pneumokokken sind die cholinbindenden Proteine (CBPs). Für einige der CBPs konnte bereits die Bedeutung für die Virulenz gezeigt werden. PspC, auch als SpsA oder CbpA bezeichnet, ist ein multifunktionales Oberflächenprotein, das als Adhesin und Faktor H-Bindungsprotein eine wichtige Rolle in der Pathogenese der Pneumokokken hat. PspC vermittelt als Adhesin die Anheftung der Bakterien an die mukosalen Epithelzellen, indem es human-spezifisch an die sekretorische Komponente (SC) des polymeren Immunoglobulinrezeptors (pIgR) bindet. SC ist die Ektodomäne des pIgR und PspC kann ebenso die freie SC binden oder an die SC des sekretorischen IgA Moleküls binden. PspC interagiert auch mit dem löslichen Komplement Faktor H. Die SC und der Faktor erkennen zwei verschiedene Epitope im bakteriellen PspC Protein. Der genaue Mechanismus der jeweiligen Interaktionen unter physiologischen- bzw. wirtspezifischen Bedingungen ist noch nicht vollständig verstanden.

In dieser Arbeit wurde die Auswirkung der PspC Interaktion mit dem humanen pIgR (hpIgR) bzw. dem Faktor H auf die Virulenz der Pneumokokken und die Wirtszellantwort, d.h. die induzierten Signalkaskaden in den eukaryotischen Zellen untersucht. Die molekulare Analyse und die Verwendung von spezifischen pharmakologischen Inhibitoren der Signalmoleküle zeigten, dass verschiedene Signalmoleküle an der PspC-pIgR vermittelten Internalisierung beteiligt sind. Die Aktivierung, d.h. die Phosphorylierung der Signalmoleküle

wurde in Immunblots demonstriert. Die Studien zeigten, dass das Aktinzytoskelett und die Mikrotubuli für die bakterielle Aufnahme essentiell sind. Es konnte auch zum ersten Mal nachgewiesen werden, dass Cdc42 die entscheidende GTPase für die Invasion der Pneumokokken in die Wirtsepithelzellen, vermittelt über den PspC-hpIgR Mechanismus, ist. Der Einsatz von PI3-kinase und Akt Kinase Inhibitoren reduzierte signifikant die hpIgR-vermittelte Aufnahme der Pneumokokken in die Wirtszelle. Zusätzlich durchgeführte Infektionen von hpIgR exprimierenden Zellen zeigten eine zeitabhängige Phosphorylierung von Akt und der p85α Untereinheit der PI3-Kinase. Damit ist neben der GTPase Cdc42 der PI3K und Akt Signalweg entscheidend für die PspC-pIgR vermittelte Invasion der Pneumokokken. Des Weiteren sind an der Infektion mit Pneumokokken auch die Protein Tyrosin Kinasen Src, ERK1/2 und JNK beteiligt. Dabei wird die Src Kinase unabhängig von der PI3K in hpIgR exprimierenden Zellen aktiviert. Inhibitionsexperimente und genetische Knockdown Versuche mit siRNA bewiesen, dass die Endozytose der Pneumokokken über PspC-pIgR ein Clathrin und Dynamin abhängiger Mechanismus ist.

Im weiterenn Teil der Arbeit wurde der Einfluss des PspC gebundenen Faktor H auf die Anheftung an und Invasion in die Epithelzellen analysiert. Die Bindung von Faktor H erfolgte unabhängig vom PspC-Subtyp. Die Bindungsversuche bewiesen, dass die Kapselmenge negativ korreliert mit der Bindung des Faktor H. Der Einsatz von Faktor H aus Maus oder Ratte zeigte keine typische Bindung. Daraus kann abgeleitet werden, dass diese Interaktion humanspezifisch ist. Die Infektionsexperimente demonstrierten, dass Faktor H die Adhärenz und die Invasion der Bakterien in die Nasenrachenraumzellen (Detroit562), alveolären Lungenepithelzellen (A549) und humanen Hirnendothelzellen (HBMEC) steigert.

Der Faktor H hat Heparin Bindestellen. Diese Bindestellen vermitteln die Adhärenz der Faktor H gebundenen Pneumokokken mit Epithelzellen. Inhibitionsstudien mit spezifischen monoklonalen Antikörpern, die gegen die short consensus repeats (SCRs) von Faktor H gerichtet waren, konnten die essentielle Bedeutung der SCR19-20 für die Anheftung der Pneumokokken über Faktor H an die Wirtszellen nachweisen. Die Faktor H vermittelte Assoziation der Pneumokokken an polymorphonukleäre Leukozyten (PMNs) erfolgt über das Integrin CD11b/CD18. Die weiteren Inhibitionsstudien zeigten dann auch zum ersten Mal den Einfluss des Aktinzytoskeletts der Wirtszelle auf die Faktor H-vermittelten bakterieller Internalisierung und den dabei bedeutsamen Signaltransduktionswegen in der eukaryotischen Zelle. Dabei wurden insbesondere die Proteintyrosinkinasen und die PI3K als wichtige Signalmoleküle für die Faktor H vermittelte Invasion der Pneumokokken identifiziert.

Die in dieser Arbeit erhaltenen Resultate belegen, dass die Faktor H vermittelte Infektion der Zellen mit *S. pneumoniae* ein konzertierter Mechanismus ist, bei dem Oberflächen-Glycosaminoglycane, Integrine und Signaltransduktionswege der Wirtsepithelzellen involviert sind. Des Weiteren wurde aufgezeigt, dass die PspC-pIgR-vermittelte Invasion in mukosale Epithelzellen unterschiedliche Signalwege wie z.B. den PI3K und Akt Weg induziert und abhängig von Cdc42 und einer Clathrin vermittelten Endozytosemechanismus ist.

2. Summary

Streptococcus pneumoniae (pneumococci) are Gram-positive human bacteria and commensals of the nasopharyngeal cavity. Besides colonization, pneumococci are responsible for severe local infections such as otitis media, sinusitis and life-threatening invasive diseases, including pneumonia, sepsis and meningitis. The initial phase of pathogenesis of mucosal microorganisms is associated with colonization followed by intimate contact with host cells, which can promote uptake into the cells. The successful conversion of a commensal to an invasive microorganism is accompanied by the transmigration of tissue barriers and the subsequent adaptation of the pathogen to different host niches. This is a multifunctional and highly regulated process.

The surface of pneumococci is decorated with proteins that are covalently or non-covalently anchored to the cell wall. The most unique group of cell wall associated proteins in pneumococci are the choline-binding proteins (CBPs). Several CBPs are implicated in virulence. PspC, also known as SpsA or CbpA, is a multifunctional surface protein that plays an essential role in pneumococcal pathogenesis by functioning as an adhesin. PspC promotes adherence of pneumococci to mucosal epithelial cells by interacting in a human specific manner with the free secretory component (SC) or to SC as part of the secretory IgA (SIgA) or polymeric immunoglobulin receptor (pIgR). PspC has also been shown to interact specifically with the soluble complement Factor H. Apparently, PspC uses two different epitopes for binding the soluble host protein Factor H and SC of pIgR. However, the mechanism by which these independent interactions facilitate pneumococcal infections under physiological and host specific conditions have not yet been completely elucidated.

The interaction of PspC with pIgR is critical for pneumococcal translocation from nasopharynx and spread to normally sterile parts of the respiratory tracts such as lungs or the blood stream during infections. This study aims to explore the impact of the PspC interaction with human pIgR (hpIgR) or complement regulator Factor H on pneumococcal virulence. Here the cellular and molecular basis of PspC-mediated adherence to and invasion of host epithelial and endothelial cells was demonstrated. The genetic approach, specific pharmacological inhibitors and immunoblot analysis demonstrated the complexity of the induced signal transduction pathways during PspC-hpIgR mediated pneumococcal uptake by host cells. Inhibition studies with specific inhibitors of actin cytoskeleton and microtubules demonstrated that the dynamics of host cell actin microfilaments and microtubules are

essential for pneumococcal uptake by mucosal epithelial cells. Moreover, this study reports for the first time that the small GTPase Cdc42 is essential for pneumococcal internalization into host epithelial cells via the PspC-hpIgR mechanism. In addition, in infection experiments performed in presence of specific inhibitors of PI3-kinase and Akt hpIgR-mediated pneumococcal uptake by host cells was significantly blocked. The pivotal impact of PI3-kinase and Akt was confirmed in kinetic infections of hpIgR expressing host cells. Both PI3-kinase p85α subunit and Akt were activated during pneumococcal uptake by eukaryotic cells with pneumococci. Taken together the results demonstrate the critical role of PI3-kinase/Akt pathways during pneumococcal infection. Likewise, the inhibition studies and kinetic infections demonstrated the importance and activation of protein tyrosine kinase (PTKs) during pneumococcal infection. Amongst PTKs the Src kinase pathway, ERK1/2 and JNK pathways were implicated during pneumococcal ingestion by hpIgR expressing cells. Moreover, inhibition experiments performed in the presence of individual inhibitors or with a combination of inhibitors suggested the independent activation of PI3-kinase/Akt and Src kinase pathways during pneumococcal infections of hpIgR expressing cells. Taken together the results revealed the complexity of PspC induced signalling events in epithelial cells via its interaction with hpIgR. By employing specific inhibitors and siRNA in cell culture infection experiments it was further demonstrated that pneumococcal endocytosis by host epithelial cells via the PspC-hpIgR mechanism depends on clathrin and dynamin.

PspC recruits also Factor H to the pneumococcal cell surface. Consequently, the impact of pneumococcal cell surface bound Factor H on adherence to host cells and the molecular mechanism facilitating the uptake of Factor H bound pneumococci by epithelial cells was investigated. Flow cytometry and immunoblots revealed that *S. pneumoniae* has evolved the ability to recruit both purified Factor H as well as Factor H from human plasma or serum. Moreover, it was demonstrated that the recruitment of Factor H is independent of the PspC-subtypes and that capsular polysaccharide (CPS) interferes with the Factor H recruitment. However, the results suggested that pneumococci interacts specifically in species specific manner with human Factor H, since binding of mouse and rat Factor H to pneumococci was significantly reduced compared to human Factor H binding. Factor H bound to pneumococci significantly increased bacterial attachment to and invasion of host epithelial cells including nasopharyngeal cells (Detroit562), lung epithelial cells (A549), and human brain-derived endothelial cells (HBMEC).

Blocking experiments demonstrated that bacteria bound Factor H interacts via the heparin binding sites on Factor H with eukaryotic cell surface glycosaminoglycans and that this interaction promotes pneumococcal adherence to host cells. In addition, inhibition studies with mAbs recognizing specifically different short consensus repeats (SCR) of Factor H suggested that SCR 19-20 of Factor H are essential for the pneumococcal interaction with host epithelial cells via Factor H. In the presence of Factor H, attachment of pneumococci to human polymorphonuclear leukocytes (PMNs) is enhanced. The integrin CD11b/CD18 was identified as the cellular receptor on PMNs. By using pharmacological inhibitors the impact of host cell cytoskeleton and signalling molecules for Factor H-mediated pneumococcal internalization into eukaryotic cells was shown. Inhibition of host cell actin cytoskeleton and not microtubules inhibited Factor H-mediated pneumococcal invasion of host cells. Finally inhibition of protein tyrosine kinase and PI3-kinase significantly blocked Factor H-mediated pneumococcal uptake by host cell. Taken together, these results revealed that Factor-H mediated pneumococcal infection requires a concerted role of host epithelial cell surface glycosaminoglycans, integrins and host cell signalling pathways.

3. Introduction

3.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae , a major cause of human disease, was one of the first pathogen that was isolated from humans and morphologically characterized. Pneumococci were probably recognized by Edwin Klebs, in 1875, in infected sputum and lung tissues, just a few years before they were isolated and independently identified by George M. Sternberg (United States) and Louis Pasteur (France) (Austrian, 1999). Sternberg called his isolate *Micrococcus pasteuri* while, Pasteur called his the "microbe septicémique du saliva". Later M. Mátray applied the term "pneumoniekokken" to this organism in 1883 and in 1886 Albert Fraenkel gave the name "pneumokokkus". The same year, Anton Weichselbaum, who established the pneumococcus as the predominant cause of bacterial pneumoniae, suggested the name *Diplococcus pneumoniae*, which became official until the organism was reclassified as *Streptococcus pneumoniae* in 1974 on the basis of its growth in chains in liquid media. During that period pneumococcal pneumonia was a driving force behind clinical and microbiological research. The insight gained from studying pneumococcus lead to the development of the concept of humoral immunity by Felix and Georg Klemperer (Austrian, 1999). However, one of the most important scientific advances of the 20th century was the recognition of DNA as the basic unit of genetic material by Avery, MacLeod and McCarty in 1944 which based on transformation studies of Griffith in 1928 with *S. pneumoniae*.

Streptococcus pneumoniae (the pneumococcus) is Gram-positive human bacteria that colonizes the upper respiratory tract and causes local infections such as otitis media and sinusitis and life threatening invasive diseases, including lobar pneumonia, sepsis and meningitis (Cartwright, 2002). The burden of disease is highest in the youngest and elderly population and in patients with immunodeficiencies (Garenne *et al.*, 1992; Leowski 1986). The pneumococcus is the prime cause of community-acquired pneumonia (CAP) in adults and accounts for 50-75 % cases. CAP is the sixth leading cause of death in the United States overall and the leading cause of infectious disease death (Bartlett and Mundy, 1995; Kozak *et al.* , 2005). Moreover, worldwide pneumococcal septicaemia is a major cause of infant mortality in developing countries, where it causes approximately 25 % of all preventable deaths in children under the age of 5 and more than 1.2 million infant deaths per year (Denny and Loda, 1986; Berkley *et al.* , 2005). In addition, community-acquired pneumococcal meningitis has a very high case-fatality rate. The survivors often develop long-term clinical

symptoms such as hearing loss, neurological disorders, and neurophysiological impairments (Koedel *et al.*, 2002).

The pneumococcus asymptomatically colonizes the mucosal surface of the upper respiratory tract. However, depending on the pathogen and host susceptibility pneumococcus have the potential to gain access to the normally sterile parts of the airways. The mechanisms promoting invasiveness are associated with the expression of virulence factors during colonization and dissemination. Pneumococci possess a wide variety of virulence factors that are thought to contribute towards its pathogenesis (Kadioglu *et al.* , 2008; Bergmann and Hammerschmidt, 2006). These factors, which are adapted successfully to different host niches, are involved either predominantly in nasopharyngeal colonization or subsequently in dissemination and transmigration of host tissue barriers (Orihuela *et al.*, 2004). However, the mechanisms involved in this transition are not yet completely explored. Therefore a comprehensive understanding of the critical steps during pneumococcal pathogenesis including colonization, progression to pneumonia, dissemination in the blood stream, and transition of the blood-brain-barrier is crucial to combat the threat of pneumococcal infections and hence, reduce the mortality due to this pathogen.

3.2. Therapy and prevention of pneumococcal infection: history and present

In the early 20th century, pneumococcal pneumonia was termed “Captain of the Men of Death” by William Osler in one of his famous textbook of medicine, because of its high case fatality rate of 30-35 % in untreated adults. In the preantibiotic era, pneumonia was exceedingly common, taking third place after heart disease and cancer as cause of death in the 1930s. The recognition that pneumococcus played a major role in pneumonia was an initial step towards its control. The era saw enormous interest by the researchers in the biology and treatment of pneumococcal infection. In 1902 the Quelling or capsular swelling test was developed by Neufeld. However, it was in 1910 that the distinct pneumococcal serotypes were recognized and divided into type I and type II by Neufeld and Haendel. This finding led to the development of a program for the serum treatment of type I and type II pneumococcal pneumonia. With the identification of additional pneumococcal serotypes, serum therapy was extended to the treatment of those infected with a number of them, reducing the case-fatality rates to the vicinity of 20 %.

In the late 1930s the search for effective anti-pneumococcal drugs started. The discovery of Prontosil in 1932 by Gerhard Domagk of I.G. Farbenindustrie in Germany opened the modern era of antimicrobial agents. Prontosil, a water-soluble salt of sulfonamide

chrysoidine, was highly effective against hemolytic streptococci in experiments with mice. Later the researchers at Pasteur Institute identified sulfanilamide as active component of Prontosil, which by itself could inhibit the growth of bacteria *in vitro*. However, it was in 1939, that sulfapyridine, a more-effective and less-toxic derivative of sulfanilamide was approved by Food and Drug Administration (FDA) for treatment of pneumonia. The sulfonamide therapy provided the physicians, for the first time, a therapeutic agent which was effective against the pneumococcus irrespective of its capsular type and reduced the case fatality rate substantially. The era of this "wonderful new drug", however, was relatively short lived; it was in 1943 that the sulfonamide resistance in the organism came to into light.

The discovery of Penicillin by Sir Alexander Fleming, and its ability to reduce the overall case-fatality rate of pneumococcal pneumonia to 5-8 %, resulted in a diminished respect and fear accorded to pneumonia amongst physicians. However, the widespread antibiotic usage and its misuse resulted in the emergence of resistance against penicillin in *S. pneumoniae* . In the early 1960s, sporadic reports of pneumococcal isolates showing increase in resistance to penicillin were available. In the late 1970s, a strain of type 19A was isolated that manifested multiple drug resistance and caused an outbreak of infection in a hospital in South Africa. Since then, the infections caused by multiple drug-resistant pneumococci have become one of the major concerns. These lead to the consideration of prophylaxis and the focus shifted to strategies for prevention of pneumococcal disease by vaccination.

The first clinical trials of a pneumococcal vaccine were conducted in 1911 amongst the native workers of gold and diamond mines in South Africa by Sir Almorth Wright, using killed bacteria. The experimental research of Avery, Heidelberger, and Goebel in the 1920s formed the essential links between pneumococcal capsules, their serotypes specificity, and the identity of the capsules with polysaccharide that could be isolated from the bacterial cultures by chemical processes. It was in 1940s that these whole-cell vaccines were replaced by the next generation of pneumococcal vaccine, which consisted of the purified capsular polysaccharide of the bacteria. After two inconclusive trials in the Civilian Conservation Corps, MacLeod, Hodges, Heidelberger, and Bernhard presented conclusive evidence in 1945 that purified pneumococcal capsular polysaccharides were effective in preventing infections with the homotypic pneumococcal strains. This success resulted in the development and marketing of two hexavalent pneumococcal polysaccharide vaccines by Squibb and Sons in the United States in 1964. But their availability coincided with the era of new antimicrobial

drugs (sulfonamides and penicillin, etc.). These drugs showed significant efficacy in the treatment of pneumococcal pneumonia and resulted in the withdrawal of these vaccines from the market in 1954. However, the interest in the prevention of pneumococcal infections using vaccines renewed during 1960s when the trials in South Africa showed high efficacy of dodecavalent vaccine in preventing pneumonia. The success leads to the licensing of Merck's 14-valent pneumococcal polysaccharide vaccine (PNEUMOVAX) in 1977 by US FDA and Health Canada's HPFB. Each 0.5 ml dose of these vaccines contained 50 µg of purified polysaccharide of pneumococcal serotype 1, 2, 3, 4, 6A, 7F, 8 9N, 12F, 14, 18C, 19F, 23F, and 25F.

The current 23-valent pneumococcal vaccine produced by Merck (PNEUMOVAX-23) was approved in the United States and Canada in 1983. The 23-valent polysaccharide vaccine remains the only pneumococcal vaccine for the immunization of adults in the early 21st century. It differs from the 14-valent vaccine as the dose is reduced from 50 to 25 µg. Secondly, serotype 25F was removed and 10 additional serotypes, 5, 9V, 10A, 11A, 15B, 17F, 19A, 20, 22F, and 33, were included. In addition, serotype 6A was replaced by 6B polysaccharide. The current 23-valent formulation is effective against approximately 90 % of disease causing serotype in United States and Europe. The capsular polysaccharide (CPS) are T-cell-independent antigens and are poorly immunogenic in young children, particularly for the five pneumococcal serotypes that cause invasive disease in children (Douglas *et al.*, 1983, Stein, 1992). In order to improve the immunogenicity of CPS antigens, new pneumococcal CPS-protein conjugated vaccines (PCV) have been developed. For children under the age of two years who fail to mount an adequate response to the 23-valent adult vaccine, a current 7-valent pneumococcal conjugated vaccine (PCV) (Prevnar, Wyeth, USA) is recommended. In Prevnar the pneumococcal CPSs are linked to CRM_{197}, a nontoxic recombinant variant of diphtheria toxin (*Corynebacterium diphtheriae*). Prevnar covers the most prevalent serotype 4, 6B, 9V, 14, 18C, 19F, and 23F that causes 80% to 90% cases of severe pneumococcal disease, and is considered to be nearly 100% effective against these strains (Pelton *et al.*, 2003). Although in developing countries the efficacy of vaccination is lower in HIV-infected children compared to uninfected, a substantial proportion of children will be protected (Zar, 2004). New 9- and 11-valent conjugate vaccines that provide more optimal serotype coverage are currently undergoing clinical trials (Girard *et al.* , 2005). Studies demonstrated that although standard PCV vaccination reduced the carriage of vaccine serotypes, the vaccinated niche was replaced and occupied by non-vaccine pneumococcal serotypes that can potentially

cause the disease (Huang *et al.*, 2005; Frazao *et al.*, 2005). Moreover, conjugate vaccines are too expensive for developing countries, where the death rate of children from invasive pneumococcal disease is highest. Consequently there is an urgent need to develop new and improved therapy and alternative pneumococcal vaccine to combat pneumococcal diseases. The most promising approach is to develop vaccines based on pneumococcal proteins that contribute to virulence and are common to all serotypes.

3.3. Cell wall structures and virulence factors of *S. pneumoniae*

S. pneumoniae is a versatile microorganism and has evolved numerous successful strategies to colonize its host and to evade host defence mechanism. Pneumococci possess a wide variety of virulence factors that are thought to contribute towards its pathogenesis. (Kadioglu *et al.*, 2008; Bergmann and Hammerschmidt, 2006)

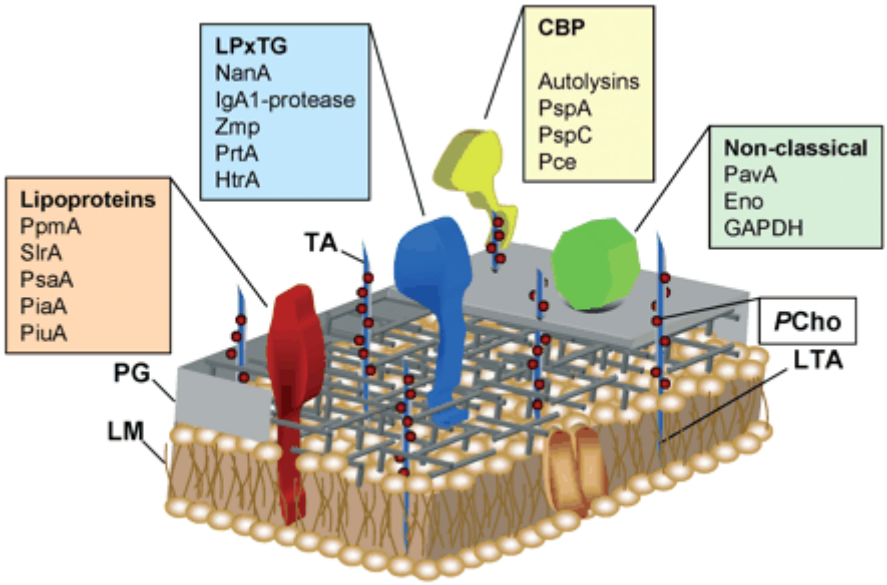


Figure 1 Schematic model of the pneumococcal outer cell wall and surface-exposed proteins. LM: phospholipid membrane, PG: peptidoglycan, TA: teichoic acid, LTA: lipoteichoic acid *P*Cho: phophorylcholine, CBP: choline-binding protein (Bergman and Hammerschmidt, 2006)

3.3.1. The pneumococcal capsule

The *Streptococcus pneumoniae* capsule forms a diverse group of polymers that are the most important and most recognized virulence factor of the organism. The polysaccharide capsule forms the outermost layer of pneumococcus and is approximately 200-400 nm thick (Sorensen *et al.*, 1990). The capsule is covalently attached to the outer surface of the cell-wall peptidoglycan, with an exception of serotype 3 (Sorensen *et al.*, 1990). The CPSs are essential for virulence and are targets for all current pneumococcal vaccines. At present a total of 91

serologically distinct CPS, that are structurally and chemically different have been described (Henrichsen, 1995; Park *et al.*, 2007). The CPS has been recognized as a *sine qua non* of virulence and is strongly anti-phagocytic in non-immune hosts (Austrian, 1981). The significance of polysaccharide capsule for pneumococcal pathogenesis has been studied in detail.

The capsule renders the pneumococcus resistant against complement-mediated opsonophagocytosis (Fine, 1975; Giebink, et al., 1977; Silvenoinen-Kassinen and Koskela, 1986). However, the degree of protection appears to be dependent not only on the biochemical structure of the CPS but also, to a lesser extent, on the thickness of the capsule (Austrian, 1981). Moreover, the interaction of pneumococci with complement system varies according to the serotype, for example, type 3 activates classical pathway, whereas type 25F exclusively activates alternative complement pathways, and type 14 activates both of them (Cheson *et al.*, 1984; Winkelstein *et al.*, 1976). In addition, the deposition and degradation of complement components on the capsule (Hostetter, 1986; Angel *et al.*, 1994), induction of protective antibodies (van Dam *et al.*, 1990), clearance mediated by lectin-like structure, for example by a C-type lectin SIGN-R1, (Ofek and Sharon, 1988; Kang *et al.*, 2004; Lanoue *et al.*, 2004) differs among the serotypes. Also, Fernebro *et al.* (2004) reported capsular serotype dependent resistance to spontaneous or antibiotic-induced autolysis, contributing to antibiotic tolerance in clinical isolates.

The CPS expression also reduces the entrapment of pneumococcus in the mucus, thereby allowing the access to epithelial surfaces (Nelson *et al.*, 2007). While most of the pneumococcal CPSs are negatively charged, they repel the sialic acid-rich mucopolysaccharides found in mucus. Moreover, the encapsulated strains were found to be at least 10^5 times more virulent than nonencapsulated strains lacking the capsule (Avery and Dubos, 1931; Watson and Musher, 1990). *S. pneumoniae* undergoes spontaneous, reversible opacity phase variation with a frequency of 10^{-3} to 10^{-6} resulting in opaque and transparent colonies (Weiser *et al.*, 1994). The transparent phenotype produces lower amount of CPS and has an enhanced ability in colonizing the mucosal surfaces of nasopharynx and in residing on surfaces whereas the opaque phenotype is more virulent in systemic infection (Kim and Weiser, 1998; Tong *et al.*, 2001). In addition, Hammerschmidt *et al.* (2005) demonstrated that pneumococci in intimate contact with cells of the murine lung tissues or cultured epithelial cells have substantially reduced amount of capsular material compared to pneumococci in spatial distance of the cells.

3.3.2. Pneumococcal cell wall

The layer underneath the capsule, the **pneumococcal outer cell wall**, is composed of peptidoglycan bearing structurally different peptides, glycolipids, teichoic (TA) and lipoteichoic acids (LTA, Forssman antigen), and phosphorylcholine (Mosser and Tomazs, 1970; Tomazs, 1981; García-Bustos et al., 1987; Fischer, 2000). TA and LTA of the pneumococcal cell wall consist of extended repeats of carbohydrates and differ only in their attachment to the cell surface. The **phosphorylcholine** (*P*Cho) is covalently linked to TA and LTA and this moiety acts further as a docking site for a class of pneumococcal surface proteins known as choline-binding proteins (CBPs) (Gosink *et al.* , 2000). Interestingly, phosphorylcholine is not unique to pneumococci but is also present on the surface of other respiratory pathogens such as *Neisseria* spp., *Haemophilus in fluenzae*, *Actinobacillus actinomycetemcomitans*, and *Pseudomonas aeruginosa* (Weiser *et al.*, 1998a, 1998b; Kolberg *et al.*, 1997; Gmur *et al.*, 1999). In pneumococci, *P*Cho is a bacterial adhesin, as it mediates pneumococcal adherence to the receptor for platelet-activating factor (rPAF) and activates host cell signaling through this receptor (Radin *et al.*, 2005). The rPAF is rapidly internalized after interaction with its ligand PAF and pneumococci have been shown to engage the upregulated rPAF for internalization (Cundell *et al.* , 1995). Moreover, the *P*Cho-rPAF interaction represents a specific mechanism for pneumococcal trafficking across the blood-brain-barrier and subsequent internalization (Ring *et al.*, 1998). A recent data indicated that the endocytosis of pneumococci requires not only rPAF but also β-Arrestin 1, and the event causes a G-protein independent activation of the MAP kinase ERK-1/ERK-2. The pneumococci are endocytosed via clathrin-coated vesicles and at least half of them proceed through Rab5 to Rab7 marked endosomes towards lysosome. Other vacuoles acquire Rab11, which is consistent with the known recycling of the bacteria to the apical surface (Radin *et al.*, 2005).

The host-mediated killing of *S. pneumoniae* is generally thought to require opsonisation by the serotype-specific antibodies together with complement, followed by phagocytosis. Interestingly, McCool and Weiser (2004) demonstrated that in mice having genetic defects in humoral immunity, serotype-specific antibodies are not required for the clearance of pneumococcal colonization. The *P*Cho is targeted by the C-reactive protein (CRP), which is an acute phase serum protein produced rapidly in response of inflammatory stimuli (Volanakis and Kaplan, 1971). This results in activation of the complement system and protection from pneumococcal infection in mice models (Szalai *et al.*, 1997; Mold *et al.*,

2002). The pneumococcal cell wall but not the CPS, *P*Cho, and purified LTA, strongly stimulate the alternative pathway of complement system (Winkelstein *et a l.*, 1976; Winkelstein and Tomasz, 1977, 1978). Studies demonstrated that highly purified pneumococcal LTA stimulates the host immune response via the TLR2 signal pathway (Schwandner *et al.*, 1999; Yoshimura *et al.*, 1999; Schroder *et al.*, 2003). In contrast, a novel study by Travassos *et al.* (2004) demonstrated that highly purified pneumococcal peptidoglycan is not detected by TLR2, TLR2/1 or TLR6/2; rather it might be detected by intracellular NOD1/NOD2. Furthermore, the lipopolysaccharide binding protein (LBP) binds the glycan backbone of the peptidoglycan and in turn facilitates the meningeal inflammation (Weber *et al.*, 2003). The pneumococcal cell wall induces CD14 dependent inflammatory response in culture monocytes (Cauwels *et al.*, 1997). The cell-wall mediated signaling induces the expression of transcription factor NF-κB and the production of TNF-α, IL-1, IL-6 and IL-8 (Bergeron *et al.*, 1998; Saukkonen *et al.*, 1990; Spellerberg *et al.*, 1996). In addition to its inflammatory activities, pneumococcal cell wall components are further involved in attachment of pneumococci to human umbilical vein endothelial cells (HUVEC) (Geelen *et al.*, 1993).

3.3.3. Pneumococcal virulence factors

In addition to the pneumococcal capsular polysaccharide and the cell wall components, the pneumococcal protein virulence factors also play a major role in the pathogenesis of pneumococcal infections. The surface proteins of *S. pneumoniae* are of special interest because of their potential role in pathogenesis and their possible usage as vaccine or part of vaccine. To date, the genomic DNA of three pneumococcal strains have been sequenced and analyzed (Tettelin *et al.*, 2001; Hoskins *et al.*, 2001; Dopazo *et al.*, 2001). This lead to the prediction of number of surface located proteins, which could be the potential vaccine or drug targets.

Pneumolysin is a sulfhydryl (thiol)-activated cytolysin which is produced by virtually all clinical isolates (Johnson *et al.*, 1980). Although its amino acid sequence is well conserved, a small number of variants have been observed (Lock *et al.*, 1996; Kirkham *et al.*, 2006). Pneumolysin is a member of the family of cholesterol-dependent cytolysin that is synthesized by Gram-positive bacteria. It binds to cholesterol in the plasma membrane of the host cells and induces the cell lysis due to its hemolytic activity (Johnson *et al.*, 1980; Alouf, 1980). As early as 1905, Libman reported for the first time the production of hemolysin by pneumococci. It is a well characterized cytosolic pneumococcal toxin of *S. pneumoniae* and is

known to interfere with eukaryotic host cell functions and the immune system. Pneumolysin is encoded as a 470 amino acid long protein with a molecular weight of 52 kDa. It oligomerizes in the membrane of the target cell to form a large ring-shaped transmembrane pore, which is 260 Å in diameter and is composed of approximately 40 monomer subunits (Morgan *et al.*, 1994, 1995). At high concentration pneumolysin has been implicated in the development of the acute inflammatory response due to its ability to activate the classical complement pathway (Paton *et al.*, 1984) and it bind nonspecifically to the Fc-fragment of IgG (Mitchell *et al.*, 1991). Interestingly, complement activation is not inhibited by free cholesterol (Paton *et al.*, 1984); however, it does inhibit the cytolyic activity of pneumolysin. Studies demonstrated that purified pneumolysin substantially increased alveolar permeability ex vivo in the isolated rat lung model, and may account for pneumococcal penetration into the bloodstream during bacteremia (Rubins *et al.*, 1993). In contrast, at very low doses (< 1 ng/ml) pneumolysin significantly inhibited respiratory burst, associated with reduced uptake and killing of pneumococci, and bactericidal activity, by inhibiting the migration of human polymorphonuclear leukocytes (PMNs) towards pneumococci (Paton and Ferrante, 1983). In addition, pneumolysin exposure stimulates the production of cytokine TNF-α and IL-1β from monocytes (Houldsworth *et a l.*, 1994) which have also been detected in experimental meningitis (McAllister *et al.*, 1975; Saukkonen *et al.*, 1990). Furthermore, pneumolysin has also been shown to be required for pneumococcal-induced deafness in meningitis and for pneumococcal-induced damage to the brain ependyma (Winter *et al.*, 1996; Hirst *et al.*, 2000, 2004). A recent study by Malley and coworkers suggested that pneumolysin recognition by TLRs induces release of TNF-α and IL-6 by macrophages (Malley *et al.*, 2003).

The pneumococcal cell-surface proteins are potential targets as vaccine antigens as they stimulate the production of opsonic antibodies. These cell-surface proteins have been classified into three major groups, the lipoproteins, proteins that are covalently linked to the bacterial cell wall by a carboxy terminal sortase (LPXTG) motif and choline-binding proteins. To date, between 42 and 45 pneumococcal cell-surface lipoproteins have been described (Bergmann and Hammerschmidt, 2006). These include the metal-binding lipoproteins pneumococcal surface antigen A (PsaA), pneumococcal iron acquisition A (PiaA) and pneumococcal iron uptake A (PiuA). In addition, the group also includes peptide isomerases putative proteinase maturation protein A (PpmA) and streptococcal lipoprotein rotamase A (SlrA). All of these proteins have been shown to be essential for substrate transport and bacterial fitness.

The **pneumococcal surface antigen A** (PsaA) is a part of divalent metal-ion-binding lipoprotein component of an ATP-binding cassette (ABC) transport system that has specificity for manganese (Dintilhac *et al.*, 1997, McAllister *et al.*, 2004). Due to its sequence homology to putative adhesin from other streptococci, PsaA was proposed to be a pneumococcal adhesin (Sampson *et al.*, 1994). Deletion of *psaA* abolished virulence in murine model of pneumonia, bacterimia and colonization (Berry and Paton, 1996; Marra *et al.*, 2002; Johnson *et al.*, 2002). Furthermore, anti-PsaA antibody has been shown to inhibit pneumococcal adherence (Romero-Steiner *et al.*, 2003). The microarray analysis demonstrated that *psaA* is upregulated during attachment of pneumococci to the nasopharyngeal cells (Orihuela e*t a l.*, 2004). Anderton *et al.* (2007) categorically demonstrated that E-cadherin is a putative eukaryotic cellular receptor for PsaA.

The **pneumococcal iron acquisition A** (PiaA) and **pneumococcal iron uptake A** (PiuA) are lipoprotein components of two separate iron uptake ABC transporters and have been shown to be required for full pneumococcal virulence (Brown *et a l.*, 2001). Immunization with PaiA and PiuA elicited protective antibodies that promote bacterial opsonophagocytosis rather than inhibiting iron transport (Jomaa *et al.*, 2005; Brown *et al.*, 2001).

Pneumococci produce two conserved surface-exposed lipoprotein belonging to a family of chaperons, the peptidyl-prolyl isomerases (PPIase), which are thought to be involved in secretion and activation of cell surface molecules. The **putative proteinase maturation protein A** (PpmA) and **streptococcal lipoprotein rotamase A** (SlrA) have been shown to be immunogenic (Adrian *et al.*, 2004). PpmA has been suggested to be involved in pneumococcal virulence, as mutation of *ppmA* in strain D39 increased the survival rate of mice (Overweg *et al.*, 2000). In addition, SlrA mutants are less efficient in nasopharyngeal colonization of mice due to their decreased capability to adhere to non-professional cells (Hermans *et al.*, 2006). However, further investigations are required to elucidate the role of PpmA and SlrA as vaccine targets are required.

Furthermore, peptide permeases are also known to influence indirectly pneumococcal virulence. The **permease-like protein A** (PlpA or AliA) belongs to the family of protein-dependent permeases for the transport of small peptides (Pearce et al., 1994). Another permease known as AmiA shows ~80 % sequence similarity to PipA. Loss of function of the AmiA has been found to increase resistance to antibiotics and to decrease pneumococcal adherence to eukaryotic cells (Alloing *et a l.*, 1990). Cundell and coworkers suggested that

peptide permeases modulate pneumococcal adherence to epithelial and endothelial cells either by acting directly as adhesins or by modulating the expression of adhesins on the pneumococcal surface during the initial stages of colonization (Cundell *et al.*, 1995).

In addition to lipoproteins, the pneumococcal surface is decorated with proteins that are covalently anchored to the peptidoglycan of the Gram-positive cell wall. These proteins possess a signal peptide required for protein export via the general secretory pathway and as a C-terminal cell wall sorting signal the conserved LPXTG anchorage motif. These cell wall anchored proteins, approximately 20, possesses often enzymatic activities and are important for colonization and immune evasion (Bergmann and Hammerschmidt, 2006). The **hyaluronate lyase** (hyaluronidase; Hyl) hydrolyzes hyaluronan of the extracellular matrix thus facilitating the pneumococcal penetration of the host tissue (Berry *et al.*, 1994). The hyaluronate lyase deficient pneumococcal strain demonstrated significantly reduced virulence compared to the wild-type strain in intraperitoneal mouse infection model (Berry and Paton, 2000; Chapuy-Regaud *et al.*, 2003).

The **neuraminidases**, also known as sialidases, are exoglycosidases which cleave terminal sialic acid residues (N-acetylneuraminic acids) from glycoproteins, glycolipids and oligosaccharides on cell surface and in body fluids. A recent study showed that neuraminidases can remove sialic acid from soluble proteins, such as lactoferrin, IgA2 and secretory component (King *et a l.*, 2004). Virtually all clinical isolates of *S. pneumoniae* produce an enzyme with neuraminidase activity (Kelly *et al.*, 1967). *S. pneumoniae* encodes at least three neuraminidases: NanA, NanB and NanC. However, while all strain encode NanA and most also encode NanB, only approximately 50 % isolates encode NanC (Pettigrew *et al.*, 2006). Although neuraminidases are secreted from the cell, only NanA contains the LPXTG sequence, suggesting differential *in vivo* roles of these enzymes. Both NanA and NanB have essential but different roles and are essential for survival during infections of respiratory tract and sepsis (Manco *et a l.*, 2006). In contrast, mouse nasopharyngeal colonization model demonstrated no significant difference in the virulence and ability of *nanA*-mutant to colonize (Berry and Paton, 2000). The precise biological role of NanC is still not known, however, its distribution among isolates from cerebrospinal fluid suggested a tissue-specific role (Pettigrew *et al.*, 2006). NanA has also been implicated in pneumococcal evasion of the adaptive immune response (King *et al.*, 2005).

S. pneumoniae strains produce an **immunoglobulin A1 (IgA1) protease** which cleaves human IgA1 but is inactive against other proteins including IgA2 (Kilian *et al.*, 1979,

Male, 1979). It cleaves the human IgA1 including secretory IgA1 in the hinge region and interfere with the function of IgA antibodies by eliminating the Fc-mediating effector function. Weiser and colleagues demonstrated markedly enhanced pneumococcal attachment during infections with pneumococci coated with human type-specific IgA1 antibodies generated against the CPS. Increased adherence was observed due to neutralization of the capsular negative charge by the Fab fragment, thus facilitating the interaction of unmasked cell wall *P*Cho with the rPAF (Weiser *et al.*, 2003).

The **zinc metallo protease C** (ZmpC) has been characterized in the Norway type 4 (TIGR4) strain as a bacterial zinc metallo protease cleaving human matrix metalloproteinase 9 (MMP-9). Further inactivation of *zmpC* in serotype 19F has been shown to impair virulence in a pneumoniae mouse model (Oggioni *et al.* , 2003). In addition intranasal infection experiments confirmed the significant contribution of **zinc metallo protease B** (ZmpB) to pneumococcal virulence (Blue *et al.*, 2003).

The **high-temperature requirement A** (HtrA) proteases are temperature-dependent molecular chaperons or heat shock-induced serine protease. They are regulated by the CiaRH two-component system. HtrA has been implicated in pneumococcal resistance against oxidative stress, nasopharyngeal colonization in rat, and pneumococcal pneumonia. Moreover, *htrA*-mutants compared to the wild-type strain induces release of cytokine IL-6 and TNF-α in the lungs during pneumonia (Sebert *et al.*, 2002; Mascher *et al.*, 2003; Ibrahim *et al.*, 2004a, 2004b).

Recently, **pili** were discovered in *S. pneumoniae*. Pilus mediates critical host-bacterial interactions, such as adherence to the epithelium and interaction with extracellular matrix proteins, and increasing virulence in mice (Barocchi *et al.*, 2006). However, pneumococcal pili is reported to be expressed in ~30% overall and 50 % among antibiotic-resistant strains. In *S. pneumoniae*, the *rlrA* pilus is encoded by a 14-kb islet, comprising of seven genes: the *rlrA* transcriptional regulator, three pilus subunits with LPXTG-type cell wall sorting signals, and three sortase enzymes involved in synthesis of the pilus polymer and in the incorporation of ancillary pilus components (Telford *et al.* , 2006; Fälker *et al.* , 2008). RrgB is the major subunit that forms the backbone of the structure, while the other two subunits, RrgA and RrgC, are ancillary proteins (Barrochi *et al.*, 2006; Hilleringmann *et al.*, 2008; LeMieux *et al.*, 2006). Recently, Nelson *et al.* (2007) showed that RrgA as the major *rlrA* pilus adhesin and that bacteria lacking RrgA are significantly less adherent to epithelial cells than wild-type organisms. Furthermore, RrgA mediates colonization of the pharyngeal epithelium of mice.

Interestingly, similar observations have been made in *Streptococcus agalactiae*, indicating that *rrgA* homologues (gbs104, gbs147*8*, gbs1467, and sak1441, and san1519) are involved in pilus-mediated adherence to human cells, while in *Streptococcus pyogenes* (*cpa*) and *Corynebacterium diphtheriae* both *rrgA* (*spaC*, *spaF*, and *spaG*) and *rrgC* (*spaB*, *spaE*, and *spaI*) homologues are defined as pilus-associated adhesins (Maisey *et al.*, 2007; Abbot *et al.*, 2007; Telford *et al.*, 2006). Moreover, piliated pneumococci evoked a higher TNF response during systemic infection, compared with nonpiliated derivatives, suggesting that pneumococcal pili not only contribute to adherence and virulence but also stimulate the host inflammatory response (Barrochi *et al.*, 2006). Additionaly, a second pilus islets, consisting of *pitA*, *sipA*, *pitB*, *srtG1*, and *srtG2*, coding for a second functional pilus in pneumococcus have been identified (Bagnoli *et al.*, 2008). Similar to the earlier known pilus this second pilus also functions as a bacterial adhesin and is found at a frequency of 16 % among the clinical isolates. The presence of different pilus types may confer a critical selective advantage to pneumococci and could be used as a potential vaccine target.

The family of **choline binding proteins** (CBPs) consists of 13-16 different proteins. CBPs have a modular organization and they are highly homologous in their C-terminal parts whereas the N-terminal parts are non-homologous. The C-terminal part consist of choline-binding repeat sequences proceeded by a proline-rich sequence. Four to five of the 20-amino acid repeat units mediate non-covalent attachment of the protein to the cell surface through *P*Cho (Yother and White, 1994). The amino-terminal parts consist of a signal peptide and the biologically functional polypeptide that is the site of the specific activities of the different proteins (Jedrzejas, 2001). To date, extensively characterized CBPs include the pneumococcal surface protein A (PspA), the pneumococcal surface protein C (also referred to as CbpA or SpsA), and four cell wall hydrolases, LytA, LytB, LytC, and the phosphorylcholine esterase (Pce or CbpE). The bacterial cell wall hydrolases are endogenous enzymes that specifically cleave covalent bond of the cell wall. To date, four cell wall hydrolases have been identified: two glycosidases, LytC, a β-N-acetylmuramidase (lysozyme) and LytB, a β-N-acetylglucosamidase (García *et a l.*, 1999), an amidase, LytA, which represents the major autolysin of pneumococci (Höltje and Tomasz, 1976), and the Pce phosphorylcholine esterase (CbpE).

LytA, the major **autolysin** is an amidase that cleaves the N-acetlymuramoyl-L-alanine bond of pneumococcal peptidoglycan (Howard and Gooder, 1974). The LytA enzyme plays a key role in pneumococcal lysis in the stationary phase as well as in the presence of penicillin

(Tomasz *et al.*, 1970). Moreover, this enzyme also participates in cell-wall growth and in daughter cell separation (Ronda *et al.*, 1987; Sánchez-Puelles *et al.*, 1986). Nevertheless, LytB is the major enzyme involved in cell separation, since *lytB*-deficiency induces the formation of pneumococcal chains with more than 100 cells per chain (García *et al.*, 1999; de las Rivas et al., 2002). Pneumococci deficient in *lytA* were shown to have reduced virulence in murine model of pneumonia and bacteremia (Canvin *et al.*, 1995; Berry *et al.*, 1989). It was suggested that the principal role of LytA in pneumococcal pathogenesis was to mediate release of pneumolysin from the bacteria to the extracellular environment (Lock *et al.*, 1992). In addition, autolysin-mediated release of bacterial components of the pneumococcal cell wall after cell death is highly inflammatory in animal infection models (Tuomanen *et al.*, 1999) However, Balachandran and colleagues demonstrated LytA, LytB and LytC independent release of pneumolysin into the extracellular environment (Balachandran *et al.*, 2001).

The **phosphorylcholine esterase** Pce belongs to the metallo-β-lactamase family, and cleaves the *P*Cho residues located at the end of the teichoic-acid chains. This ability to change *P*Cho decoration on the bacterial surface has relevant implications for the host-pathogen interactions. Vollmer and Tomasz, 2001 demonstrated that the inactivation of phosphorylcholine esterase caused a striking increase in pneumococcal virulence when pneumococci were injected into the peritoneal cavity of mice. The inactivation of *pce* gene might have increased the number of choline residues thereby facilitating the interaction with rPAF during infection. In contract, the *pce*-mutant showed significant reduction in colonization at 48 h in the infant rat colonization model (Gosink *et al.*, 2000). In addition, loss of function of Pce also reduced adherence to nasopharyngeal epithelial cell to 68 % of that of the wild-type (Gosink *et al.*, 2000).

In addition to CbpE (Pce), the genes encoding CbpF, CbpJ, **CbpD**, and **CbpG** were identified in the TIGR4 strain by a search of the pneumococcal genome (Gosink *et al.*, 2000). Both CbpD and CbpG are suggested to have a role in pneumococcal colonization. The CbpD functions as a murein hydrolase and has been demonstrated to be a competence-stimulating-peptide-inducible protein and it assists LytA in competence-induced cell lysis (Kausmally *et al.*, 2005). In addition, study by Guiral and colleagues demonstrated that CbpD is involved in the ability of competent bacteria to trigger release of virulence factors from non-competent *S. pneumoniae* (Guiral *et al*., 2005)

Another serologically variable CBP protein is the **pneumococcal surface protein A** (PspA), which is expressed in all clinical important capsular serotypes (Crain *et al.*, 1990). Its

highly electronegative properties are thought to inhibit complement binding (Jedrzejas *et al.*, 2001). PspA is a highly variable molecule that, based on the N-terminal sequence, can be grouped into three families that, in turn, can be subdivided into six different classes (Hollingshead *et al.*, 2000). PspA interferes with the binding of complement component C3 on the pneumococcal cell surface, and thus inhibits complement-mediated opsonization (Ren *et al.* , 2003, 2004; Tu *et al.* , 1999). Moreover, PspA protects pneumococci from the bactericidal activity of apolactoferrin because of its ability to bind lactoferrin (Shaper *et al.*, 2004; Hammerschmidt *et al.*, 1999). Therefore, the PspA-lactoferrin interaction might play a significant role in nasopharyngeal colonization, which is a prerequisite for invasive infection. In addition, *pspA*-mutant showed substantially reduced virulence in a mouse sepsis model as compared to the wild-type strain (McDaniel *et al.*, 1987).

A new class of cell-surface adhesins and virulence factors lacking typical signal peptide and/or a membrane anchor such as the LPXTG motif or choline binding repeats has been identified for *S. pneumoniae* (Chhatwal, 2002). These include, the pneumococcal adherence and virulence factor A (PavA) and two glycolytic enzymes including enolase and GAPDH. *S. pneumoniae* interacts with a variety of proteins of the extracellular matrix (ECM), including the fibrinectin, thrombospondin, and vitronectin (Pracht *et al* ., 2005; Rennemeier *et a l.*, 2007; Bergmann *et a l.*, in press). Pneumococci interact with the immobilized form rather than the soluble form of fibronectin (van der Flier *et al.* , 1995). Holmes and colleagues identified the PavA protein (**Pneumococcal adherence and virulence factor A**) as a pneumococcal adhesin for fibronectin (Holmes *et al.*, 2001). Although PavA lacks a signal peptide, it is localized on the pneumococcal outer cell surface (Holmes *et al*., 2001). PavA interacts via its C-terminal part with immobilized fibronectin and in turn modulates pneumococcal adherence to epithelial and endothelial cells (Pracht *et al*., 2005). In addition, PavA also functions as a virulence factor, as a *pavA*-mutant is highly attenuated in a mouse sepsis and meningitis model, respectively (Holmes *et al.*, 2001; Pracht *et al.*, 2005). However, the expression and functional activity of other known pneumococcal virulence factors such as pneumolysin and CBPs was not affected in *pavA* knockout strains (Holmes *et al.*, 2001; Pracht *et al.*, 2005).

The glycolytic enzymes **glyceraldehyde-3-phosphate dehydrogenase** (GAPDH) and **α-enolase** have been identified as plasminogen (PLG) binding proteins of *S. pneumoniae*. Both enzymes are essential for pneumococcal viability and are located in the cytoplasm as well as on the bacterial cell surface (Bergmann *et al.*, 2001 and 2004). In the presence of a

host-derived plasminogen activator recruitment of PLG facilitates pneumococcal transmigration through reconstituted basement membranes (Eberhard *et al.*, 1999). Enolase and its PLG binding are the key factors to potentiate degradation of ECM, dissolution of fibrin and pneumococcal transmigration (Bergmann *et al.*, 2005).

3.4. PspC: a multifunctional virulence factor of *S. pneumoniae*

One of the important virulence factors of *S. pneumoniae*, the **pneumococcal surface protein C** (PspC) (also designated as CbpA or SpsA) is a multifunctional choline-binding protein. PspC is a multifunctional protein that plays an important role in virulence and pathogenesis of this versatile pathogen. The functions attributed to PspC include binding of the free secretory component (SC) or SC as part of the secretory IgA (SIgA) and polymeric immunoglobulin receptor (pIgR), respectively, (Hammerschmidt *et al.*, 1997; Zhang *et al.*, 2000; Elm *et al.*, 2004). In addition, PspC contributes to pneumococcal binding to epithelial cells (Rosenow *et al.*, 1997), is suggested to bind complement component C3 (Cheng *et al.*, 2000; Smith and Hostetter, 2000) and was shown to interacts specifically with the complement regulator Factor H (Dave *et al.*, 2001; Durthy *et al.*, 2002). A *pspC*-knockout mutant showed less binding to epithelial cells and sialic acid *in vitro*, and shows reduced nasopharyngeal colonization compared with the wild-type (Rosenow *et al.*, 1997).

Although PspC proteins are highly polymorphic, they share a common organization that includes a 37 amino acid long signal peptide, the mature N-terminal domain, a proline-rich domain, and the choline binding repeats. The N-terminal domain is associated with multiple biological functions of PspC. So far 11 different subtypes of PspC proteins are identified and based on their different anchorage in the bacterial cell wall they are divided into two subgroups (Ianelli *et al.*, 2002). The classical PspC proteins (subtypes 1 to 6) are choline-binding proteins (CBPs) and constitute subgroup 1. The C-terminal choline-binding domain (CBD) attaches the classical PspC proteins non-covalently to cell wall via an interaction with the phophorylcholine of lipoteichoic and teichoic acids. The second subgroup representing atypical or PspC-like proteins (subtypes 7 to 11) are anchored in a sortase-dependent manner to the peptidoglycan of the cell wall by an LPXTG motif. The N-terminal regions of the first PspC subgroup show a common structure and organization. All proteins have a leader peptide and an N-terminal domain which is followed by one or two repetitive sequences (termed R1 and R2) and a proline-rich sequence (Ianelli *et al.*, 2002; Brooks-Walter *et al.*, 1999; Hammerschmidt *et al.*, 1997; Luo *et al.*, 2005). The *pspC*-like gene of serotype 3 strain A66

was demonstrated to encode a Factor H-binding inhibitor of complement, (Hic, PspC11.4) (Janulczyk *et al.*, 2000).

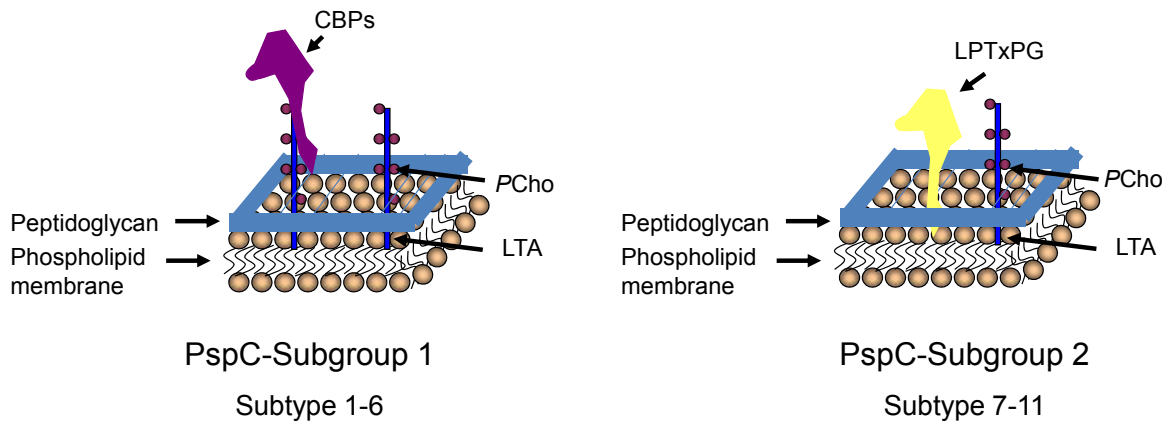


Figure 2 Schematic representations of PspC subgroups based on their different anchorage in the bacterial cell wall. LTA: lipoteichoic acid *P*Cho: phophorylcholine, CBPs: choline-binding proteins.

The PspC protein interacts with the SC of the polymeric immunoglobulin receptor (pIgR) and this interaction was shown to mediate adherence to and transmigration of pneumococci through human epithelial cells (Elm *et al.*, 2004; Zhang *et al.*, 2000). PspC protein is also known as SpsA (**Streptococcus pneumoniae secretory IgA binding protein**) because of its ability to bind pIgR that normally transports SIgA. To date, *S. pneumoniae* is the only bacterium known to interact with SC for virulence and this interaction could be critical for pneumococcal translocation from nasopharynx and spread to normally sterile parts of the respiratory tracts such as lungs or the blood stream during infections. The binding domains in the bacterial adhesin and host receptor were identified. The binding domain for SIgA and SC was mapped to a hexapeptide motif YRNYPT in the R domain of PspC protein (Hammerschmidt *et al.*, 2000; Elm *et al.*, 2004). Moreover, only one SC-binding motif, either R1 or R2, is sufficient for PspC to bind SC with high affinity (Elm *et al.*, 2004; Luo *et al.*, 2005). Interestingly PspC interacts in a species-specific manner with human SC or SIgA and not with SC or SIgA derived from animals, specifically those from bovine, canine, equine, guinea pig, hamster, rabbit, rat and mouse (Hammerschmidt *et al.*, 2000; Elm *et al.*, 2004). Likewise, Zhang *et al.* (2000) demonstrated that human-pIgR but not rabbit-pIgR expressed by MDCK cells enhances pneumococcal invasion. PspC interacts with the SC via the ectodomains D3 and D4 of the human-pIgR (hpIgR) and mediates invasion of pneumococci into the epithelium (Lu *et al.*, 2003; Elm *et al.*, 2004).

3.5. The polymeric immunoglobulin receptor

The **polymeric immunoglobulin receptor** (pIgR), which is broadly expressed by epithelial cells of the respiratory tract, is involved in the transport of immunoglobulins (IgA and IgM) across the mucosal epithelial barriers from the basolateral to apical surface (Mostov and Kaetzel 1999, Johansen *et al.*, 1999, Shimada *et al.*, 1999). At the apical cell surface, the extracellular binding domain of the pIgR complex is proteolytically cleaved off allowing the release of secretory component, either free or bound covalently to IgA, forming SIgA (Mostov *et al.*, 1984; Mostov, 1994; Piskurich *et al.*, 1995; Luton and Mostov, 1999). The association of SC to dIgA has been shown to protect SIgA antibodies from proteolytic degradation. SIgA represents the first line of defence on mucosal surfaces (Heremans, 1974; Underdown and Schiff, 1986; Kramer and Cebra, 1995; Lamm, 1997; Brandtzaeg *et al.*, 1999) and protects the mucus membrane from inhaled or ingested pathogens such as bacteria, viruses, parasite and toxins (Fubara and Freter, 1973; Outlaw and Dimmock, 1990; Mazanec *et al.*, 1993, Enriquez and Riggs, 1998). SIgA also prevents colonization and invasion of pathogens into mucosal surfaces by interfering with their motility and by competing with pathogens for adhesion sites on the apical surface of the epithelial cells (Giugliano *et al.*, 1995; Wold *et al.*, 1990; Dallas and Rolfe, 1998; Williams and Gibbons, 1972). In addition, pIgR transports immune complexes, microorganisms and antigens coated with IgA from the basolateral surface of mucosal epithelial to the apical surface, thereby providing a mechanism for a safe disposal of potential pathogens and harmful antigens (Mazanec *et al*., 1992; Kaetzel, 2001). Finally, luminal SIgA can neutralize the toxic activity of pathogen products such as bacterial toxins (Vaerman *et al.*, 1985). However, despite its role in host defence, some pathogens and viruses have developed strategies to exploit pIgR for their invasion into the epithelium (Sixbey *et.al.*, 1992, Gan *et.al.*, 1997, Lin *et.al.*, 1997, Lin *et.al.*, 2000; Zhang *et al.*, 2000). Under *in vivo* conditions in secretions covering the mucosal lining, binding of free SC or SIgA to PspC is able to inhibit pneumococcal internalization into host cells. In addition, binding of free SC and SIgA may also confer pneumococci protection against the immune defence on mucosal surface (Hammerschmidt *et al.*, 1997). Therefore, it seems clear that the balance between free SC and / or SIgA in secretion and uncleaved pIgR on cells most likely determine the outcome of the PspC-SC/pIgR interaction (Zhang et al., 2000; Kaetzel, 2001).

The intracellular pathways for pIgR transcytosis after binding of its ligand pIgA have been clearly documented. The basolateral to apical cell surface and corresponding retrograde

transport across epithelium has been thoroughly explored using the polarized monolayer of rabbit (rb)-pIgR transfected MDCK cells as model cell line (Song *et al.*, 1994, Cardone *et al.*, 1996). The vast knowledge regarding the pIgR-dIgA traffic has provided important insight into receptor sorting, intracellular compartments involved and the modulating receptor signal transduction pathways (Rojas and Apodaca, 2002). The initial process of basolateral to apical transcytosis involves the internalization of rb-pIgR-pIgA complex through clathrin-coated pits, which is then delivered at the apical surface via various sorting endosomes (Hoppe *et al.,* 1985; Limet *et al.,* 1985). Although unloaded-pIgR transcytosis is a constitutive process, it is subjected to regulation by various mechanisms. These include cell cytoskeleton (Hunziker *et al.*, 1990; Maples *et al*., 1997), along with small GTPases such as Rho family GTPases (Leung *et al.*, 1999; Jou *et al.,* 2000; Rojas *et al.,* 2001) and Rab GTPases (Hunziker and Peters, 1998; Casanova *et al.*, 1999; Wang *et al.,* 2000; van IJzendoorn *et al.*, 2002), intracellular host cell signalling molecules like the heterotrimeric G-protein (Bomsel and Mostov, 1993, Hansen and Casanova, 1994), phosphatidylinositol-3-kinase (PI3 kinase) (Hansen *et al.*, 1995, Tuma *et al.*, 2001), SNAREs (Low *et al.*, 1998, Calvo *et al.*, 2000, Apodaca *et al.*, 1996), protein kinase C (Cardone *et al.*, 1996, Cardone *et al.*, 1994), $p62^{yes}$ (Luton *et al.*, 1999), phospholipase Cγ (Luton *et al.*, 1998), cyclic AMP (Hansen and Casanova, 1994), intracellular calcium (Cardone *et al.*, 1996, Luton *et al.*, 1998), receptor phosphorylation of serine 664 (Low *et al.*, 1998, Apodaca *et al.*, 1996, Casanova *et al.*, 1990), receptor dimerization (Singer and Mostov, 1998), and ligand binding (Song *et al*., 1994, Giffroy *et al.*, 1998).

The stimulation of pIgR transcytosis upon pIgA binding and the induction of receptor signal transduction pathways has been well demonstrated for the rabbit (Song *et al.*, 1994, Cardone *et al.*, 1996) and the rat receptor (Luton *et al.*, 1998). In contrast, this process might not be true for human pIgR, as in human Calu-3 cells, and hpIgR transfected MDCK cells, pIgA binding fails to induce transcytosis, even though induced intracellular signalling pathways are similar to rb-pIgR (Giffroy *et al.* 2001). It is known that PspC-SC/h-pIgR interaction mediates adherence to and invasion of mucosal epithelial cells. However, the cellular and molecular basis of PspC-hpIgR mediated pneumococcal infections of host epithelial cells and the initiated signal transduction pathways are not explored. Nevertheless, Zhang *et al.* (2000) and colleagues hypothesized that *Streptococcus pneumoniae* may utilize the apical recycling pathway of hpIgR i.e. the transport in the retrograde fashion to the basolateral surface, for bacterial translocation across human epithelial barriers. However,

whether this apical to basolateral pneumococcal translocation occurs by utilizing the hpIgR-transcytosis machinery in reverse or by other mechanisms is still not clear.

In addition to its role as an adhesin, PspC also mediates immune evasion by binding the C3 or the host complement and innate immune regulator Factor H. C3 is produced by alveolar macrophages (Cole *et al.*, 1983), pulmonary fibroblasts and epithelial cells (Rothman *et al.*, 1989; Strunk *et al.*, 1988). PspC-deficient mutants fail to bind to a C3 matrix (Smith and Hostetter, 2000). The PspC uses two different epitopes for binding the soluble host protein Factor H and SC (Dave *et al.*, 2004). Hic (PspC sub-type 11.4) protein of subgroup II of PspC molecule also interacts with Factor H and shows considerable sequence homology with the N-terminal sequence of the subgroup I PspC proteins (Janulczyk *et al.*, 2000; Iannelli *et al.*, 2002). Recruitment of Factor H to the surface of pneumococci efficiently prevents activation of C3b and complement mediated opsonophagocytosis of pneumococci (Jarva *et al.*, 2004, Quin *et a l.*, 2005). Moreover, role of PspC protein has been implicated in a pneumococcal induced pulmonary inflammation (Madsen *et al.*, 2000).

3.6. Complement system

Complement system is a crucial component of the innate immunity and plays a central role in the elimination of microbes, clearing of immune complexes and damaged self cells and also in modulating the adaptive immune response (Walport, 2001). The complement system is highly regulated but excessive or uncontrolled complement activation on self-tissues has severe effects and can cause various diseases (de Córdoba and de Jorge, 2008; Markiewski and Lambris, 2007). The complement system consists of ~40 proteins that are present in body fluids or on cell and tissue surfaces and is activated in a cascade-like manner by three major pathways (Walport, 2001). Based on the activation mechanism, which differ considerably, the complement system has been classified as the classical, lectin and alternative pathways. The classical pathway is activated by binding of C1q to antigen bound immunoglobulins, the lectin pathway is stimulated by structurally similar pattern-recognition receptors, mannose binding lectin or ficolins that recognize microbial carbohydrates and the alternative pathways is activated continuously at a low rate by the spontaneous hydrolysis of the central component C3. The three pathways converge at the level of C3-convertase (C3bBb for alternative pathway; and C2a4b for classical or lectin pathways) which cleaves C3 into C3a and C3b. While C3a acts as an anaphylatoixn and antimicrobial substance, C3b binds covalently to surfaces and aids phagocytosis of target cells. C3b interacts with C3-convertase to generate

C5-convertase, which binds and cleaves C5 and initiates the terminal pathway leading to assembly of the lytic membrane attack complex (MAC) (Morgan, 1999).

Excessive complement activation on self tissue has severs effects and can lead to the development of various diseases (de Córdoba and de Jorge, 2008; Markiewski and Lambris, 2007). To prevent this, the human body uses fluid phase and membrane anchored complement regulators. Several of these regulatory proteins interact with C3 or C4 derivatives and are encoded by closely linked genes that constitute the Regulator of Complement Activation (RCA) gene cluster on human chromosome 1q32. The glycoproteins involved in regulation include fluid phase regulators such as Factor H, Factor H-like protein 1 (FHL-1), C4b binding protein (C4BP), C1 inhibitor, and cell membrane bound regulators like CR1/CD35, CR2/CD21, MCP/CD46, DAF/CD55, and protectin/CD59 as well (Morgan and Harris, 2003).

3.6.1. The complement and immune regulator Factor H

The complement and immune regulator Factor H belongs to the human Factor H protein family, which consists of seven structurally and immunologically related members. The other members of this protein family are the Factor H-like protein 1 (FHL-1) and five Factor H-related proteins proteins (FHR-1, -2, -3, -4 and -5) (Józsi and Zipfel, 2008). All proteins of the Factor H protein family are predominately synthesized in the liver and the secreted proteins are composed exclusively of globular protein domains termed as short consensus repeats (SCR) or complement control protein module (CCP). Although members of this group differ in the number of SCRs, the individual SCR domains show a high degree of identity to each other, thus explaining the immunological cross-reactivity and the common functions of the members of this protein family.

Factor H was first identified by Nilsson and Müller Eberhard (1965) as β1H globulin. Factor H is one of the most abundant human plasma proteins, with a concentration of 300-800 µg/ml, and is an important complement regulator. It is essential to regulate complement activation and to restrict the action of complement to activating surfaces. Factor H is single polypeptide chain plasma glycoproteins of approximately 150 kDa and is composed of 20 repetitive units of 60 amino acids (Ripoche *et al.*, 1988). The SCRs are comprised of highly conserved residues including four cysteines, two prolines, one tryptophan and several other partially conserved glycines and hydrophobic residues.

Factor H is the central soluble activation inhibitor of the alternative complement pathway and regulates complement both in fluid-phase and on cellular surfaces. The protein prevents binding of Factor B to C3b, accelerates the decay of alternative pathways C3-

convertase (C3bBb) (decay accelerating activity) and acts as a cofactor for Factor I-mediated proteolytic inactivation of C3b (Weiler *et a l.*, 1976; Whaley and Ruddy, 1976; Pangburn *et al.* , 1977). The members of human Factor H protein family represent multifunctional, multidomain proteins, where the complementary regulatory activity is displayed by the N-terminal four SCRs (SCRs 1-4). The C-terminus of the protein (SCRs 18-20) mediates surface binding and target recognition (Oppermann *et al.* , 2006). This C-terminal includes binding sites for several ligands, such as C3b, C3d, heparin, cell surface glycosaminoglycans and microbial virulence factors (Rodríguez de Córdoba *et al.*, 2004, Zipfel *et al.*, 2002).

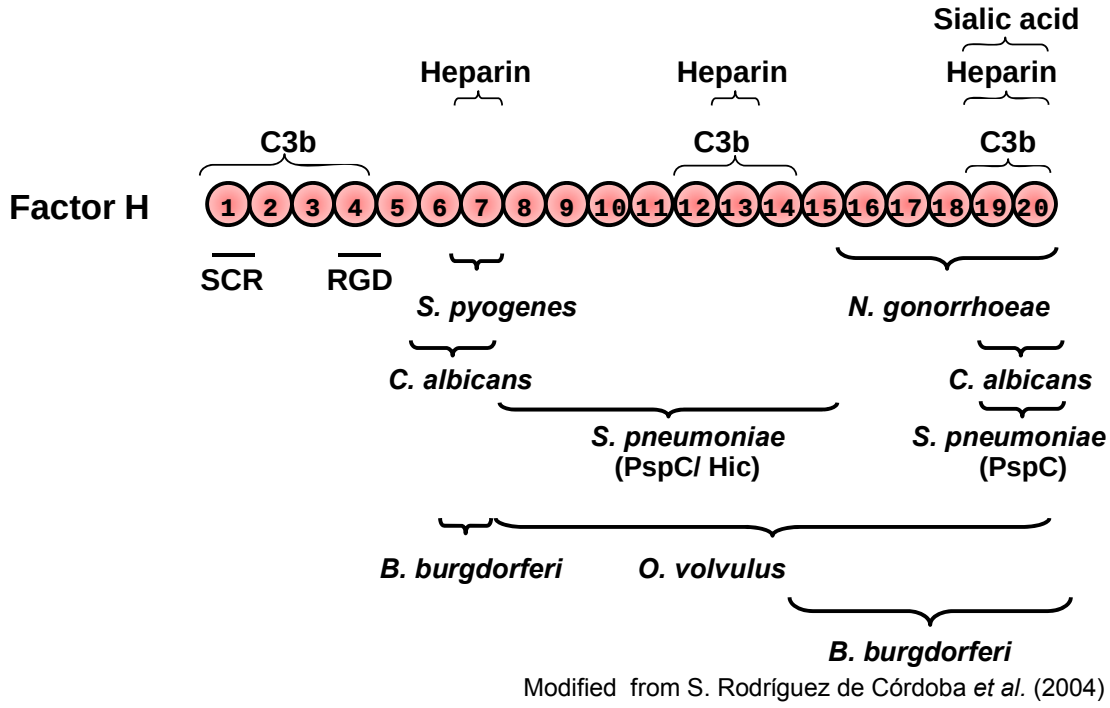


Figure 3 Schematic representation of Factor H molecule and its functional domains.

The Factor H like protein 1 (FHL-1 or reconectin), which is derived from the Factor H gene by means of alternative splicing, is identical with the seven N-terminal SCRs of Factor H and includes an extension of four amino acids at it C-terminal end. Although FHL-1 is present in the plasma at a concentration of approximately 10-50 µg/ml, it acts as a complement regulator and displays cofactor and decay-accelerating activity similar to Factor H (Zipfel and Skerka, 1999). In addition, FHL-1 has unique functions. It acts as an adhesin protein and this function is mediated by the RGD domain located within SCR4 (Hellwage *et al.*, 1997).

The Factor H related proteins (FHR-1, -2, -3, -4 and -5), which are comprised of four to nine SCR domains, show two major conserved regions. The N-terminal SCRs of all five FHRs are related to each other, and show homology to SCRs 6-9 of Factor H, while the C-

termini of FHR proteins show homology to the C-terminal surface binding region of Factor H and to each other (Józsi and Zipfel, 2008). Although FHR proteins lack the potent complement regulatory activity of Factor H, they possess a complement modulatory activity in the form of a Factor H cofactor enhancing activity for example FHR-3 and FHR-4 (Hellwage *et al.*, 1999; Timmann *et al.*, 1991). In addition, a relatively weak cofactor and decay accelerating activities have been described for FHR-5 (McRae *et al.*, 2005). The homology of FHR domains with SCRs of Factor H suggests similar ligand binding and functional activities. The C3b binding was reported for FHR-3, -4 and -5, heparin binding was reposted for FHR-3 and FHR-5, whereas the C-reactive protein (CRP) binding was reported for FHR-5 ((Hellwage *et al.*, 1999; McRae *et al.*, 2005).

Factor H and FHL-1 are soluble regulators that bind to and protect cells and tissues that lack endogenous membrane regulators. However, mutations, polymorphisms and large deletions within the gene clusters are associated with a wide spectrum of severe diseases including kidney disease atypical haemolytic uremic syndrome (aHUS) and membrane nonproliferative glomerulonephritis type II (MPGN II) and the retinal disease age-related macular degeneration (AMD) (Józsi and Zipfel, 2008; Noris and Remuzzi, 2005, Appel *et al.*, 2005; Hageman et al., 2005). Atypical HUS is a severe kidney disease characterized by microangiopathic haemolytic anaemia, thrombocytopenia and acute renal failure. Approximately 50 % of aHUS are caused by mutation in complement genes coding for Factor H membrane cofactor proteins such as Factor I, Factor B and C3 (Noris and Remuzzi, 2005; Kavanagh *et al.*, 2008). The majority of these mutations are heterozygous and result in defective alternative pathway regulation, which leads to complement mediated tissue damage in the kidney. MPGN II is a severe kidney disease characterized with electron-dense deposits within the glomerular-basement membrane and mesangial cell proliferation. It is associated with inappropriate complement regulation with low levels of C3 and enhanced amount of C3 activation product in plasma (Appel *et al.*, 2005). AMD, which is a leading cause for irreversible vision loss in developed countries, affects millions of elderly individuals worldwide. AMD is associated with immune deposits formed between retinal pigment epithelial cells and Bruch's membrane (Anderson *et al.*, 2002). A Tyr402His exchange within SCR7 of Factor H and FHL-1 strongly increases the risk for AMD (Hageman *et al.*, 2005). In addition, mutations in the complement components C3, Factor B and C2 have also been reported for AMD. The complement activation products C3a and C5a were shown to contribute to neovascularisation in the diseased eye (Józsi and Zipfel, 2008).

The host cells use these soluble immune-regulators to control complement activation directly on their surfaces. However, pathogenic microorganisms exploit this strategy in order to establish an infection and to counteract complement attacks. Numerous pathogenic microorganisms including bacteria, fungi, viruses and parasites express surface proteins which mimic the binding characteristics of host surface proteins and recruits Factor H and/or FHL-1 for complement and immune evasion (Lambris *et al.*, 2008). These includes group A streptococci (Horstmann *et al.*, 1988), group B streptococci (Areschoug *et al.*, 2002; Jarva *et al.*, 2004), *Yersinia enterocolitica* (China *et al.*, 1993), HIV-1 (Pinter *et al.*, 1995a, 1995b; Sadlon *et al.*, 1994), *Onchocerca volvulus* (Meri *et al.*, 2002), *Echinococcus* spp. (Inal, 2004; Diaz *et al.*, 1997), *Borrelia burgdorferi* (Hellwage *et al.*, 2001; Kraiczy *et al.*, 2004), *Borrelia afzelii* (Wallich *et al.*, 2005), *Borrelia hermsii* (Hovis *et al.*, 2006), *Borrelia sp ielmanii* sp. nov. (Herzberger *et al.*, 2007), *Candida albicans* (Meri *et al.*, 2002, 2004), *Aspergillus fumigatus* (Behnsen *et al.*, 2008), *Neisseria m eningitides* (Madico *et al.*, 2006), *Neisseria gonorrhoeae* (Ngampasutadol *et al.*, 2008), *Leptospira interrogans* (Verma *et al.*, 2006), West Nile virus (Chung *et al.*, 2006), *Pseudomonas aeruginosa* (Kunert *et al.*, 2007). In addition, *Streptococcus pneumoniae* also acquire Factor H, fluid phase regulator of alternative pathways, via PspC and Hic (Dave *et al.*, 2001, 2004a, b; Duthy *et al.*, 2002; Neeleman *et al.*, 1999, Janulczyk *et al.*, 2000, Jarva *et al.*, 2002, 2004). In addition, FHR-3 and FHR-4 were shown to bind to C3b-opsonized pneumococci (Hellwage *et al.*, 1999), and may cooperate with Factor H for complement inhibition. A wide range of structurally and functionally different microbial surface molecules bind Factor H family proteins for complement evasion. However, additional aspects such as cellular adhesion of pathogens in the presence of recruited Factor H are still poorly explored. For group A streptococci it was shown that recruitment of FHL-1 promotes intracellular invasion of host cells (Pandiripally *et al.*, 2003).

Similarly Factor H is also sequestered of to the cell surface of some cancer cells, thus inhibiting the complement-mediated lysis. A number of proteins belonging to the small integrin binding ligand N-linked glycoproteins (SIBLING) family such as bone-sialoprotein (BSP), osteopontin (OPN) and dentin-matrix protein 1 (DMP-1) are upregulated by many tumors (Fisher *et al.*, 2004). These proteins form a rapid and tight complex with Factor H and confer these cells the ability to evade the complement-mediated attack (Fedarko *et al.*, 2000; Jain *et al.*, 2002). In addition, tumor cells like the H2 glioblastoma cells produce and bind Factor H and FHL-1, and are able to promote cleavage of surface-bound C3b to iC3b (Junnikkala *et al.*, 2000).

3.7. Bacterial strategies to interact with eukaryotic cells

Infectious diseases are major threat to human health and are one of the leading causes of morbidity and mortality worldwide. For the past 60 years, antimicrobial chemotherapy has been in forefront of medical intervention against infectious diseases caused by bacterial pathogens. The extensive use of antimicrobial chemotherapy and the emergence of new multi-drug resistant pathogens has reduced the therapeutic effectiveness and increased the burden due to infectious diseases. Therefore, a detailed understanding of the individual processes and the underlying interactions on a molecular level is essential to describe the mechanisms of infectious diseases and the development of new therapeutic interventions.

Bacterial infections depend on both bacterial virulence factors and host susceptibility. During the infection, bacterial components can directly or indirectly contribute to pathogenesis and thereby function as virulence factors. Both extracellular and intracellular pathogens employ variety of strategies to subvert and control normal host cellular functions. The majority of bacterial pathogens specifically attack key intracellular-signaling and cytoskeletal pathways and alters host responses in a way that outcome is advantageous for the pathogen (Bhavsar *et al.* , 2007). These pathogens employ a wide range of effectors or virulence factors, which are either injected directly into the host cells or utilized by engaging host cell surface receptors. Effectors are usually specialized proteins that are injected directly into the cytosol of the host cell by a type III secretion system (T3SS) or a type IV secretion system (T4SS). Such secretion system consist of structurally conserved proteinaceous apparatus that is shaped like a needle (Galan and Wolf-Watz, 2006). The most commonly described and extensively investigated cellular target of pathogens is the cytoskeleton and many pathogenic microorganisms utilize its components to gain entry in the host cells and/or for moving within host cells (Alonso and García-del Portillo, 2004; Stevens *et al.*, 2006).

3.7.1. Interaction of bacterial pathogens with the host cell cytoskeleton

The cytoskeleton is a rigid cellular scaffolding or skeleton present within the cytoplasm of all cells. It is a dynamic structure that plays a major role in virtually all biological process in eukaryotes, from maintenance of cellular integrity and shape to cellular motions (by formation of cellular extensions in form of flagella, cilia and lamellipodia), intracellular trafficking of organelles, mitosis, cytokinesis and secretions. The cytoskeletal network is principally composed of three types of protein filaments including actin filaments, intermediate filaments and microtubules each presenting unique biophysical and biochemical properties. The remodeling of these protein filaments by multiple intrinsic and extrinsic cues,

which act through conserved signaling pathways, enables the cytoskeleton to control the amazing diversity of eukaryotic cell shapes, and moreover, to modify dynamic cellular behavior.

Actin is the most abundant intracellular protein in a eukaryotic cell and in terms of bacterial pathogenesis actin is the most extensively studied cytoskeletal component. It is a moderate-sized protein encoded by a highly conserved gene family and consists of approximately 375 residues. In human beings six actin genes encoding various isoforms are present including the four α-actin isoforms in various muscle cells and the β- and γ-actin isoforms in nonmuscle cells. The α-actin is associated with contractile structures, while the β-actin is at the front of the cell where actin filaments polymerize. Actin exists as a globular monomer called G-actin and as a filamentous polymer called F-actin, which is a linear chain of G-actin subunits. The mechanisms controlling the actin equilibrium in response to external stimuli and the signaling cascades leading to the regulation of actin cytoskeleton dynamics have been intensively investigated. These include regulation of actin polymerization dynamics by activation of specific actin binding proteins, activation of protein kinases or phosphatases and activation of signal transduction pathways involving Rho family of small GTPases (Hall, 1998). Under physiological conditions these intracellular signal transducers can be activated by extracellular stimuli that include hormones, growth factors, or cytokines (Narumiya, 1996; Hilpelä *et al.*, 2004; Myers and Casanova, 2008). In cultured cells three assemblies of actin are known, the stress fibers, lamellipodia and filopodia. The stress fibers, activated by Rho GTPases are large bundles of actin filaments in the cytoplasm of the cells and terminate in focal adhesions where integrins mediate cell attachment to extracellular matrix (ECM) proteins including collagen, laminin and fibronectin (Parsons *et al.*, 2000). The lamellipodium or leading edge is a characteristic, extensional structure for the spreading cells at the cell periphery, whereas filopodia or microspikes are an actin-rich element seen either as an extension from lamellipodia or found within this assembly (Mattila and Lappalainen, 2008; Naumanen *et al.* , 2008). Lamellipodia and filopodia are required for the spreading and motility of cells (Ladwein and Rottner, 2008). Actin filament polymerization is a controlled process and several proteins regulate this process (Antón et al., 2007; Hilpelä *et al.*, 2004; Myers and Casanova, 2008).

Cytoskeletal intermediate filaments (IF) are organized into a dynamic nanofibrillar complex that extend throughout mammalian cells. These filaments act as response elements in the subcellular transduction of mechanical perturbations initiated at cell surfaces. They also

provide a scaffold for other types of signal transduction that together with molecular motors translocates signaling molecules from the cell periphery to the nucleus (Goldman *et a l.*, 2008). Microtubules are highly dynamic tubular structures composed of α- and β-tubulin dimers. Besides actin cytoskeleton, microtubules have an important role in the generation and maintenance of polarity in epithelial cells. Microtubules also regulate the intracellular trafficking of proteins and lipids from the Golgi complex to the apical plasma membrane domain of the cells and target the transport vesicles between the basolateral and apical membrane domains (Apodaca, 2001). Microtubules and mitotic motors are major components of the spindle fibers hence; they play a major role in mitosis as they coordinate chromosomal movements in dividing cell (Scholey *et al.* 2001).

Bacterial pathogens have the ability to manipulate the cytoskeleton which helps to invade a host cell and/or to move within the cell (Stevens *et al.* , 2006). Pathogens do generally not interact directly with actin filament. Instead they modulate the actin polymerization by interacting with regulators such as Rho family GTPases (Finlay, 2005). Striking examples are observed during the invasion and intracellular motility by *Shigella flexneri* and *Listeria* spp., respectively. With the help of specific effector IcsA and ActA for *Shigella flexneri* or *Listeria* spp. respectively, they indirectly modulate actin filament polymerization (Egile *et al.*, 1999; Chakraborty *et al.*, 1995; Welch *et al.*, 1997). In addition, extracellular pathogens such as enterohaemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) hijack host actin cytoskeleton during their attachment and ingestion by utilizing effector protein Tir (Gruenheid *et a l.*, 2001). Tir is delivered into the target cells by T3SS and modulates the host cell cytoskeleton resulting in the formation of pedestal on the host cell surface (Campellone *et al.*, 2004; Garmendia *et al.*, 2004). Likewise, microtubules are also targeted by microorganisms. The pathogens can modify or control the cargo transport and the microtubule assembly and/or disassembly dynamics. Prototypes are the VirA protein of *Shigella* spp. or EspG of EPEC which destabilize the host cell microtubules through interaction with heterodimers of α-tubulin and β-tubulin (Yoshida *et al.*, 2002; Hardwidge *et al.*, 2005). In contrast, a strain of *Campylobacter jejuni* has been shown to use microtubules and their associated molecular motors to support host cell invasion (Hu and Kopecko, 2002).

3.7.2. Bacterial interference with host cell signaling pathways

The ability of the host cell to perceive and appropriately respond to their respective microenvironment is essential for the basic development and maintenance of normal tissue

homeostasis. Eukaryotic cell signalling is a highly complex mode of communication that governs the basic cellular activities and coordinates the cellular actions. Cells use a large number of clearly defined signalling pathways to regulate cellular processes such as cell proliferation, differentiation, gene activation, metabolism and death. Signal transduction refers to a process by which a cell converts one kind of signal or stimulus into another and it involves ordered sequence of biochemical reactions carried out by enzymes, second messengers and/or protein-protein interactions within the cell. Although cell signaling is a tightly controlled and regulated process, a defect in signalling pathways results in large number of diseases such as cancer, autoimmune diseases, diabetes etc.

The ability to modify central host cellular functions, as part of their virulence mechanisms, is a major advantage to many bacterial pathogens. Bacterial pathogens have a variety of cell-surface adhesins that enable them to attach to host cells. Some of these adhesins can bind to host cell receptors on non-phagocytic cells, thereby allowing the uptake of bound bacteria into the host cells. Although pathogen internalization mechanisms differ amongst pathogens, they share common features such as the ability to engage and modulate host intracellular-signalling pathways. Moreover, small GTPases, including **Rho GTPases**, are particularly attractive targets for pathogens as they play a central role in modulating cellular functions such as cytoskeletal control. Rho proteins belong to the super-family of Ras proteins. They cycle between an active, GTP-bound, and an inactive, GDP-bound, state. The transition between two states is catalyzed by GTPase-activating proteins (GAPs) that accelerate the hydrolysis of bound GTP (Moon and Zheng, 2003) and guanine nucleotide exchange factors (GEFs) that substitute GDP for GTP (Rossman *et al.*, 2005). Rho-GTPases interact with their effectors mostly in their GTP-bound states, thereby relaying incoming signals to downstream signalling pathways. In mammalian cells, several Rho subfamily proteins (RhoA, B, C, Rac1 and 2, Cdc42 (G25K), RhoG, RhoD, and RhoE) have been identified. However, Rho, Rac, and Cdc42, which are the most extensively studied GTPases, play a crucial role in actin cytoskeleton regulation. The Rho subtype proteins are involved in formation of stress fibers and focal adhesion complexes whereas Rac proteins induce lamellipodia formation and membrane ruffling (also induced by Rho in some cell types). Cdc42 has been shown to induce formation of filopodia or microspikes. However, in some cell types these GTPases act on the actin cytoskeleton in a cascade-like manner.

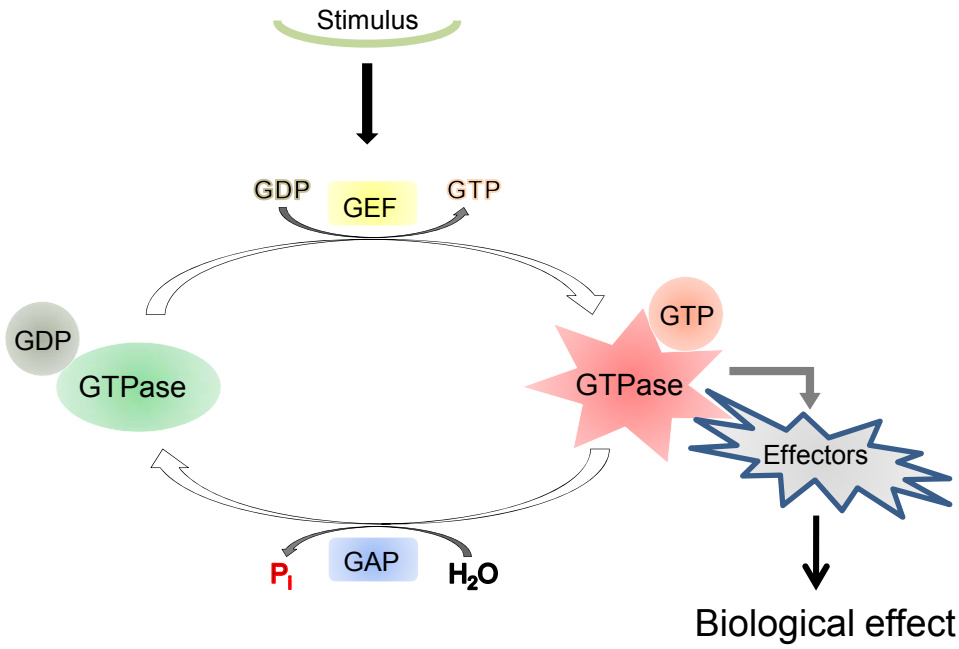


Figure 4 Rho GTPases cycle. GEF: guanine neucleotide exchange factor, GAP: GTPase-activating protein, GDP: guanidine diphosphate, GTP: guanidine triphosphate.

Notably, the activity of one or more members of the small Rho family GTPases are required for host cell invasion by pathogenic bacteria (Cossart and Sansonetti, 2004; Rottner *et al.* , 2004). The modulation of these GTPases can include either direct chemical modification of the GTPase or interaction with other regulatory elements associated with GTPase control. Pathogens use these alterations in GTPase functions for a variety of functions, including killing the host cell, mediating bacterial uptake into the host cell, intracellular survival by affecting intracellular trafficking, or providing polymerized actin mechanisms for microbial motility inside host cells and into adjacent cells (Boquet and Lemichez, 2003). Rho GTPases are important and play a crucial role in host cell invasion of many pathogenic bacteria including *Mycobacterium avium* and *Pseudomonas areuginosa* , *Salmonella enterica* , *Shigella flexn eri* and *Campylobacter jejuni* (Sangari *et al .,* 2000; Kazimierczak *et al.* , 2001; Hardt *et al.* , 1998; Tran Van Nhieu *et al.* , 1999; Krause-Gruszczynska *et al.* , 2007). Moreover, Rho family GTPases have also been shown to be involved in the regulation of rabbit-pIgR-dimeric IgA transcytosis across mucosal epithelium (Leung *et a l.*, 1999; Jou *et a l.*, 2000; Rojas *et al.* , 2001). In addition to actin cytoskeleton regulation, Rho GTPases also act as molecular switches in various signal transduction processes, such as, integrin signalling, endocytosis, transcriptional activation, proliferation, and apoptosis (Bishop *et al.*, 2000, Kaibuchi *et al.*, 1999). Rho GTPases also functions as a

switch in protein kinase cascades, resulting in activation of JNK/SAPK (c-Jun NH2-terminal kinase) and p38 kinase.

Pathogens are known to interfere with the phosphorylation cascade in the intracellular-signalling pathways of the host cell. These phosphorylation events are catalyzed by host cell protein kinases and phosphates. Phosphatidylinositol, a cellular phospholipid, is an important precursor of several second-messenger molecules in cellular signalling. The **PI3-kinases** are heterodimeric proteins consisting of a catalytic subunit (110 kDa, p110) associated with an 85 kDa noncatalytic regulatory subunit designated as p85 (Vanhaesebroeck *et al.*, 2001). PI3-kinase phosphorylates the 3´ hydroxyl position of the inositol ring of phosphatidylinositol, phosphatidylinositol-4-phosphate or phosphatidylinositol-4, 5-bisphosphate (PIP_2). PI3-kinase regulate many biological activities, such as DNA synthesis, cell survival, differentiation, phagocytosis, pseudopod formation and membrane ruffling, cell survival pathways, gene regulations, actin cytoskeleton, vesicle transport, and cell metabolism (Cox *et al.* , 1999; Pizarro-Cerda & Cossart, 2004; Stokoe, 2005; Fruman *et al.*, 1998) The PI 3-kinase family of enzymes plays a central role in growth factor receptor signal transduction and is involved in the signalling of F-actin polymerization (Chodniewicz and Zhelev, 2003 a, b).

A key downstream effector of PI3-kinase is the serine-threonine kinase **Akt** (protein kinase B) which in response to PI3-kinase activation is phosphorylated and regulates the activity of a number of targets including kinases, transcription factor and other regulatory molecules (Scheid & Woodgett, 2003; Milburn *et al.* , 2003; Song *et a l.*, 2005). The role of phosphoinositide 3-kinase (PI3-kinase) and phosphoinositide metabolism is being increasingly acknowledged in bacterial pathogenesis. A high number of pathogens were identified that require PI3-kinase activity during bacterial host cell invasion, such as group B streptococci (Burnham *et a l.*, 2007), group A streptococci (Purushothaman *et al.* , 2003), *Pseudomonas aeruginosa* (Kierbel *et al.* , 2005), *Helicobacter pylori* (Kwok *et al.* , 2002), *Chlamydia pneumoniae* (Coombes & Mahony 2002), *Escherichia coli* K1 (Reddy *et al.* , 2000) and *Listeria monocytogenes* (Ireton *et al.* , 1999). Recently the role of the PI3-kinase/Akt pathway has been demonstrated to be important for vitronectin mediated pneumococcal invasion of host epithelial cells (Bergmann *et al.*, in press).

In addition, **protein tyrosine kinases** (PTKs), especially the *Src* family of protein tyrosine kinases and mitogen-activated protein kinases (MAPKs) have been implicated in bacterial pathogenesis. Tyrosine phosphorylation is a central event in the regulation of a variety of biological processes such as cell proliferation, migration, differentiation and

survival. Several families of receptor and non-receptor tyrosine kinases, that control these events by catalyzing the transfer of phosphate from ATP to a tyrosine residue of specific target protein(s), have been identified. MAPKs include the extracellular signal-regulated kinases 1 and 2 (ERK1 [p44 MAPK] and ERK2 [p42 MAPK]) and two other groups of stress-activated protein kinases: c-Jun N-terminal kinases (JNK), also known as stress-activated protein kinase (Kyriakis and Avruch, 2001) and p38 MAP kinase, also known as hyperosmolarity glycerol (HOG) kinase (Shi and Gaestel, 2002). The MAPKs phosphorylate specific serines and threonines of other protein kinases, phospholipases, and cytoskeletal proteins thereby regulating various cellular processes (Johnson and Lapadat, 2002). Src tyrosine kinase is also a critical signal transducer modulating a wide variety of cellular functions. Activities of *Src* family of protein tyrosine kinases and MAPKs play a critical role in various bacterial and viral infections. The activities of *Src* PTKs are important for infections with *Staphylococcus aureus* , *Listeria monocytogenes* , *Helicobacter pylori* or *Neisseria meningitidis* and pathogenic fungus *Paracoccidioides brasiliensis* (Agerer *et al.*, 2003; Sousa *et al.*, 2007; Kwok *et al.*, 2007; Hoffman *et al.*, 2001; Maza *et al.*, 2008). Several MAPKs activation was found in response to epithelial cell infection with *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium and EPEC (Hobbie *et al.*, 1997; Tang *et al.*, 1998; Czerucka *et al.*, 2001). Moreover, JNK activation has been associated with the invasion *Porphyromonas gingivalis* in gingival cells, *Neisseria gonorrhoeae* in epithelial cells, and *Neisseria meningitidis* infection of HBMEC cells (Watanabe *et al.*, 2001; Ellington *et al.*, 2001; Naumann *et al.*, 1998; Sokolova *et al.*, 2004).

Many intracellular pathogens have evolved multiple strategies to interfere with normal cellular processes in order to promote their entry and survival within the host. Bacterial entry has been intensively analyzed in non-phagocytic cells, also known as “non-professional phagocytes”. Since non-phagocytes do not ingest microbes or other particles, they are excellent models for exploring the pathogen derived ingestion mechanism. Most microorganisms or toxins penetrate into the cells through an existing entry mechanism, for example, clathrin-mediated endocytosis, phagocytosis and macropinocytosis. Only a few of these ingested microorganisms can replicate and move within the vacuolar compartments or escape the killing within the host cells. Many intracellular pathogenic bacteria, based on the invasion mechanism have been classified into two well differentiated groups, namely “zippering” and “triggering” mechanism (Cossart and Sansonetti, 2004; Veiga and Cossart, 2006). Although it has been hypothesized that *Streptococcus pneumoniae* may utilize the

apical recycling pathway of hpIgR for its translocation across human epithelial barriers, it is still not clear whether this apical to basolateral pneumococcal translocation occurs by utilizing the hpIgR-transcytosis machinery in reverse or by other mechanisms.

3.8. Objectives of the project

It is known that PspC functions as an adhesin and that the PspC-SC/h-pIgR interaction mediates adherence to and invasion of mucosal epithelial cells. However, the mechanisms by which this interaction facilitates pneumococcal internalization of epithelial cells and the induced signal cascades have not been explored. Therefore, the aim of this study was to understand the PspC-hpIgR mediated pneumococcal adherence to and internalization of host epithelial cells. In addition, the intracellular signals governing PspC-hpIgR mediated internalization of *S. pneumoniae* and the endocytotic machinery utilized by pneumococci for their uptake by pIgR expressing host epithelial cells were analyzed. Additionally, the impact of pneumococcal cell surface bound Factor H on adherence to host cells and the molecular mechanism facilitating the uptake of Factor H bound pneumococci by epithelial cells was elucidated.

4. Results

4.1. Interaction of the pneumococcal surface protein C (PspC) with hpIgR

Streptococcus pneumoniae is one of the major pathogen that colonizes the upper and lower respiratory tract of humans and penetrates the epithelium of the nasopharynx or lungs to gain access to vascular compartments. *S. pneumoniae* possesses a variety of virulence factors that are involved in the infectious process. One important virulence factor of *S. pneumoniae* is the pneumococcal surface protein C (PspC). Remarkably, PspC is a multifunctional protein and various functions have been attributed to this protein.

PspC functions as an adhesin and the PspC-SC/hpIgR interaction mediates pneumococcal adherence to and invasion of mucosal epithelial cells (Hammerschmidt *et al.*, 1997; Zhang *et al.* , 2000; Elm *et al.* , 2004). However, the mechanism by which this interaction facilitates pneumococcal internalization of epithelial cells and the subsequent host signal transduction cascades induced by pneumococci are still poorly understood. A better understanding of the pneumococcal pathogenesis requires the determination of the cellular and molecular basis of mucosal epithelial invasion. Thus, in this study the pneumococcal adherence to host epithelial cells and the resulting intracellular signals governing PspC-hpIgR mediated internalization were investigated. In order to understand these fundamental events, infections were synchronized by centrifuging the bacteria onto the cells. Unless otherwise specified, all the infection assays were performed using *S. pneumoniae* serotype 35A (NCTC 10319) with a MOI of 50 bacteria per host cell and infections were carried out for 1 h at 37°C under 5 % CO_2.

4.1.1. PspC-hpIgR mediated pneumococcal adherence to and internalization into host epithelial cells

PspC interacts with the human polymeric immunoglobulin receptor on the host epithelial cells and facilitates pneumococcal colonization and uptake into host cells (Hammerschmidt *et al.*, 1997; Zhang *et al.*, 2000; Elm *et al.*, 2004). However, in order investigate the host cell signalling pathways involved in the PspC-hpIgR mediated uptake mechanism of *S. pneumoniae* , synchronized infection assays were performed after centrifugation of the bacteria onto the cells. Bacterial adherence to and internalization into pIgR producing host cells were determined using *S. pneumoniae* serotype 35A (NCTC10319) and the isogenic *pspC*-mutant (Δ*pspC*). To monitor the number of attached plus intracellular pneumococci, sample aliquots of the infected cells were plated onto blood agar plates after 1 h

of infection. While the number of recovered intracellular bacteria was enumerated by the antibiotic protection assays followed by plating on blood agar plates.

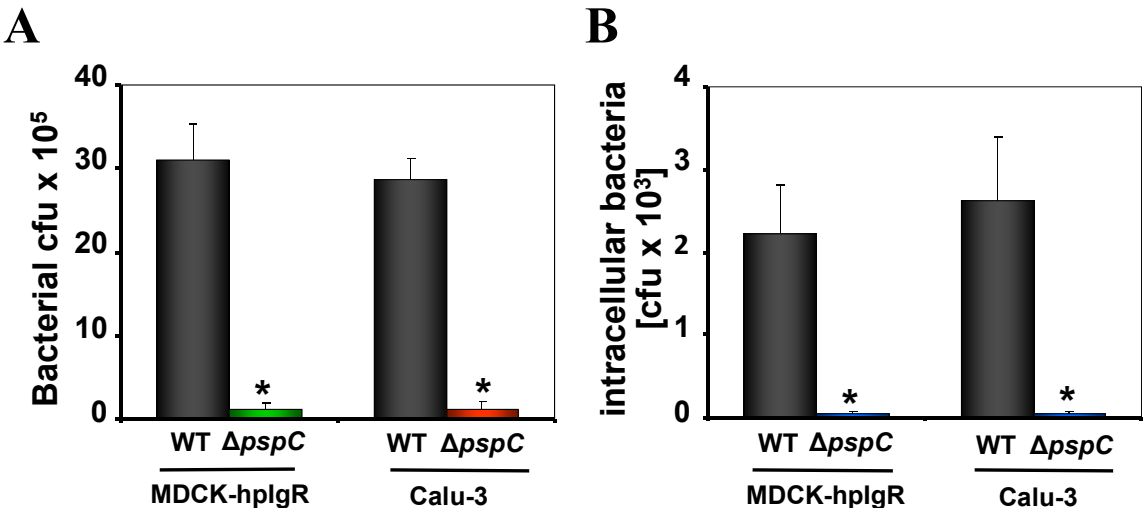


Figure 5 PspC-hpIgR mediated adherence and invasion of MDCK-hpIgR and Calu-3 cells by *S. pneumoni ae* serotype 35A (NCTC10319) (WT) and isogenic *pspC*-mutant. (A) Attachment of pneumococci was determined by counting the cfu (colony forming unit) per well obtained from the sample aliquots plated onto blood agar plates after 1 h of infection. (B) Invasive and recovered intracellular survivors were determined by the antibiotic protection assay. * $P< 0.02$ relative to infections carried out with the wild-type strain.

Bacterial strain	**adherent bacteria [cfu x 10^5] per 1x 10^5 cells**		**intracellular bacteria [cfu x 10^3] per 1x 10^5 cells**	
	MDCK-hpIgR	**Calu-3**	**MDCK-hpIgR**	**Calu-3**
S.p.type 35A	31.03 ± 4.26	28.64 ± 2.52	2.22 ± 0.59	2.62 ± 0.76
S.p. type 35A Δ*pspC*	1.11 ± 0.81	1.08 ± 1.1	0.05 ± 0.02	0.05 ± 0.03
p value	0.01	0.004	0.035	0.041

Table 1 Number of attached and recovered intracellular pneumococci estimated from cfu plated on blood-agar plates after 1 h infection of MDCK-hpIgR and Calu-3 cells. *P* value less than 0.05 was taken as statistically significant.

As compared to wild-type pneumococci, the PspC-deficient strain had significantly lost their ability to adhere to and invade human pIgR producing MDCK-hpIgR and Calu-3 cells (Figure 5A and 5B). Calu-3 is human lung epithelial cell line, naturally expressing the pIgR, whereas MDCK-hpIgR is stably a transfected canine kidney epithelial cell that produces the human pIgR. The *pspC*-mutant showed a significant reduction of about 96 % in the adherence to MDCK-hpIgR and Calu-3 cells as compared to the wild-type strain. Similarly, the internalization of PspC-deficient strain was significantly reduced. The results are in accordance to previously published data and clearly suggest that synchronized infection

assays do not alter the behaviour of the isogenic *pspC*-mutant in our cell culture infection assays.

4.1.2. Inhibition of PspC-hpIgR mediated pneumococcal internalization into host epithelial cells

PspC interacts with the secretory component (SC) via the ectodomains D3 and D4 of the human-pIgR and mediates invasion of pneumococci into the epithelium (Elm *et al.*, 2004). However, in order to verify the role of hpIgR in PspC dependent invasion and to corroborate our previous findings, inhibition assays using anti-SC antibody were performed. This antibody recognizes the human SC, which is the ectodomain of pIgR. The epithelial cells, MDCK-hpIgR and Calu-3, were preincubated for 20 min with anti-SC antibodies prior to bacterial infections. The intercellular survival of pneumococci was determined after 1 h of infection using the synchronized approach.

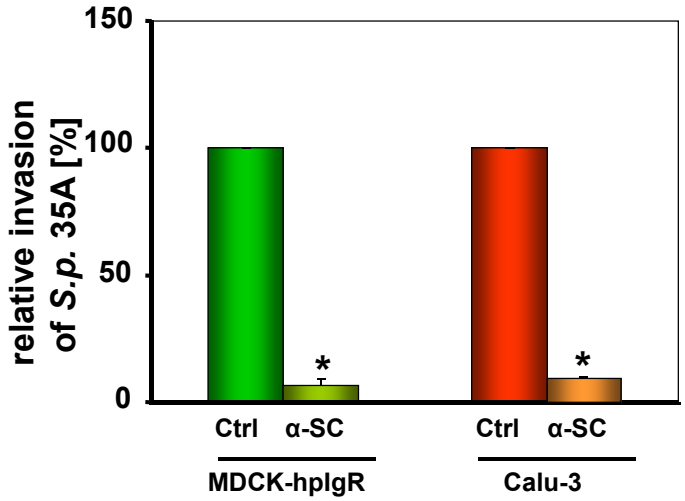


Figure 6 Inhibition of PspC-hpIgR mediated uptake of *S. pneumoniae* serotype 35A (NCTC10319) into MDCK-hpIgR and Calu-3 cells after preincubation the cells with antibodies recognizing the secretory component of hpIgR (α-SC). The invasion and intracellular survival of pneumococci in host cells was determined in the presence of α-SC (8µg/well) or absence (Ctrl) of antibody using the antibiotic protection assay. Invasion of *S. pneumoniae* in the absence of α-SC was set to 100 %. * $P < 0.001$ relative to infections carried out in absence of antibodies.

Treatment of cells	relative invasion by *S. p.* serotype 35A [%]	
	MDCK-hpIgR	Calu-3
control	100 ± 0	100 ± 0
α-SC (8µg/ml)	6.42 ± 2.39	9.26 ± 2.39
p value	2.95×10^{-10}	8.16×10^{-13}

Table 2 Pneumococcal invasion of MDCK-hpIgR and Calu-3 cells in the presence or absence of blocking α-SC antibody. The results are demonstrated as percentage invasion compared to infection assay performed in absence of blocking antibody. *P* value less than 0.05 was taken as statistically significant.

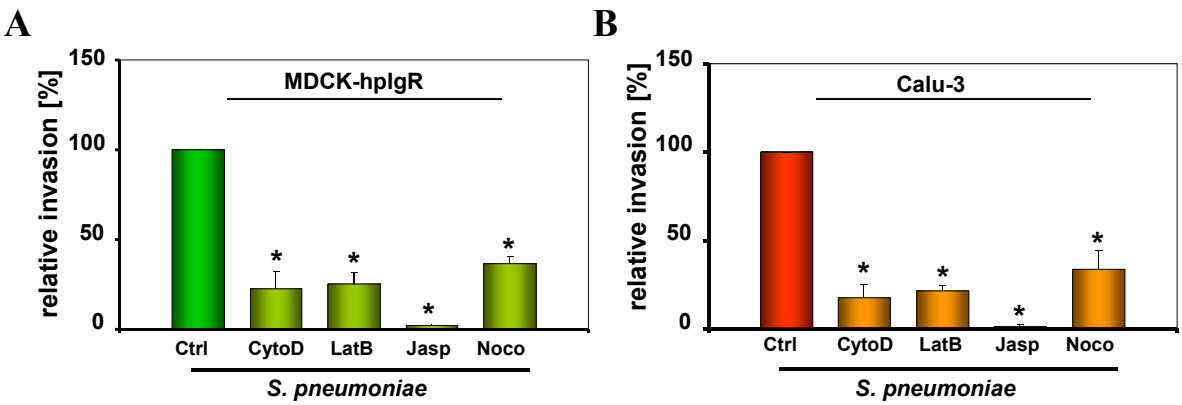
The results revealed that blocking of the receptor by anti-SC antibodies significantly reduced uptake of wild-type pneumococci by MDCK-hpIgR and Calu-3 cells (Figure 6). The internalization of wild-type *S. pneumoniae* by MDCK-hpIgR cells pre-treated with anti-SC antibodies was diminished and a 94 % reduction as compared to untreated cells was calculated. For Calu-3 cells a reduction of 90 % was observed. In the presence of host cell-bound anti-SC antibodies the levels of bacterial uptake are comparable to that measured for the isogenic *pspC*-mutant (Figure 5B). The result further confirms that PspC mediated pneumococcal internalization of mucosal host cells occurs in hpIgR-dependent manner. In addition, these inhibition assays also demonstrate that the specificity of PspC-hpIgR interaction is not altered upon synchronization of the infections.

4.2. Role of host cell cytoskeleton dynamics on PspC-hpIgR mediated ingestion of *S. pneumoniae* by epithelial cells

The transcytosis of pIgR and dimeric IgA was extensively investigated using rabbit-pIgR-dimeric IgA and polarized MDCK cells as a model cell line (Song *et al.*, 1994; Cardone *et al.*, 1996). Both microtubules and microfilament have been shown to regulate transcytosis of rabbit-pIgR-dimeric IgA from the basal to apical site (Hunziker *et al.*, 1990; Maples *et al.*, 1997). Therefore, the contribution of host cytoskeleton towards to the internalization of pneumococci via the PspC-hpIgR mediated pathway was investigated.

The impact of the actin cytoskeleton and microtubules on pneumococcal invasion was investigated in the presence of pharmacological inhibitors such as cytochalasin D, latrunculin B, jasplakinolide and nocodazole. Cytochalasin D and latrunculin B inhibit actin polymerization whereas jasplakinolide induces actin polymerization and nocodazole inhibits the polymerization of microtubules. Both MDCK-hpIgR and Calu-3 cells were preincubated with these inhibitors prior to bacterial infections and assays were performed in the presence of the inhibitors. The host cells were preincubated with 125 nM cytochalasin D, 50 nM

latrunculin B, and 100 nM jasplakinolide, respectively for 30 min at 37°C under 5 % CO_2. Treatment of host cells with 10 µM nocodazole was done for 1 h at 4°C followed by 30 min incubation at 37°C under 5 % CO_2. The antibiotic protection assay was performed using *S. pneumoniae* serotype 35A in order to ascertain the potential effect of cytoskeleton inhibitors upon pneumococcal uptake. To determine that treatment with these inhibitors does not have any significant influence upon the adherence of pneumococci, adherence was monitored by immunofluorescence staining of attached pneumococci.



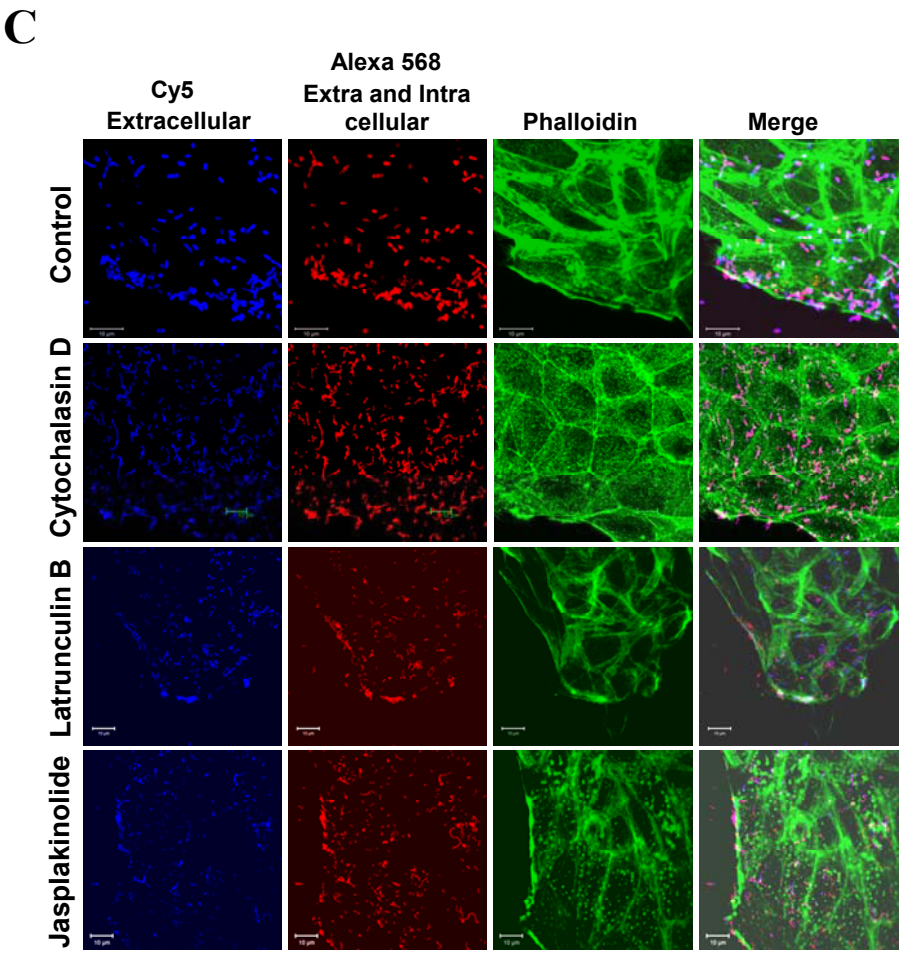


Figure 7 PspC-hpIgR mediated invasion of MDCK-hpIgR and Calu-3 cells by *S. p neumoniae* serotype 35A (NCTC10319) requires the host cell cytoskeleton dynamics. hpIgR mediated invasion and intracellular survival of the bacteria in MDCK-hpIgR (A) and Calu-3 (B) cells was followed in the absence (control) or presence of inhibitors of actin filaments and microtubules including cytochalasin D (CytoD, 125 nM), latrunculin B (LatB, 50nM), jasplakinolide (Jasp, 100nM) and nocodazole (Noco, 10µM) by the antibiotic protection assay. Invasion of *S. pneumoniae* in the absence of inhibitors was set to 100 %. * $P< 0.001$ relative to infections carried out in absence of inhibitors. (C) Immunofluorescence microscopy of pneumococcal adherence to MDCK-hpIgR cells in absence (Control) or presence of inhibitors.

Inhibitor	**relative invasion by *S. p.* serotype 35A [%]**			
	MDCK-hpIgR		**Calu-3**	
		p value relative to control		*p* value relative to control
control	100 ± 0	-	100 ± 0	-
125 nM Cytoskeleton D	22.84 ± 9.18	0.00013	17.86 ± 7.89	5.56×10^{-5}
50 nM Latrunculin B	25.28 ± 6.5	3.74×10^{-5}	21.6 ± 3.4	2.33×10^{-6}
100 nM Jasplakinolide	2.38 ± 0.28	4.24×10^{-11}	1.37 ± 0.9	4.70×10^{-9}
10 µM Nocodazole	36.69 ± 3.86	9.11×10^{-6}	34.22 ± 10.53	0.00041

Table 3 Relative invasion (in %) of MDCK-hpIgR and Calu-3 cells by *S. p.* 35A in the absence or presence of cytoskeleton inhibitors. *P* value less than 0.05 was taken as statistically significant.

The presence of cytoskeleton inhibitors significantly blocked pneumococcal invasion in both MDCK-hpIgR and Calu-3 cells, as determined by bacterial plating on blood agar plates after infection experiments (Figure 7A and 7B). In the presence of cytochalasin D, latrunculin B, jasplakinolide, and nocodazole the uptake of pneumococci by MDCK-hpIgR was significantly reduced relative to pneumococcal ingestion by host cells in untreated epithelial cells (Figure 7A). Similar results were obtained for Calu-3 cells (Figure 7B). However, no significant difference was observed for pneumococcal adherence to inhibitors treated cells when compared to the untreated host cells (Figure 7C). The results demonstrate that the host cell cytoskeleton dynamics plays an important role in uptake of pneumococci by host cells via the PspC-hpIgR mechanism.

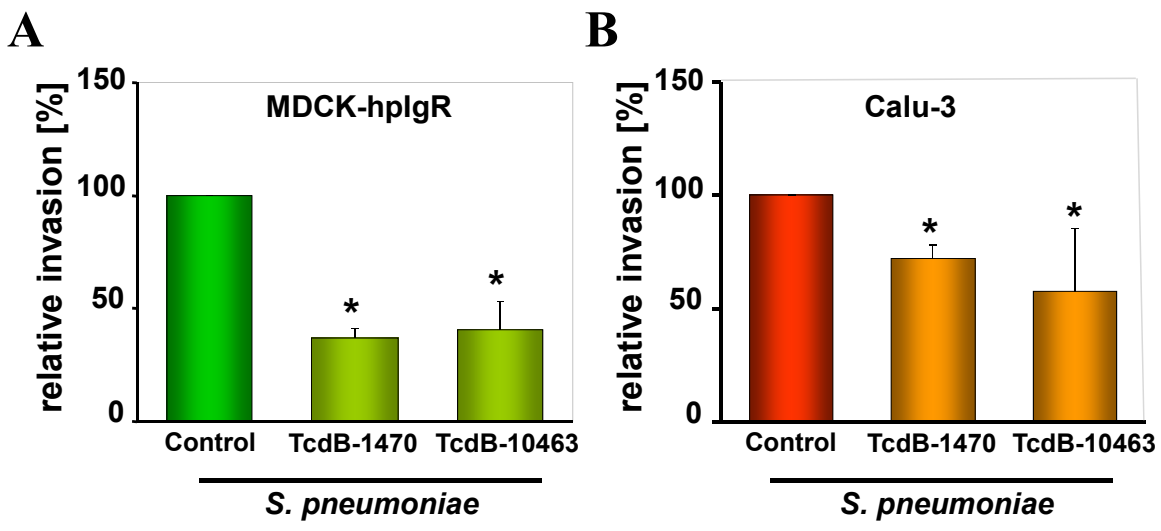
4.3. Identification of small GTPase Cdc42 as a key player in PspC-hpIgR mediated internalization of *S. pneumoniae* by epithelial cells

The Rho family of small GTPases regulate a variety of cellular processes including cytoskeleton dynamics and cell shape, cell adhesion, cell motility, membrane trafficking and gene expression (Bishop *et al.*, 2000; Kaibuchi *et al.*, 1999). Among these GTPases, Rho, Rac and Cdc42 are the most extensively characterized GTPases and are thought to be the most

important GTPases involved in actin cytoskeleton signalling pathways. Apart from these, Rho family of small GTPases have been suggested to be involved in the regulation of rabbit-pIgR-dimeric IgA transcytosis across mucosal epithelium (Leung *et al.*, 1999; Jou *et al.*, 2000; Rojas *et al.*, 2001).

4.3.1. Inhibition of Rho family of small GTPases and its effect on internalization process

In order to elucidate the impact of Rho family of small GTPases on PspC-hpIgR mediated pneumococcal internalization into host cell, *Clostridium difficile* toxin B, TcdB-10463 and a variant of toxin B from *C. difficile* strain 1470 (TcdB1470) were employed prior to bacterial infections. TcdB-10463 glucosylates Rho family of small GTPases Rho (A/B/C), Rac1, RhoG, TC10, and Cdc42 (Genth *et al.*, 2008). Glucosylation of Rho proteins causes their functional inactivation due to impaired coupling to effector and regulatory proteins (Aktories and Just, 2005; Just and Gerhard, 2004). Toxin TcdB1470 was employed to glucosylate Rac1, RhoG, TC10, and Cdc42 but not Rho (A/B/C) (Genth *et al.*, 2008). The MDCK-hpIgR and Calu-3 cells were preincubated for 1 h with 30 ng/ml of TcdB10463 and for 4 h with 100 ng/ml of TcdB1470 at 37°C under 5 % CO_2 followed by 1 h infections with *S. pneumoniae* serotype 35A for 1 h. The antibiotic protection assay was performed to ascertain the effect of toxins on the ingestion of pneumococci by host epithelial cells. In addition, immunofluorescence staining was performed to ensure that pretreatment of the eukaryotic cells did not affect adherence of pneumococci to these host cells.



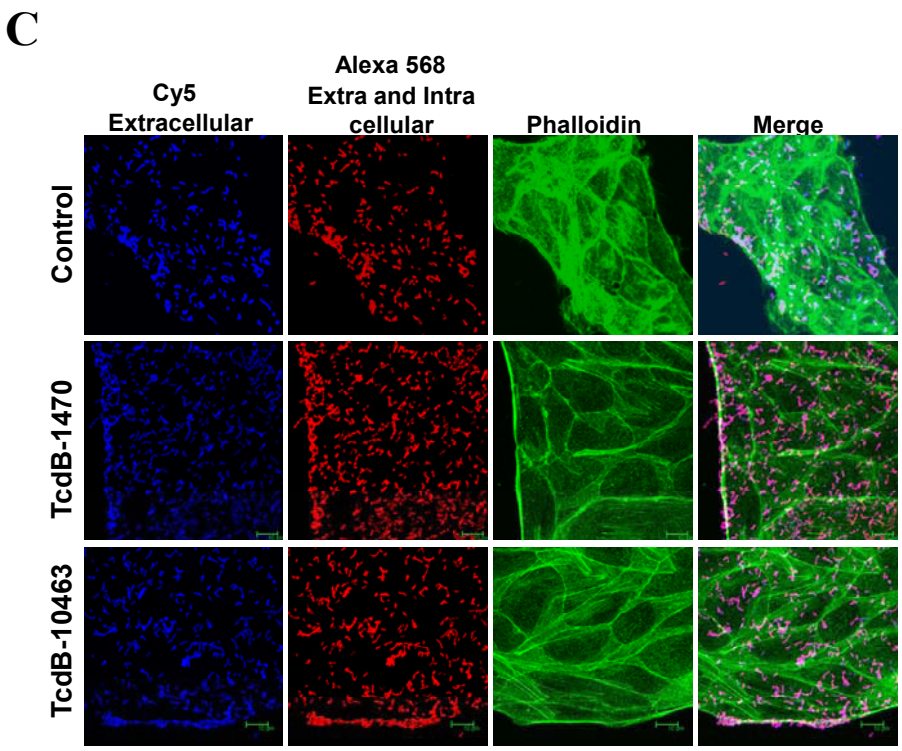


Figure 8 Impact of Rho family GTPases during PspC-hpIgR mediated invasion of MDCK-hpIgR and Calu-3 cells by *S. pneumonia e* serotype 35A (NCTC10319). Invasion and intracellular survival of the bacteria in MDCK-hpIgR (A) and Calu-3 (B) cells in the absence (control) or presence of *Clostridium difficile* toxin B, TcdB-10463 (30 ng/ml) or TcdB-1470 (100 ng/ml) monitored by the antibiotic protection assay. Invasion of *S. pneumoniae* in the absence of toxin was set to 100 %. * $P< 0.002$ relative to infections carried out in absence of toxin. (C) Immunofluorescence microscopy of pneumococcal adherence to MDCK-hpIgR cells in absence (control) or presence of toxins.

Inhibitor	**relative invasion by *S. p.* serotype 35A [%]**			
	MDCK-hpIgR		**Calu-3**	
		p value relative to control		*p* value relative to control
control	100 ± 0	-	100 ± 0	-
30 ng/ml TcdB1470	37.23 ± 3.58	7.04×10^{-6}	72.03 ± 5.74	0.001
100 ng/ml TcdB10463	40.69 ± 12.67	8.44×10^{-5}	57.49 ± 27.57	0.0087

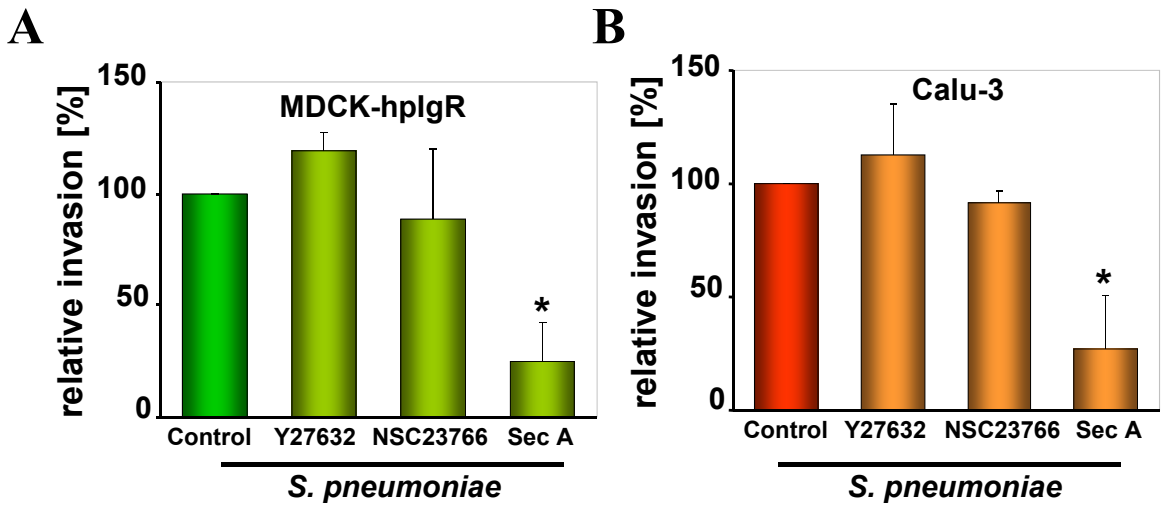
Table 4 Pneumococcal invasion of MDCK-hpIgR and Calu-3 cells in the absence or presence of *Clostridium difficile* toxin B. The results shows the percentage invasion compared to infection assay performed in absence of toxins. *P* value less than 0.05 was taken as statistically significant.

The data shows a significant reduction in the number of internalized pneumococci in both MDCK-hpIgR and Calu-3 cells upon pretreatment with TcdB10463 (Figure 8A and 8B).

Interestingly, TcdB1470 pretreatment of host cells also significantly reduced the number of internalized pneumococci (Figure 8A and 8B). These results suggest that Rac1 and Cdc42 but not RhoA are involved in the PspC-hpIgR mediated internalization of

pneumococci by mucosal epithelial cells. In addition, immunofluorescence microscopy indicated that pneumococcal adherence to pIgR expressing host cells was not affected in the presence of these toxins (Figure 8C).

To corroborate these results, infection studies were performed in presence of Y27632, a specific Rho-associated protein kinase inhibitor, NSC23766, a specific Rac1 inhibitor, or secramine A, which is a potent inhibitor of Cdc42 activation. NSC23766 is a cell-permeable pyrimidine compound that specifically and reversibly inhibits Rac1 GDP/GTP exchange activity by interfering Rac1 interaction with the Rac-specific GEF (guanine nucleotide exchange factor). Secramine A stabilizes the association of Cdc42 with RhoGDI1 (Rho GDP dissociation inhibitor 1), thereby decreasing the availability of Cdc42 for activation and downstream signalling (Pelish *et al.*, 2006). The eukaryotic host cells were preincubated with 50 µM Y27632 or 50 µM of NSC23766 for 30 min at 37°C under 5 % CO_2, whereas 10 µM secramine A was used 10 to 15 min prior to bacterial infections. The cells were infected for 1 h with *S. pneumoniae* serotype 35A and the invasion of pneumococci was determined by executing the intracellular survival assay. As a control for bacterial adherence immunofluorescence staining was performed.



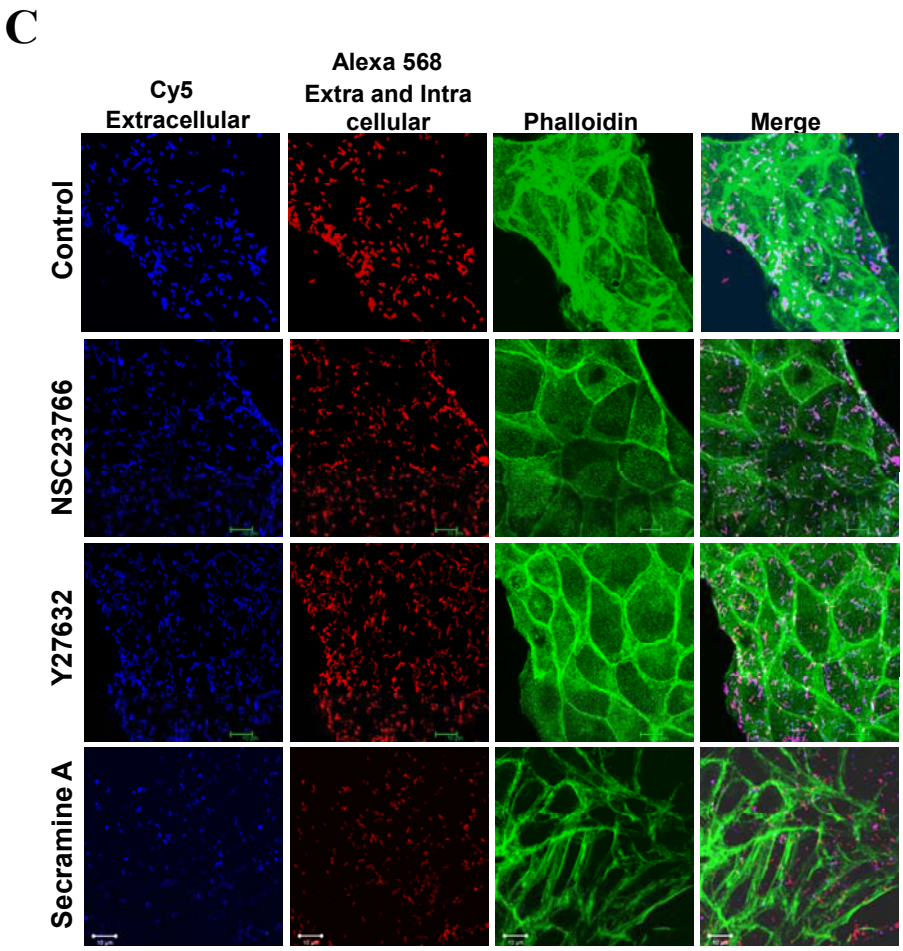


Figure 9 Small GTPases Cdc42 is key player during PspC-hpIgR mediated invasion of MDCK-hpIgR and Calu-3 cells by *S. p neumoniae* serotype 35A. Pneumococcal invasion in MDCK-hpIgR (A) and Calu-3 (B) cells was determined in the absence (control) or presence of specific individual inhibitors of Rho family GTPases Y27632 (50 µM), NSC23766 (50 µM) or secramine A (10 µM) by the antibiotic protection assay. Invasion of *S. p neumonia*e in the absence of toxin was set to 100 %. * $P< 0.05$ relative to infections carried out in absence of specific inhibitor. (C) Pneumococcal adherence to MDCK-hpIgR cells in absence (control) or presence of specific inhibitors.

Inhibitor	**relative invasion by *S. p.* serotype 35A [%]**			
	MDCK-hpIgR		**Calu-3**	
		p value relative to control		*p* value relative to control
control	100 ± 0	-	100 ± 0	-
50 µM Y27632	119.14 ± 8.48	0.004	112.80 ± 22.71	0.38
50 µM NSC23766	88.27 ± 32.03	0.56	91.67 ± 5.22	0.051
10 µM Secramine A	24.67 ± 17.17	0.0016	26.89 ± 23.89	0.006

Table 5 Percentage invasion of MDCK-hpIgR and Calu-3 cells by *S. p.* 35A in the presence of specific individual inhibitors of Rho family GTPases. *P* value less than 0.05 was taken as statistically significant.

Treatment of MDCK-hpIgR and Calu-3 cells with Y27632 and NSC23766 did not block pneumococcal internalization (Figure 9A and 9B). In contrast, pretreatment by secramine A significantly reduced invasion of pneumococci (Figure 9A and 9B). Secramine A reduced pneumococcal uptake by approximately 70 % for MDCK-hpIgR and Calu-3 cells. However, no significant change in the adherence of pneumococci between treated and untreated host cells were observed (Figure 9C). The result demonstrates the importance to Cdc42 for pneumococcal internalization into host epithelial cells via the PspC-hpIgR mechanism.

4.3.2. Functionally active Cdc42 is essential for pneumococcal internalization

In order to confirm the role of Cdc42 in PspC-hpIgR mediated pneumococcal internalization into host epithelial cells, genetic approach to interfere with small GTPase functions was employed. The pIgR expressing MDCK-hpIgR cells were transiently transfected with dominant-negative (dn) alleles of Rac1 (Rac1-T17N), Cdc42 (Cdc42-T17N) or Rho (Rho-T19N). The ingestion of pneumococci by transiently transfected MDCK-hpIgR was evaluated by infecting the host cells for 1 h with *S. pneumoniae* serotype 35A.

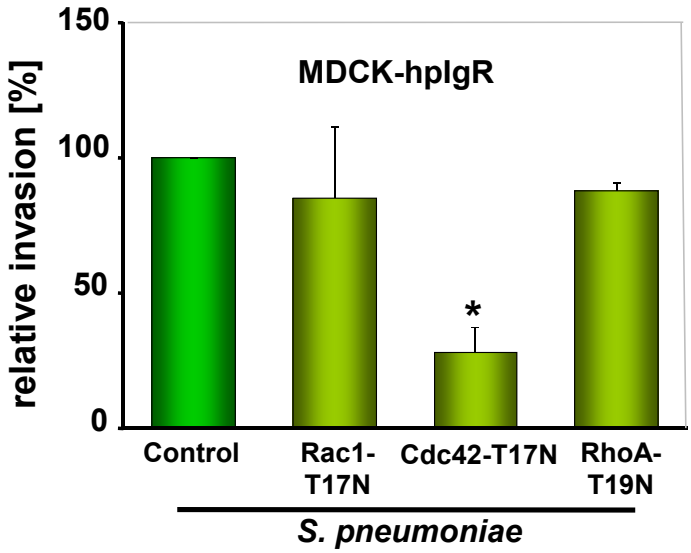


Figure 10 Activity of small GTPases Cdc42 is essential for PspC-hpIgR mediated invasion of MDCK-hpIgR cells by *S. pneumo niae* serotype 35A. Pneumococcal invasion of transiently transfected MDCK-hpIgR cells, with dominant-negative (dn) alleles of Rac1 (Rac1-T17N), Cdc42 (Cdc42-T17N) or Rho (Rho-T19N) was determined by the antibiotic protection assay. Invasion by *S. pneum oniae* in non-transfected host cells (control) was set to 100 %. * $P < 0.002$ relative to infections carried out in non-transfected cells.

Transfection	relative invasion by *S. p.* serotype 35A [%]	
	MDCK-hpIgR	
		p value relative to control
control	100 ± 0	-
dn-Rac1 (Rac1-T17N)	84.96 ± 26.38	0.38
dn-Cdc42 (Cdc42-T17N)	27.86 ± 9.56	0.00019
dn-Rho (Rho-T19N)	87.74 ± 2.99	0.002

Table 6 Relative invasion (in %) of MDCK-hpIgR cells transiently transfected with dominant-negative (dn) alleles of Rac1 (Rac1-T17N), Cdc42 (Cdc42-T17N) or Rho (Rho-T19N) by *S. p.* 35A. *P* value less than 0.05 was taken as statistically significant.

The intracellular survival assay demonstrated that over-expression of dn-Cdc42 (Cdc42-T17N) significantly reduced pneumococcal uptake by pIgR expressing host epithelial cells (Figure 10). In contrast, over-expression of dn-Rac1 (Rac1-T17N) or dn-Rho (Rho-T19N) did not influence pneumococcal internalization (Figure 10). The data confirmed that Cdc42 activity is essential for pneumococcal uptake by host epithelial cells via the PspC-hpIgR mechanism.

4.3.3. Cdc42 and not RhoA and Rac1 are activated upon pneumococcal ingestion by pIgR-expressing epithelial cells

The Rho GTPases cycle between an active GTP-bound state and an inactive GDP-bound state. To analyze the activation of Rho family of small GTPase during pneumococcal infection of pIgR expressing host epithelial cells, pull-down assays were performed. The GTP loading onto small GTPase was determined by specific binding of the active GTPase, Cdc42 and/or Rac1, to the p21 binding domain of PAK1 fused to glutathione S-transferase (GST-PBD) and active RhoA to the Rho binding domain of Rhotekin fused to glutathione S-transferase (GST-RBD) (Bernard *et al.*, 1999 and 2002). The kinetics of GTP-loaded Rac1, Cdc42 or RhoA was assessed for the indicated time points by precipitating the desired GTPase complex using the specific binding domains conjugated to glutathione sepharose beads in a pull-down assay. The precipitates lysates prepared from uninfected host cells were used as control. Activated GTPases were detected by western blot analysis. As a control total amount of Rac1, Cdc42 and Rho A were detected in lysates of sample aliquots from indicated time points.

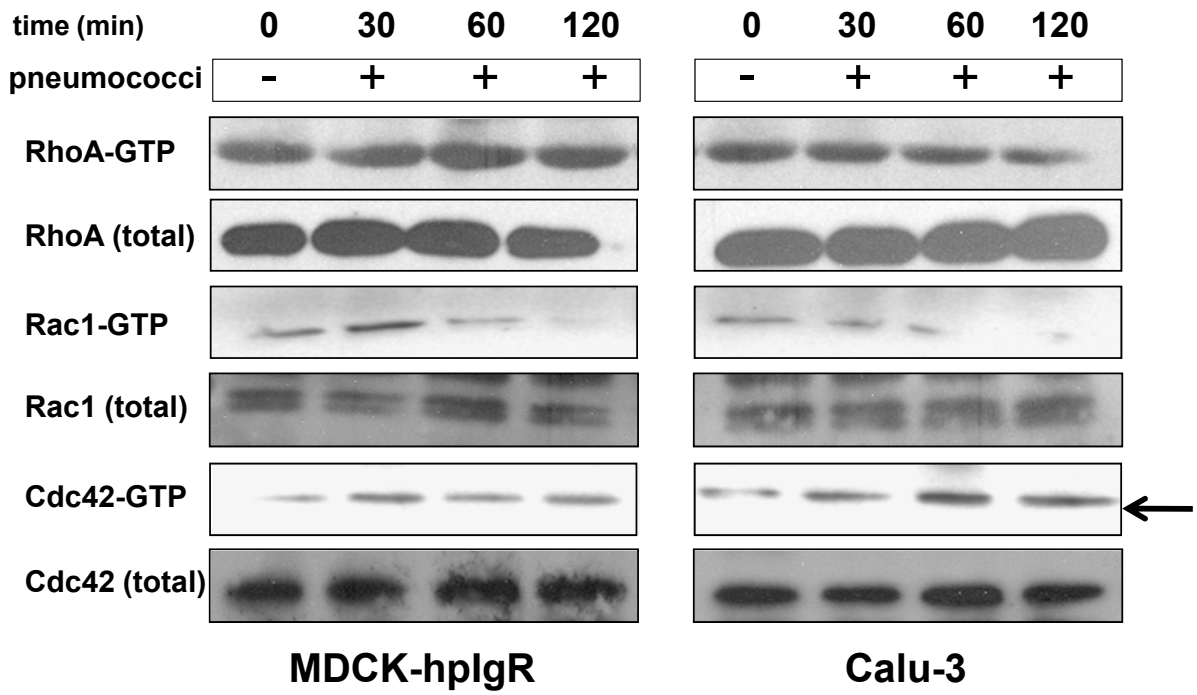


Figure 11 Activation of Rho family GTPases. Host cell lysates of MDCK-hpIgR and Calu-3 cells prepared after infection with *S. pneumoniae* serotype 35A for indicated time points were employed in pull-down of small GTPases (upper panel). The p21 binding domain of PAK1 fused to glutathione S-transferase (GST-PBD) for Rac1 (A) or Cdc42 (B), or the RhoA binding domain of Rhotekin fused to glutathione S-transferase (GST-RBD) for RhoA (C) were used. Precipitates were separated by 14 % SDS-PAGE and analyzed using GTPase specific antibodies. The pull-downs from lysate that was prepared from uninfected host cells were used as controls (0 min). Total amounts of Rac1, Cdc42 or RhoA for each sample were analyzed using aliquots of the lysates from indicated time points (lower panel).

The pull-down assays revealed a time dependent increase in Cdc42 activation following pneumococcal infections of pIgR expressing MDCK-hpIgR and Calu-3 cells (Figure 11). In contrast, no activation was detected for Rac1 and RhoA (Figure 11). A gradual decrease in Rac1 activation was observed that reduced to undetectable levels between 60 and 120 min post-infection. Taken together the results confirm the critical role of Cdc42 in PspC-hpIgR mediated pneumococcal ingestion by host epithelial cells.

4.3.4. PspC-hpIgR mediated pneumococcal infections of host epithelial cells induces Cdc42 dependent microspike like structure

Rho GTPases regulates the assembly and organization of the actin cytoskeleton. Here it was shown that Cdc42 activity is indispensable for PspC-hpIgR mediated pneumococcal uptake by epithelial cells. Moreover Cdc42 is known to regulate the microspikes (filopodium) formation in cells (Kozma *et al.*, 1995). Therefore, formation of microspike like structures in

pIgR expressing cells following pneumococcal infections was followed. MDCK-hpIgR and Calu-3 cells were infected for 3 h with *S. pneumoniae* serotype 35A and the induction of microspikes like structures was illustrated by immunofluorescence staining.

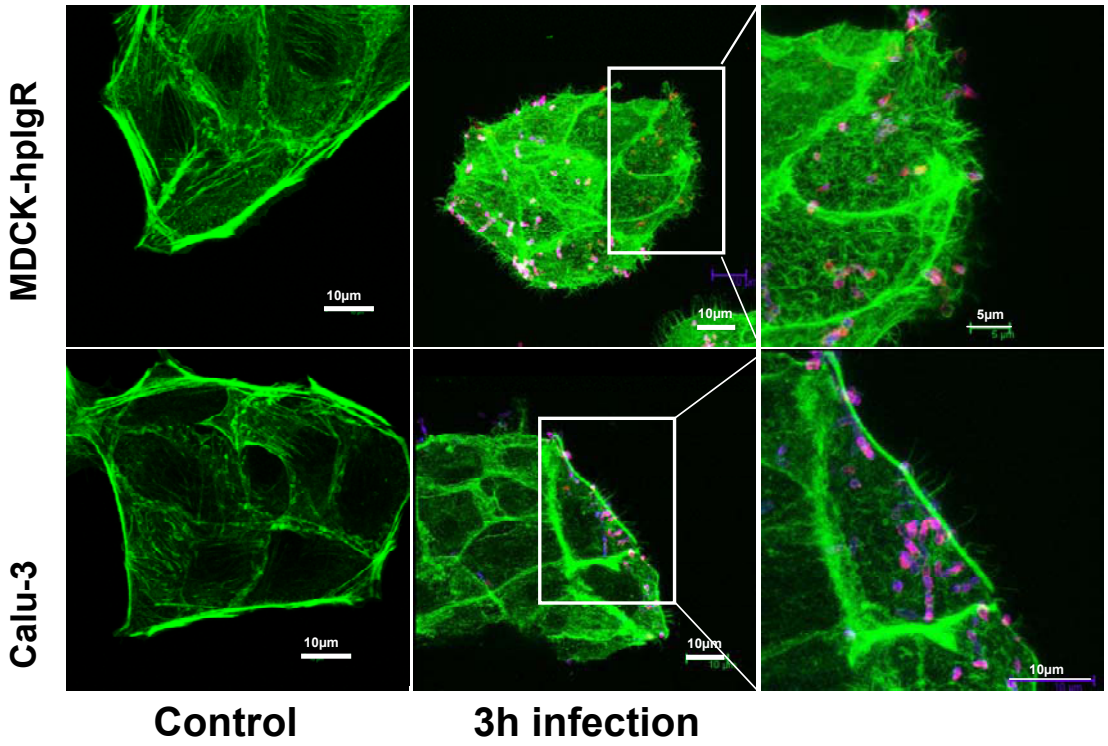


Figure 12 Microspike-like structures formation by MDCK-hpIgR and Calu-3 cells infected with *S. pneumoniae* serotype 35A (NCTC10319) after 3 h. After 3 h of infection, the host cell attached and intracellular pneumococci were stained with Cy5 (blue) and Alexa-568 (red) respectively, whereas the actin cytoskeleton was stained green using phalloidin (Alexa-488). Bar equal 10 µm.

The confocal laser scanning microscopic (CLSM) images taken during the process of pneumococcal invasion of pIgR expressing MDCK-hpIgR or Calu-3 cells demonstrated the induction of microspike-like structures at the site of bacterial attachment.

To investigate the role of Cdc42 during induction of these microspike-like structures, infection studies were performed in presence of the specific Cdc42 inhibitor secramine A. The eukaryotic host cells were preincubated with 10 µM secramine A prior to bacterial infections. The host cells were infected for 3 h with *S. pneumoniae* serotype 35A and the microspike-like formation was analysed.

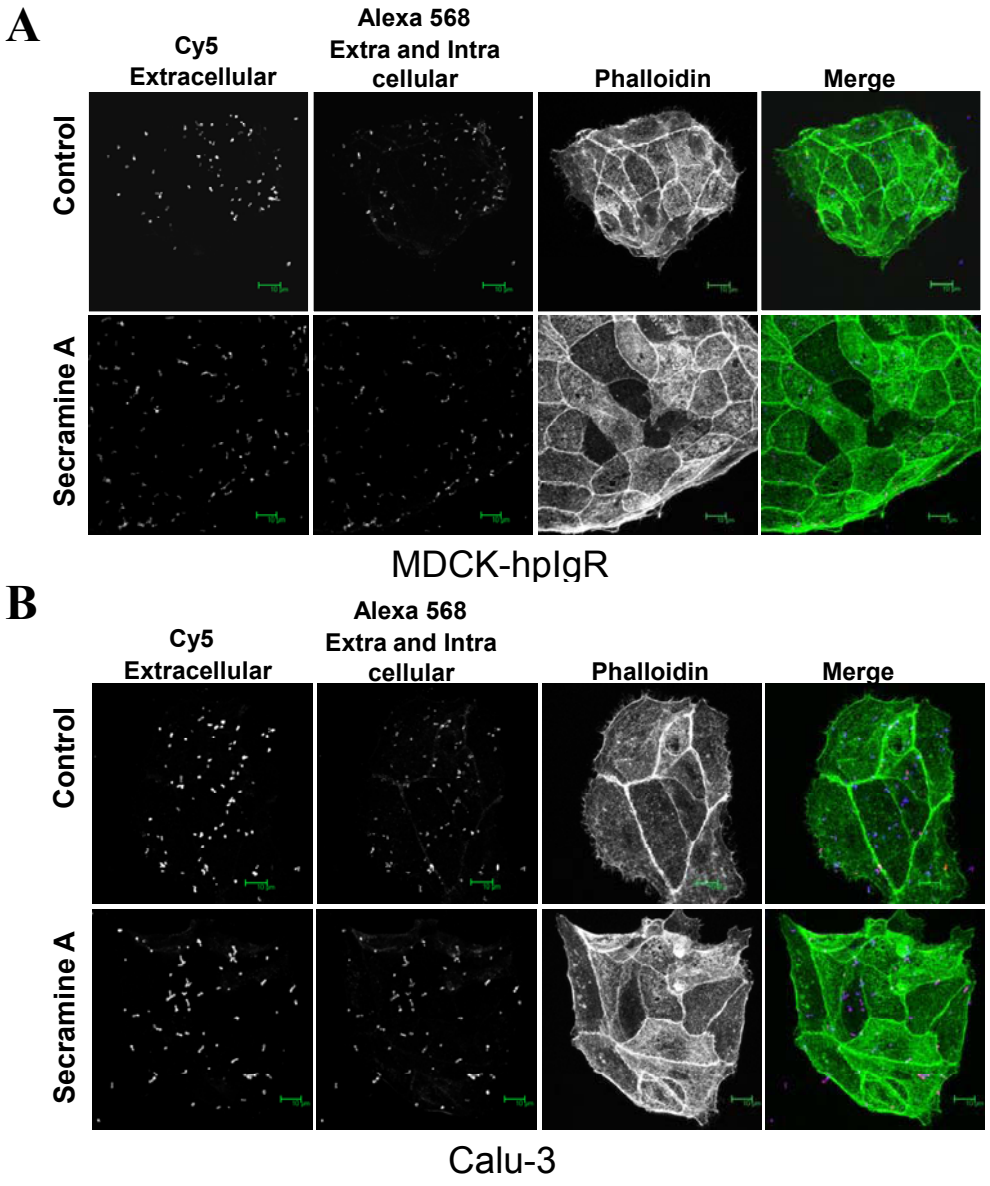


Figure 13 Cdc42 mediated formation of microspike-like structures. The induction of microspike-like structures after pneumococcal infection of MDCK-hpIgR (A) and Calu-3 (B) cell was analyzed in the absence (Control) or presence of secramine A (10 µM) by CLSM. The host cell attached and intracellular pneumococci were stained with Cy5 (blue) and Alexa-568 (red) respectively, whereas the actin cytoskeleton was stained green using phalloidin (Alexa-488). Bar equal 10 µm.

Immunofluoresence microscopy demonstrated the inhibition of microspike-like structure formation when Cdc42 activity was inhibited by secramine A (Figure 13A and 13B). In conclusion, the pneumococcal infection of host cell via the PspC-hpIgR mechanism induces Cdc42 dependent microspike-like structure formation.

4.4. PspC-hpIgR mediated pneumococcal ingestion by pIgR expressing epithelial cells relies on PI3-kinase and Akt

The role of phosphoinositide 3-kinase (PI3-kinase) and phosphoinositide metabolism is being increasingly acknowledged in bacterial pathogenesis. A high number of pathogens were identified that require PI3-kinase activity during bacterial host cell invasion, such as group B streptococci (Burnham *et al.* , 2007), group A streptococci (Purushothaman *et al.* , 2003), *Pseudomonas aeruginosa* (Kierbel *et al.* , 2005), *Helicobacter pylori* (Kwok *et al.* , 2002), *Chlamydia pneumoniae* (Coombes & Mahony 2002), *Escherichia coli* K1 (Reddy *et al.*, 2000) and *Listeria monocyto genes* (Ireton *et al.* , 1999). The role of PI3-kinase was highlighted in cell survival pathways, gene regulations, cell metabolism, and in host cell cytoskeleton rearrangements.

4.4.1. PI3-kinase is important for pneumococcal uptake by host epithelial cells

The importance of host cytoskeleton dynamics for the pneumococcal uptake was demonstrated by using cytoskeleton inhibitors. Since the PI3-kinase pathway is implicated in host cell cytoskeleton rearrangements, the role of PI3-kinase signalling pathway in PspC-hpIgR mediated pneumococcal ingestion by pIgR expressing host epithelial cells was explored. The invasion of pneumococci into MDCK-hpIgR and Calu-3 cells was determined in the presence of wortmannin, a fungal metabolite that inhibits PI3-kinase, mitogen-activated protein kinase and myosin light chain kinase (Davies *et a l.*, 2000) or LY294002, a specific inhibitor of PI3-kinase (Vlahos *et al.* , 1994). The eukaryotic host cells were pretreated with 50 nM wortmannin or 50 µM LY294002 for 30 min at 37°C under 5 % CO_2 prior to bacterial infections. The antibiotic protection assay was performed using *S. pneumoniae* serotype 35A to determine the role of PI3-kinase on pneumococcal uptake by pIgR expressing cells. In addition, immunofluorescence staining was performed to ensure that pretreatment of the eukaryotic cells with inhibitors do not affect pneumococcal adherence to host cells.

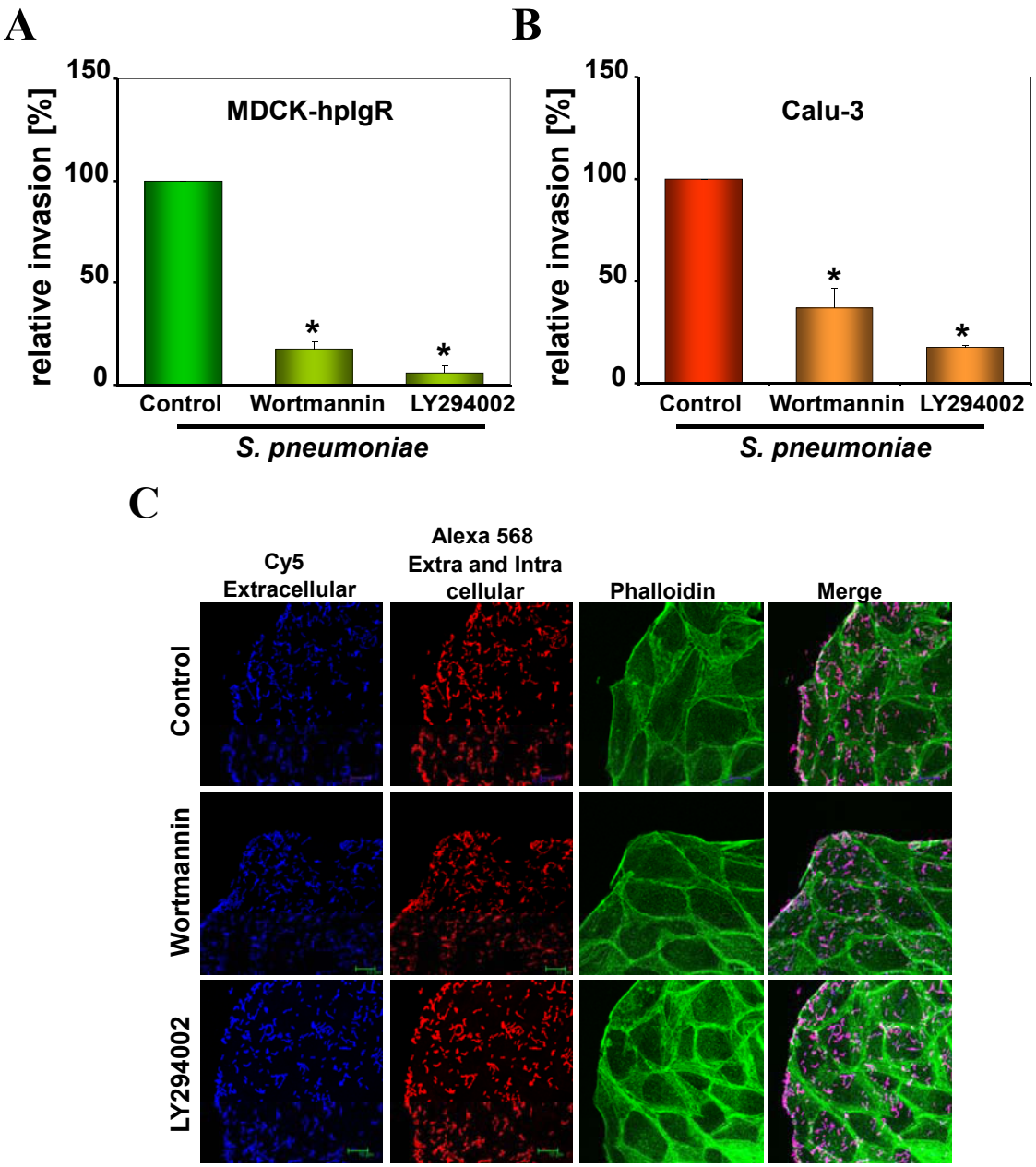


Figure 14 PI3-kinase pathway plays an essential role during PspC-hpIgR mediated invasion of MDCK-hpIgR and Calu-3 cells by *S. pneumo niae* serotype 35A. Invasion and intracellular survival of the bacteria in MDCK-hpIgR (A) and Calu-3 (B) cells was determined in the absence (control) or presence of PI3-kinase inhibitors wortmannin (50 nM) or LY294002 (50 µM) by the antibiotic protection assay. Pneumococcal invasion of host cells in the absence of inhibitor was set to 100 %. * $P< 0.001$ relative to infections carried out in the absence of inhibitors. (C) Immunofluorescence microscopy of pneumococcal adherence to MDCK-hpIgR cells in the absence (control) or presence of inhibitors.

Inhibitor	relative invasion by *S. p.* serotype 35A [%]			
	MDCK-hpIgR		Calu-3	
		p value relative to control		*p* value relative to control
control	100 ± 0	-	100 ± 0	-
50 nM Wortmannin	17.65 ± 3.79	9.96×10^{-9}	36.95 ± 9.51	1.14×10^{-5}
50 µM LY294002	5.73 ± 3.71	3.87×10^{-9}	17.36 ± 1.01	3.50×10^{-12}

Table 7 Pneumococcal uptake by MDCK-hpIgR and Calu-3 cells in the absence or presence of PI3-kinase inhibitor. The results are shown as percentage invasion of pneumococci in the presence of inhibitor relative to infection assays in the absence of inhibitor. *P* value less than 0.05 was taken as statistically significant.

The result showed a significant decrease in the PspC-hpIgR mediated pneumococcal internalization into pIgR expressing cells upon pretreatment of the host cells with wortmannin and LY294002 (Figure 14A and 14B). In presence of wortmannin pneumococcal uptake by MDCK-hpIgR and Calu-3 cells was reduced by approximately 80 % and 60 % respectively, compared to untreated host cell. Similar results were obtained with LY294002. However, no significant differences were observed for pneumococcal adherence to inhibitor treated host cells in comparison to untreated host cells (Figure 14C).

4.4.2. The PI3-kinase/Akt pathway is activated upon PspC-hpIgR mediated internalization of pneumococci into host cells

The Class I_A PI3-kinases are heterodimeric proteins consisting of a catalytic subunit (110 kDa, p110) associated with an 85 kDa noncatalytic regulatory subunit designated as p85 (Vanhaesebroeck *et al*., 2001).The PI3-Ks from class I_A are involved in the signalling of F-actin polymerization (Chodniewicz and Zhelev, 2003). They are commonly recruited to the membrane and activated via their p85 subunit (Fruman *et al.*, 1998). An important target of activated PI3-K is the serine/threonine kinase Akt (Protein Kinase B/PKB), which is phosphorylated at threonine-308 and serine-473 (Scheid and Woodgett, 2003; Milburn *et al.*, 2003; Song *et al.*, 2005). The activation of PI3-kinase and subsequent phosphorylation of Akt was implicated in the pathogenesis of various microorganisms (Burnham *et al.* , 2007; Purushothaman *et al.* 2003; Kierbel *et al.* , 2005; Kwok *et al.* , 2002; Coombes and Mahony, 200; Reddy *et al.* , 2000; Ireton *et al.* , 1999). Therefore, the involvement of PI3-kinases of class I_A, the phosphorylation status of p85 regulatory subunit and Akt, was assessed upon pneumococcal infections of pIgR expressing epithelial cells.

To determine the PI3-kinase p85 subunit and Akt phosphorylation levels, pIgR expressing MDCK-hpIgR and Calu-3 cells were infected for indicated time points with *S. pneumoniae*

serotype 35A and whole cell lysates were prepared. The lysates were analyzed via Western blotting using antibodies recognizing the activated form of p85 phosphorylated at tyrosine-508 (Tyr-508) or Akt that is phosphorylated at serine-473 (Ser-473). The lysates of uninfected host cells were taken as control. To assess the equal loading of protein, the blot was stripped and reprobed for total Akt.

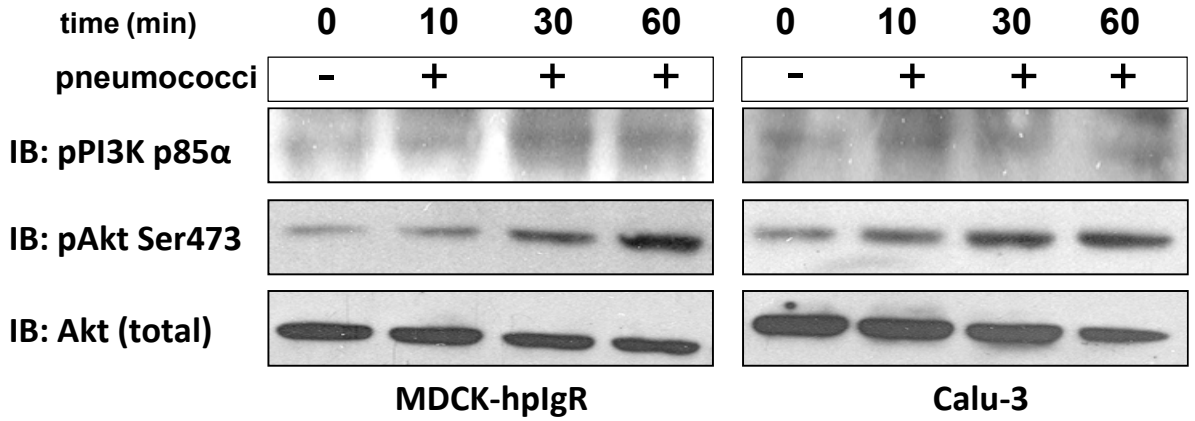


Figure 15 Activation of PI3-kinase and Akt following pneumococcal infections of pIgR expressing cells. Host cell lysates of MDCK-hpIgR and Calu-3 cells prepared after infection with *S. pneumoniae* serotype 35A for indicated time were separated by10 % SDS-PAGE. The activation of kinases were analyzed using antibodies against phosphorylated form of PI3K p85α (upper panel) or Akt (pAkt) (middle panel). The membrane was stripped and reprobed with total Akt antibody as a loading control (lower panel)

The immunoblot analysis indicated that the PI3-K p85α subunit and Akt were time-dependently phosphorylated after infecting pIgR expressing host cells with pneumococci (Figure 15).The result demonstrated the involvement of PI3-K/Akt pathway in PspC-hpIgR mediated pneumococcal infection of host cells. To explore the role of PI3-K as a direct upstream modulator of Akt phosphorylation, cells were pretreated with 50 μM LY294002 for 30 min prior to pneumococcal infection of host cells. Host cell lysates were subjected to Western blotting and activation of Akt was analyzed.

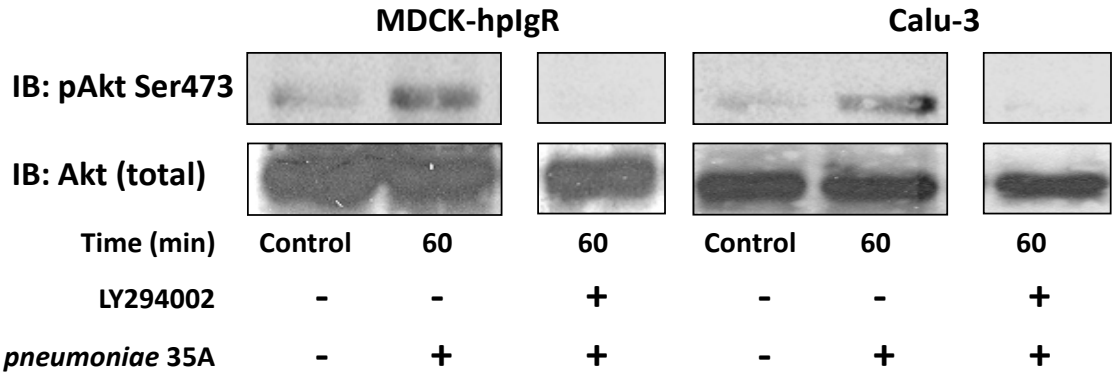


Figure 16 PI3-kinase mediates phosphorylation of Akt upon pneumococcal host cell infection. Phosphorylation of Akt (upper panel) was analysed in the absence (control) or presence of LY294002 (50 µM), in MDCK-hpIgR and Calu-3 cells after 60 min of infection with *S. pneumoniae* serotype 35A. The membrane was stripped and reprobed with total Akt antibody for loading control (lower panel)

The activation of Akt during pneumococcal infection of pIgR expressing host cells was completely abolished in presence of 50 µM LY294002 (Figure 16). The results demonstrate that induction of Akt phosphorylation, upon PspC-hpIgR mediated pneumococcal infection of host cells, occurs downstream of PI3-kinase activation.

4.4.3. Akt activation is essential for PspC-hpIgR mediated pneumococcal internalization into host epithelial cells

Protein kinase B/Akt is activated downstream of PI3-kinase. To determine whether Akt activation is required for pneumococcal internalization by host cell, Akt was inhibited using a specific Akt Inhibitor VIII (Isozyme-Selective, Akti-1/2). This inhibitor is a cell-permeable quinoxaline compound that potently and selectively inhibits Akt1/Akt2 activity, dependent on the pleckstrin homology (PH) domain. The host cells were pretreated with 10 µM Akt Inhibitor VIII for 30 min at 37°C under 5 % CO_2 prior to bacterial infections. Pneumococcal uptake by pIgR expressing epithelial cells was determined via the antibiotic protection assay. To ensure that pretreatment with Akti-1/2 did not affect pneumococcal adherence, immunofluorescence staining of attached bacteria was performed.

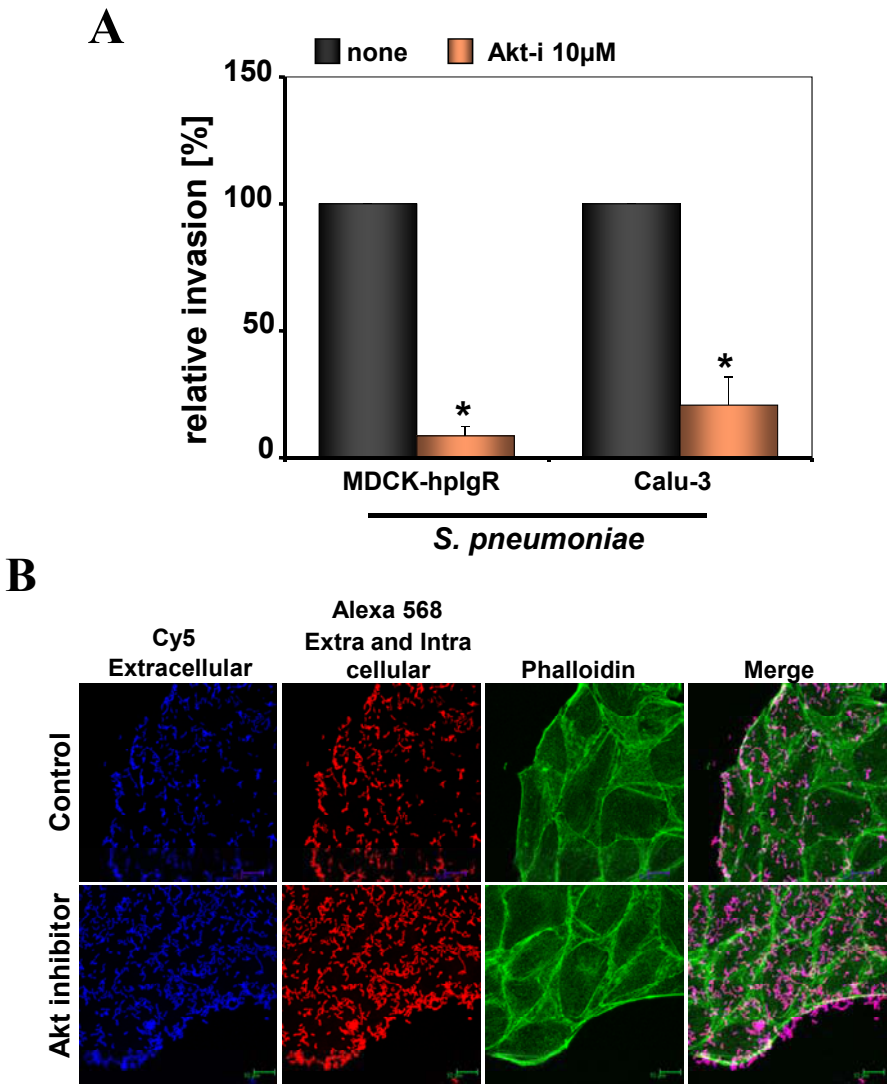


Figure 17 Activation of Akt is required for PspC-hpIgR mediated invasion of MDCK-hpIgR and Calu-3 cells by pneumococci. (A) Pneumococcal invasion of MDCK-hpIgR and Calu-3 cells was monitored in the absence (none) or presence of Akt Inhibitor VIII (Akt i, 10 µM) by the antibiotic protection assay. Invasion of *S. pneumoniae* serotype 35A in the absence of the inhibitor was set to 100 %. * $P < 0.001$ relative to infections carried out in the absence of inhibitor. (B) Pneumococcal adherence to MDCK-hpIgR cells in the absence (control) or presence of Akt inhibitor VIII.

Inhibitor	**relative invasion by *S. p.* serotype 35A [%]**			
	MDCK-hpIgR		**Calu-3**	
		p value relative to control		*p* value relative to control
control	100 ± 0	-	100 ± 0	-
10 nM Akt inhibitor VIII	8.49 ± 3.5	0.0007	20.83 ± 11.33	0.00027

Table 8 Relative invasion (in %) of MDCK-hpIgR and Calu-3 cells by *S. p.* 35A in the presence or absence of Akt inhibitor VIII. *P* value less than 0.05 was taken as statistically significant.

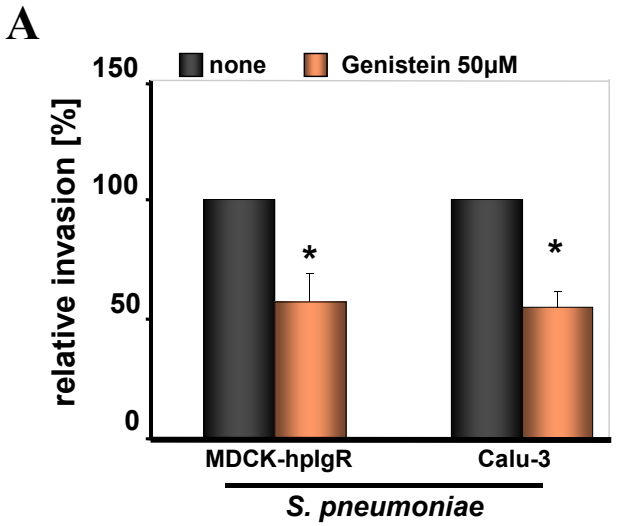
Inhibition of Akt by its specific inhibitor resulted in a significant reduction in the number of internalized pneumococci (Figure 17A). Similar to PI3-kinase inhibitors, pneumococcal adherence to host cells expressing pIgR was not affected in the presence of the inhibitor as determined by immunofluorescence staining (Figure 17B).

4.5. Function of protein tyrosine kinases during PspC-hpIgR mediated internalization of *S. pneumoniae* by epithelial cells

So far the results demonstrated the involvement of Cdc42 and PI3-kinase/Akt pathways in PspC-hpIgR mediated pneumococcal internalization. However the rabbit-pIgR-dimeric IgA transcytosis across mucosal epithelia is also regulated by other signalling molecules. Given the complexity of signal transduction pathways, other molecules involved have yet to be identified. Among the signalling molecules, protein tyrosine kinases (PTKs) form a very diverse family of proteins that modulates a variety of cellular events including cell proliferation, cytoskeleton rearrangements, adhesion, metabolism, and apoptosis.

4.5.1. Activation of protein tyrosine kinases is essential during pneumococcal internalization into host cells

To investigate the contribution of protein tyrosine kinases during PspC-hpIgR mediated pneumococcal infections, genistein, a broad spectrum inhibitor of protein-tyrosine kinase activity was employed. Both MDCK-hpIgR and Calu-3 cells were preincubated with 50 µM genistein for 30 min at 37°C under 5 % CO_2 and bacterial infections were performed in the presence of inhibitor. The antibiotic protection assay was performed to determine the number of intracellular plus recovered pneumococcal survivors. Pneumococcal adherence was monitored by immunofluorescence staining of attached pneumococci.



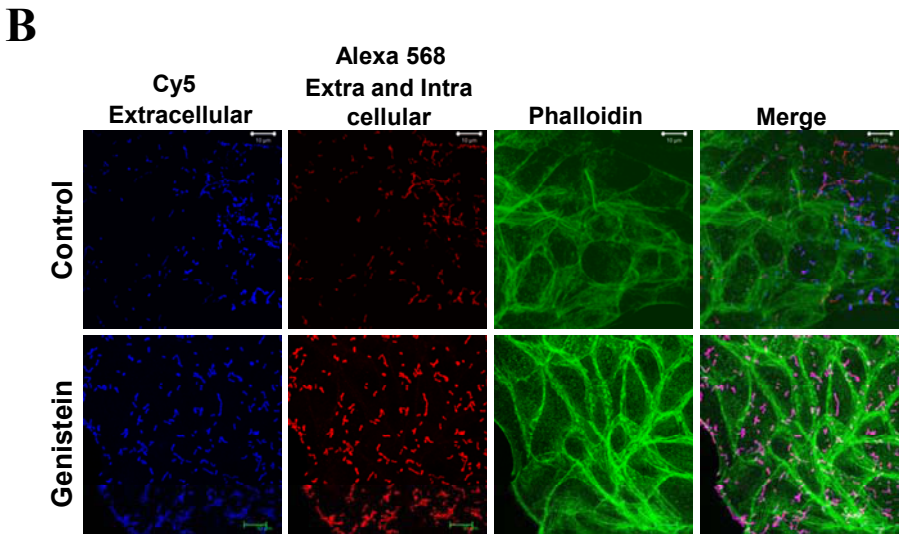


Figure 18 Impact of protein tyrosine kinases during PspC-hpIgR mediated invasion of MDCK-hpIgR and Calu-3 cells by pneumococci. (A) *S. pneumoniae* serotype 35A invasion of MDCK-hpIgR and Calu-3 cells was determined in the absence (none) or presence of protein tyrosine kinase inhibitor genistein (50 µM) by the antibiotic protection assay. Invasion of *S. p neumoniae* in the absence of inhibitor was set to 100 %. * $P< 0.001$ relative to infections carried out in the absence of inhibitor. (B) Immunofluorescence microscopy of pneumococcal adherence to MDCK-hpIgR cells in absence (control) or presence of genistein.

Inhibitor	**relative invasion by *S. p.* serotype 35A [%]**			
	MDCK-hpIgR		**Calu-3**	
		p value relative to control		*p* value relative to control
control	100 ± 0	-	100 ± 0	-
50 µM Genistein	57.35 ± 11.75	3.93×10^{-5}	55.03 ± 6.45	2.86×10^{-7}

Table 9 Percentage internalization of *S. p.* 35A into MDCK-hpIgR and Calu-3 cells in the absence or presence of protein tyrosine kinase inhibitor genistein. *P* value less than 0.05 was taken as statistically significant.

Treatment of MDCK-hpIgR and Calu-3 cells with 50 µM genistein significantly reduced the number of internalized bacteria (Figure 18A). The number of intracellular bacteria was reduced by approximately 45 % in both MDCK-hpIgR and Calu-3 cells, compared to untreated host cells. However, no significant alteration was observed for pneumococcal adherence (Figure 18B). The results suggested the implication of PTKs in PspC-hpIgR mediated pneumococcal uptake by host epithelial cells.

The host cell contains several protein-tyrosine kinases. In order to investigate the particular kinase or family of kinases involved in the pIgR-mediated pneumococcal infection, the *Src* family of protein-tyrosine kinase and bcr/abl kinase were blocked with the specific

inhibitors PP2 and AG957 respectively. The cells were preincubated with 5 µM PP2 or 10µM AG957 for 30 min at 37°C under 5 % CO_2, followed by 1 h infection with *S. pneumoniae* serotype 35A. To determine the effect of the inhibitors on the ingestion of pneumococci by host epithelial cells, the antibiotic protection assay was performed. Pneumococcal adherence to pIgR expressing host cells, in the absence or presence of inhibitors, was analysed by immunofluorescence staining.

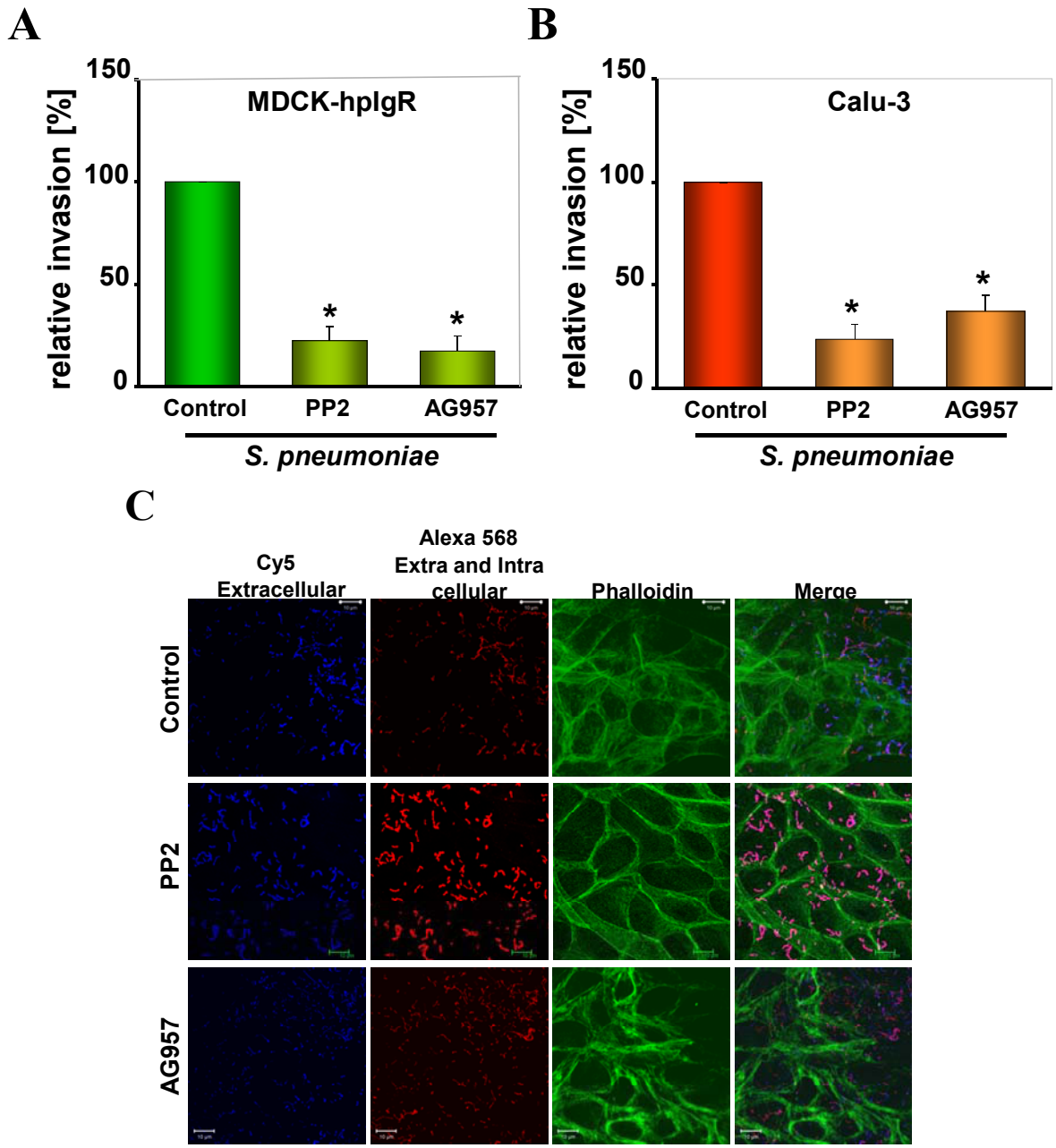


Figure 19 Involvement of *Src* family of protein-tyrosine kinase and bcr/abl kinase in PspC-hpIgR mediated invasion of MDCK-hpIgR and Calu-3 cells by pneumococci. Invasion and intracellular survival of *S. pneumoniae* serotype 35A in MDCK-hpIgR (A) and Calu-3 (B) cells was monitored in the absence (control) or presence of *Src* family of protein-tyrosine kinase inhibitor PP2 (5 µM) or bcr/abl kinase inhibitor AG957 (10 µM) by the

antibiotic protection assay. Pneumococcal invasion in the absence of inhibitor was set to 100 %. * $P<$ 0.001 relative to infections carried out in the absence of inhibitors. (C) Immunofluorescence microscopy of pneumococcal adherence to MDCK-hpIgR cells in the absence (control) or presence of kinase inhibitors.

Inhibitor	relative invasion by *S. p.* serotype 35A [%]			
	MDCK-hpIgR		Calu-3	
		p value relative to control		*p* value relative to control
control	100 ± 0	-	100 ± 0	-
5 µM PP2	22.47 ± 7.02	4.41×10^{-5}	23.83 ± 7.48	6.05×10^{-5}
10 µM AG957	17.38 ± 7.77	5.11×10^{-5}	37.21 ± 7.88	0.00016

Table 10 Pneumococcal ingestion by MDCK-hpIgR and Calu-3 cells in the presence or absence of *Src* family of protein-tyrosine kinase inhibitor PP2 or bcr/abl kinase inhibitor AG957. The results are shown as percentage invasion of pIgR expressing epithelial cells relative to pneumococcal uptake by untreated cells. *P* value less than 0.05 was taken as statistically significant.

Inhibition of Src protein-tyrosine kinases and bcr/abl kinase by PP2 and AG957, respectively, strongly impaired hpIgR-mediated pneumococcal uptake by host cells (Figure 19A and 19B). Pretreatment of MDCK-hpIgR and Calu-3 cells with 5 µM PP2 resulted in approximately 75 % reductions in number of intracellular bacteria compared to untreated host cells. Similar results were obtained for AG957. In addition, immunofluorescence microscopy indicated that pneumococcal adherence to host cells was not altered in the presence of these inhibitors (Figure 19C). Taken together these results demonstrated that *Src* family of kinases and bcr/abl kinases are both involved in the hpIgR-mediated pneumococcal invasion of host epithelial cells.

4.5.2. Functionally active Src kinase is important for pneumococcal ingestion by pIgR-expressing host epithelial cells

To confirm the role of Src protein-tyrosine kinase in pIgR-mediated internalization of pneumococci, the genetic approach to interfere with *Src* kinase function was exploited. The plasmids encoding the wild-type C-terminal Src kinase (Csk WT), which is a negative regulator of Src protein-tyrosine kinase, and a kinase-inactive form of Csk (Csk K222M) were used to transfect transiently host cells. The wild-type C-terminal Src kinase inhibits Src protein-tyrosine kinase activity and hence Src kinase dependent cellular events. Approximately 48 h after the transfection, transfected cells were infected for 1 h with *S. pneumoniae* wild-type strain 35A. Invasion of pneumococci was calculated by executing the intracellular survival assay.

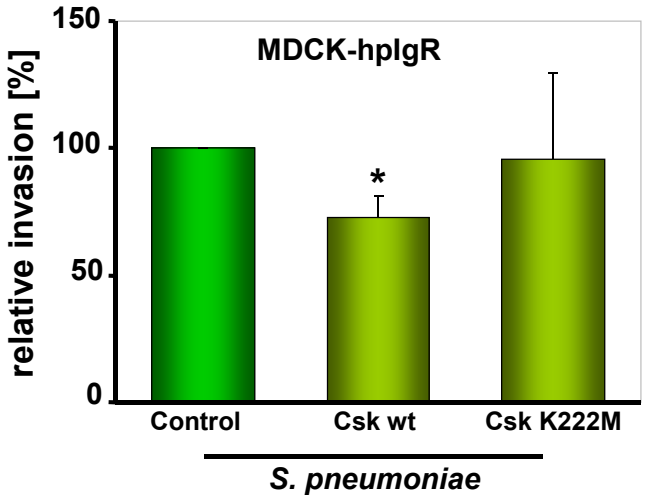


Figure 20 Interference with Src family kinsae function blocks PspC-hpIgR mediated invasion by pneumococci. MDCK-hpIgR cells were transfected with constructs encoding wild-type C-terminal Src kinase (Csk wt) or a kinase-inactive form of Csk (Csk K222M). Transfected cells were employed in gentamicin protection assay with *S. p neumoniae* serotype 35A. Pneumococcal invasion of non-transfected cells was set to 100 %. * $P < 0.05$ relative to infections carried out in non-transfected cells.

Transfection	**relative invasion by *S. p.* serotype 35A [%]**	
	MDCK-hpIgR	
		p value relative to control
control	100 ± 0	-
Csk wt	72.67 ± 8.5	0.0051
Csk K222M	95.67 ± 34	0.84

Table 11 Percentage ingestion of pneumococci by MDCK-hpIgR cells transfected with constructs encoding wild-type C-terminal Src kinase (Csk wt) or a kinase-inactive form of Csk (Csk K222M). *P* value less than 0.05 was taken as statistically significant.

The intracellular survival assay demonstrated that over-expression of Csk WT significantly reduced pneumococcal uptake via the PspC-hpIgR mechanism (Figure 20). In contrast, the kinase-inactive form of Csk had no influence on the pneumococcal internalization (Figure 20). These data support our previous inhibition data and showed that Src protein-tyrosine kinase activity is essential for hpIgR mediated pneumococcal invasion of host cells.

To corroborate the above results dominant-negative, kinase-inactive form of Src (Src K297M) was transiently over-expressed in MDCK-hpIgR cells. The antibiotic protection assays was performed to examine the effect Src kinase mutant on pneumococcal uptake by host cells.

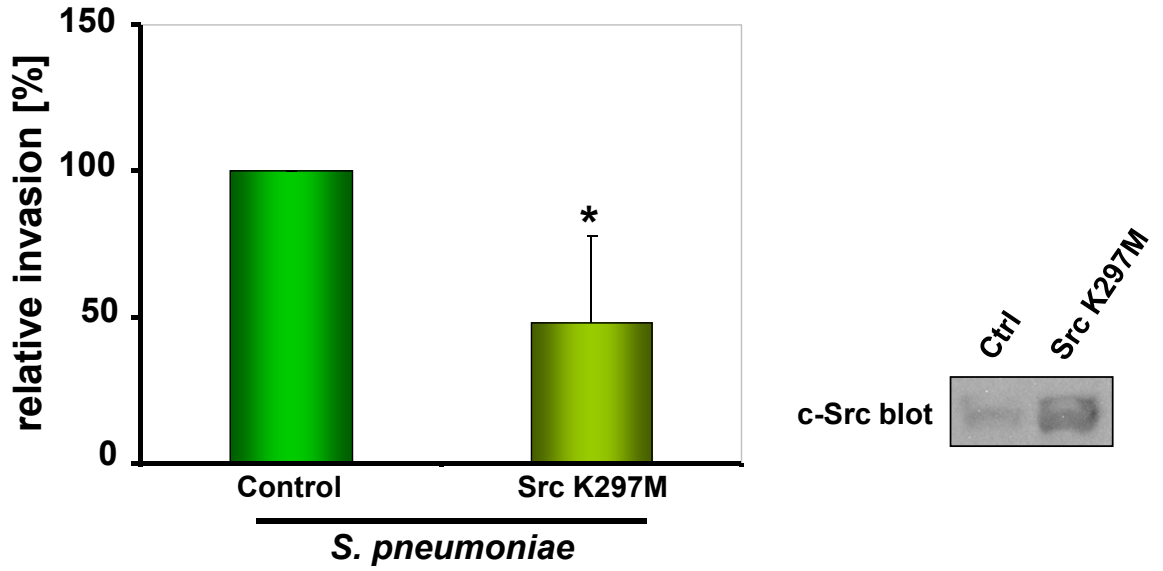


Figure 21 Src kinsae activity is essential for PspC-hpIgR mediated invasion of pneumococci. MDCK-hpIgR cells were transfected with constructs encoding a kinase-inactive form of c-Src (Src K297M). Transfected cells were employed in gentamicin protection assay with *S. pneumoniae* serotype 35A. Pneumococcal invasion of non-transfected cells was set to 100 %. * $P < 0.05$ relative to infections carried out in non-transfected cells.

Transfection	**relative invasion by *S. p.* serotype 35A [%]**	
	MDCK-hpIgR	
		p value relative to control
control	100 ± 0	-
Src K297M	48 ± 29.72	0.038

Table 12 Pneumococcal uptake by MDCK-hpIgR cells transfected with constructs encoding kinase-inactive form of c-Src (Src K297M) compared to invasion of nontransfected cells. *P* value less than 0.05 was taken as statistically significant.

The results revealed a significant reduction in the uptake of pneumococci, further confirming the critical role of Src kinase activity in the internalization of pneumococci via PspC-hpIgR mechanism. While the data, in figure 20 and 21, are statistically significant and the trend was in concurrence with the pharmacological inhibitor studies, the level of reduction in uptake was not comparable to that observed with inhibitors. This variation in the reduction was likely due to the fact that this being transient transfection assay and thus only a subset of cells would have taken up the desired plasmid and would have expressed them.

4.5.3. Role of Mitogen activated protein kinases in PspC-hpIgR mediated pneumococcal infection of host epithelial cells

Mitogen activated protein kinases (MAPK) family members are involved in host cell invasion by several pathogenic bacteria. To date, six distinct groups of MAPKs have been

characterized in mammals: extracellular regulated kinases 1 and 2 (ERK1 [p44 MAPK] and ERK2 [p42 MAPK]), c-Jun NH2 terminal kinases (JNK1/2/3), p38 (p38 α/β/γ/δ), ERK7/8, ERK3/4 and ERK5 (Krishna and Narang, 2008). The most extensively studied groups are ERK1/2, JNKs and p38 kinases. The MAPKs phosphorylate specific serines and threonines of other protein kinases, phospholipases, transcriptional factors and cytoskeletal proteins, thereby regulating various cellular processes (Krishna and Narang, 2008).

4.5.3.1. ERK and JNK MAPK pathways are activated during PspC-hpIgR mediated pneumococcal infection of host cells

To assess whether MAPKs are activated, MDCK-hpIgR and Calu-3 cells were infected with *S. pneumoniae* serotype 35A and phosphorylation of kinases was analysed by western blotting. At indicated time points MAPKs activation was assessed using antibodies that specifically detects the phosphorylated forms of ERK1/2, JNK1 / JNK2 and p38 MAPK, which is also the activated form of these enzymes. For loading control the blot was stripped and reprobed for total protein.

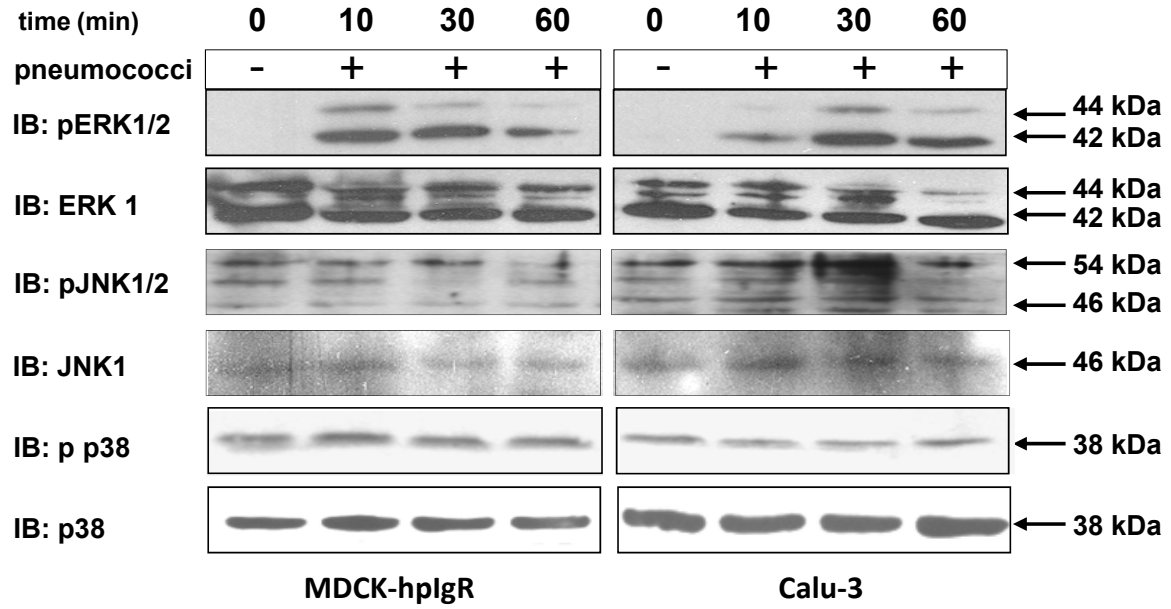


Figure 22 Activation of MAP kinases following pneumococcal infection of host epithelial cells. Cell lysates of infected MDCK-hpIgR and Calu-3 cells were separated by 10 % SDS-PAGE and analyzed using antibody against phosphorylated form of ERK1/2, JNK1 /JNK2 and p38 MAPK. The membrane was stripped and reprobed with total ERK1/2, JNK1 and p38 MAPK antibody for loading control.

A time-dependent increase in phosphorylation of ERK1 and ERK2 was observed in kinetic experiment after infecting pIgR expressing MDCK-hpIgR and Calu-3 cells with pneumococci (Figure 22). Moreover, immunoblot analysis showed phosphorylation of JNK isoforms p54

and p46; however activation was not as high as ERK1 and ERK2 activation. In contrast, the phosphorylation of p38 MAPK was not induced during pneumococcal infection of pIgR-expressing epithelial cells.

4.5.3.2. Transcription factor c-Jun is activated during uptake of pneumococci via PspC-hpIgR mechanism

Although immunoblot analyses indicated activation of ERK1 and ERK2, and JNK isoforms p54 and p46 following pneumococcal infections of pIgR expressing host epithelial cells, activation of JNK was not as high as for ERK1 and ERK2. One of the most important and extensively studied nuclear substrate of JNK is c-Jun, which when phosphorylated at Ser 63 and 73 results in enhancement of AP-1 transcriptional activity (Bogoyevitch and Kobe, 2006). To assess pneumococci-mediated JNK activation, c-Jun activation in pIgR expressing host epithelial cells was monitored in the kinetic infection experiments.

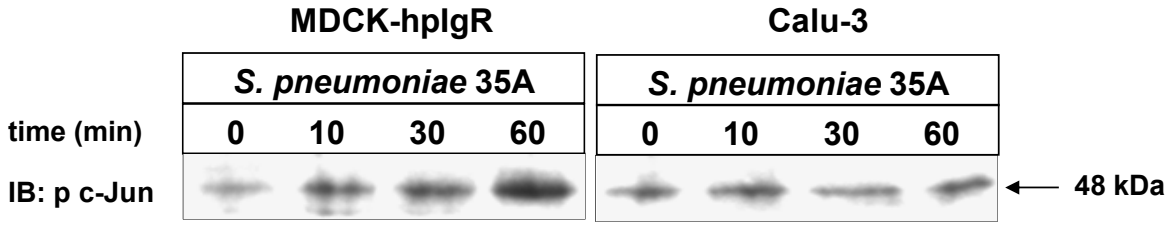


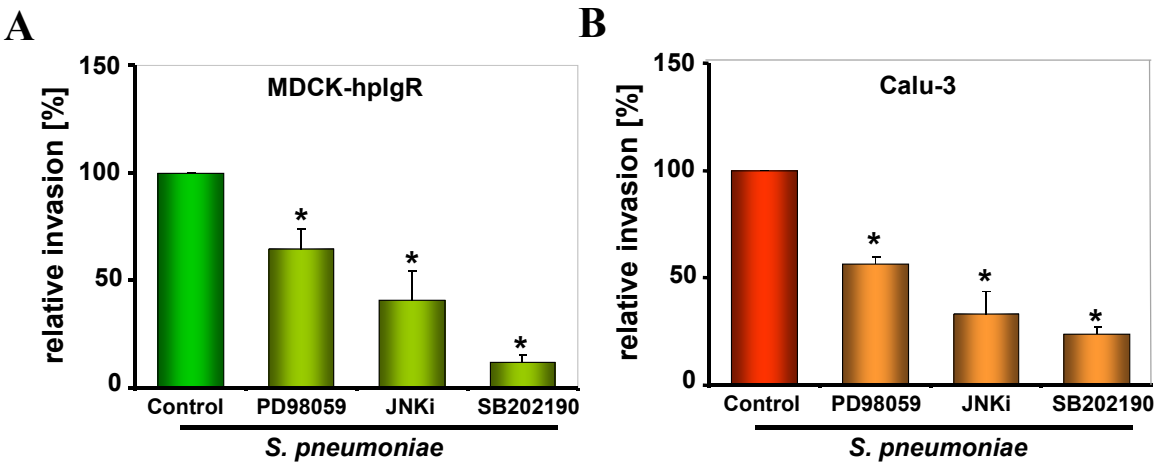
Figure 23 Activation of transcription factor c-Jun during pneumococcal infection. The lysates prepared at indicated time points post infection of MDCK-hpIgR and Calu-3 cells by *S. pneumoniae* serotype 35A were separated by 10 % SDS-PAGE and analyzed using antibody against phosphorylated form of c-Jun (Ser 63).

Immunoblot analyses demonstrated increase in c-Jun phosphorylation, in both MDCK-hpIgR and Calu-3 cells, in response to *S. pneumoniae* infection (Figure 23). Already 10 min post infection, c-Jun was phosphorylated and phosphorylation reached its maximum level by 60 min post infection in MDCK-hpIgR. Activation of c-Jun data indicates towards the role of JNK MAPK pathway in PspC-hpIgR mediated pneumococci ingestion by of host epithelial cells.

4.5.3.3. Mitogen Activated Protein Kinase activity is essential for hpIgR-mediated pneumococcal invasion of host cells

Immunoblot analyses indicated activation of ERK1/2 and JNK1 / JNK2 during pneumococcal infection of pIgR-expressing host cells. However, these data do not provide evidence regarding the importance of MAPKs for pneumococcal internalization into host

cells. In order to analyse the role of MAPKs, pneumococcal invasion of pIgR expressing cells was monitored in the presence of PD98059, a specific inhibitor of MAP kinase kinase (MEK) or JNK inhibitor II, a selective and reversible inhibitor of JNK MAKK. SB202190, a specific inhibitor of p38 MAPK pathway was also employed in pneumococcal invasion assays. Eukaryotic host cells were preincubated with 100 µM PD98059 for 1 h, with 5 µM JNK inhibitor II or 10 µM SB202190 for 30 min at 37°C under 5 % CO_2 prior to bacterial cell infections. Antibiotic protection assay was performed in order to determine the role of MAPKs in pneumococcal invasion of host cell. To negate the effect of inhibitors on the pneumococcal adherence to pIgR expressing cells, immunofluorescence staining of attached pneumococci was performed.



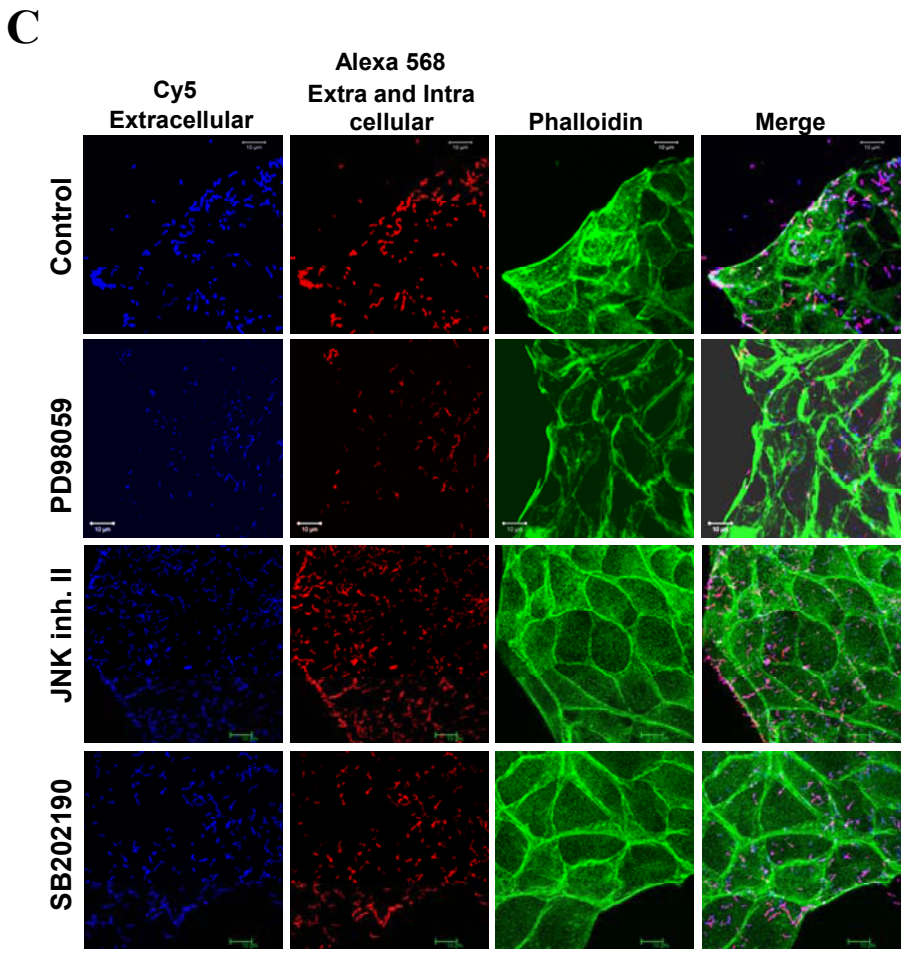


Figure 24 Mitogen Activated Protein Kinases (MAPKs) activity is essential for pneumococcal uptake by host cell. Invasion and intracellular survival in MDCK-hpIgR (A) and Calu-3 (B) cells, of *S. pneumoni ae* serotype 35A, was monitored in the absence (control) or presence of MAP kinase kinase (MEK) inhibitor (PD98059, 100 µM), c-Jun N-terminal kinase inhibitor (JNKi, 5 µM) or p38 MAP kinase inhibitor (SB202190, 10 µM) by the antibiotic protection assay. Pneumococcal invasion in the absence of inhibitor was set to 100 %. * $P<$ 0.005 relative to infections carried out in the absence of inhibitor. (C) Immunofluorescence microscopy of pneumococcal adherence to MDCK-hpIgR cells in absence (Control) or presence of MAPK inhibitors.

Inhibitor	**relative invasion by *S. p.* serotype 35A [%]**			
	MDCK-hpIgR		**Calu-3**	
		p value relative to control		*p* value relative to control
control	100 ± 0	-	100 ± 0	-
10 µM PD98059	64.63 ± 9.28	0.0027	56.45 ± 3.38	2.39×10^{-5}
5 µM JNK inhibitor II	40.95 ± 13.37	0.0001	32.95 ± 10.88	1.73×10^{-5}
10 µM SB202190	11.74 ± 3.66	3.89×10^{-6}	23.6 ± 3.14	1.9×10^{-6}

Table 13 Percentage internalization of *S. p.* 35A into MDCK-hpIgR and Calu-3 cells in the absence or presence of MAPK inhibitors, respectively. *P* value less than 0.05 was taken as statistically significant.

The antibiotic protection assay shows that PD98059 and JNK inhibitor II significantly decreased the hpIgR mediated pneumococcal ingestion by host epithelial cells (Figure 24A and 24B). Surprisingly blockade of p38 MAPK by a selective inhibitor, SB202190, reduced significantly pneumococcal invasion (Figure 24A and 24B), although immunoblots showed no activation of p38 MAPK. A plausible explanation for this effect could be non-specificity of pharmacological inhibitors (Bain *et al.*, 2007; Davies *et al.*, 2000).

4.6. Cross-talk between signalling pathways induced during pIgR mediated pneumococcal infections of host cells

4.6.1. Src kinase facilitates ERK activation during PspC-hpIgR mediated pneumococcal infections

Src protein-tyrosine kinases and MAPKs are essential for hpIgR-mediated pneumococcal uptake by host epithelial cells. Various studies have suggested that ERK1/2 is a downstream target of Src kinases (Schlaepfer *et al.*, 1999; 1997). Therefore, the relationship between Src kinase and ERK1/2 during the pneumococcal infections of pIgR expressing epithelial cells was investigated. After preincubation of eukaryotic cells with 5 µM PP2 for 30 min the host cells were infected for 1 h with pneumococci and activation of ERK1/2 was monitored by Western blotting. The specificity of PD98059 was assessed by pretreating the cells and determining the phosphorylation status of ERK1 and ERK2 following pneumococcal infection.

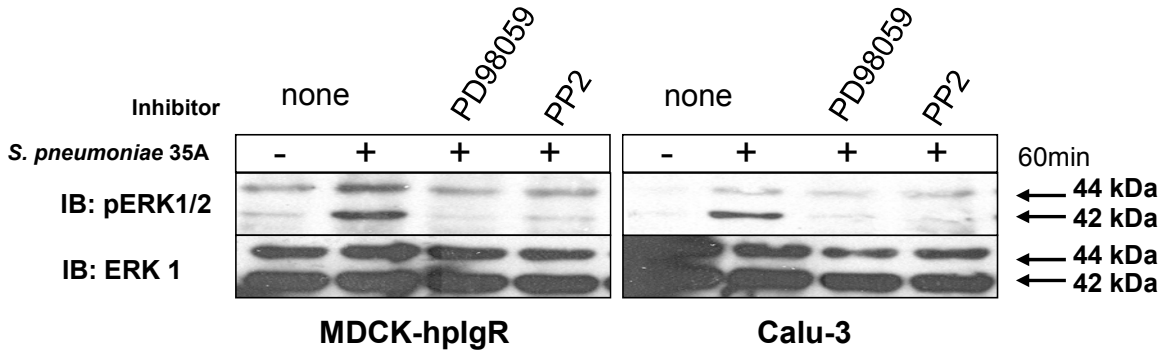


Figure 25 Src kinase facilitates ERK 1/2 activation upon pneumococcal infection. Phosphorylation of ERK (upper panel) was analyzed in the absence (none) or presence of PD98059 (100 µM) and PP2 (5 µM), respectively, after 60 min of infection with *S. pneumoniae* serotype 35A. As a loading control total ERK was detected (lower panel).

In presence of PP2, phosphorylation of ERK1/2 was comparable to phosphorylation levels of uninfected host cells (Figure 25). Similarly, treatment with PD98059 also inhibited ERK1/2

phosphorylation, thereby demonstrating the specificity of PD98059 (Figure 25). However, pretreatment of cells with either PP2 or PD98059 had no effect on the total ERK levels of the cells.

Moreover, activation of ERK1/2 was also investigated in cells over-expressing the kinase-inactive mutant of Src (Src K297M).

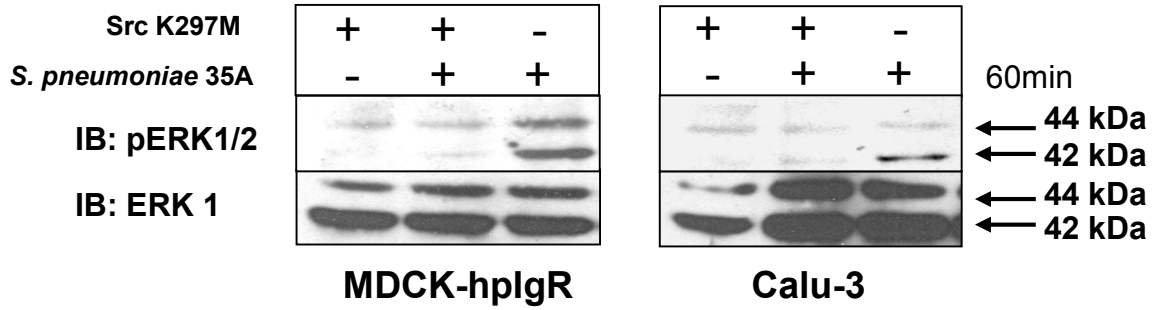


Figure 26 Src kinase activity is required for ERK 1/2 activation in pneumococcal infected host cells. MDCK-hpIgR and Calu-3 cells were transiently transfected with plasmid encoding the kinase-inactive c-Src (Src K297M). Phosphorylation of ERK was analyzed (upper panel) by western blotting. As a loading control total ERK was detected (lower panel)

Notably, cells expressing Src KM showed no activation of ERK1/2 after infection with pneumococci (Figure 26) confirming that Src kinase is involved in activation of ERK in pneumococcal infections of host epithelial cells via the PspC-hpIgR uptake mechanism.

4.6.2. Activation of JNK during pneumococcal invasion relies on Src kinase

To investigate the interplay between Src kinase and the JNK MAPKs pathway, invasion of pneumococci in pIgR expressing epithelial cells was determined in presence of combination of Src kinase and JNK inhibitors. As a control each inhibitor was employed separately and the individual effect on pneumococcal invasion of was assessed. In principle simultaneous inhibition of two independent signalling pathways is thought to cause additive effects on the internalization of pneumococci by host cells. Consequently, inhibition of signalling pathways belonging to same cascade should not show any additive effect. To understand the cross-talk between Src kinase and JNK pathways, pneumococcal ingestion by MDCK-hpIgR cells was monitored after preincubating host cells with 5 µM PP2, 5 µM JNK inhibitor II or combination of both. Host cells were then infected for 1 h with pneumococci and the antibiotic protection assay was performed.

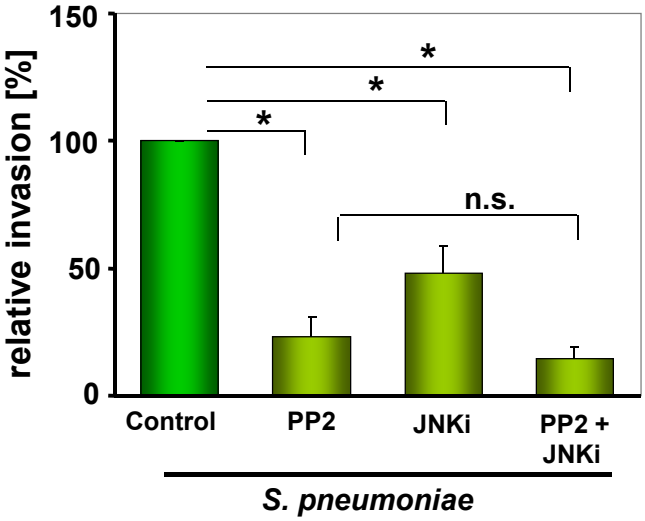


Figure 27 Sequential activation of Src and JNK MAPK during pneumococcal infection of pIgR expressing host cells. Pneumococcal invasion of MDCK-hpIgR cells was monitored in the absence (control) or presence of Src kinase inhibitor (PP2, 5 µM), c-Jun N-terminal kinase inhibitor (JNKi, 5 µM) or combination of both inhibitors by the antibiotic protection assay. Invasion of *S. pneumoniae* in the absence of inhibitor was set to 100 %. * $P < 0.005$ relative to infections carried out in the absence of inhibitors.

Inhibitor	**relative invasion by *S. p.* serotype 35A [%]**	
	MDCK-hpIgR	
		p value relative to control
control	100 ± 0	-
5 µM PP2	23.07 ± 7.89	7.22×10^{-5}
5 µM JNKi	47.96 ± 10.65	0.001
PP2 + JNKi	14.42 ± 4.49	5.01×10^{-6}
p value relative to PP2 alone	0.17	

Table 14 Relative uptake (in %) of *S. p.* 35A by MDCK-hpIgR in the absence or presence of Src family of protein-tyrosine kinase inhibitor PP2, c-Jun N-terminal kinase inhibitor or combination of both inhibitors, respectively. *P* value less than 0.05 was taken as statistically significant.

The results confirmed that the inhibition of Src protein-tyrosine kinase or JNK MAPK pathway impaired the PspC-hpIgR mediated pneumococcal invasion of MDCK-hpIgR cells. The simultaneous inhibition Src kinase and JNK MAPK pathways resulted in reduced bacterial internalization rates, which were similar to those measured by inhibiting Src kinase pathway alone (Figure 27). In conclusion the results revealed a cross-talk between these two pathways and suggest that activation of Src kinase occurs upstream of JNK activation during PspC-hpIgR mediated pneumococcal infection of host epithelial cells.

4.6.3. PI3-kinase and Src kinase are activated separately during pneumococcal infection

Inhibition studies demonstrated the critical role of PI3-kinase and Src kinase for PspC-hpIgR mediated pneumococcal invasion of host epithelial cells. To determine a putative cross-talk between PI3-kinase and Src kinase pneumococcal invasion of host cells was determined in the presence of individual or combined PI3-kinase and Src kinase inhibitors. MDCK-hpIgR cells were treated with 50 nM wortmannin, 5 µM PP2 or with a combination of both inhibitors prior to infections with *S. pneumoniae*.

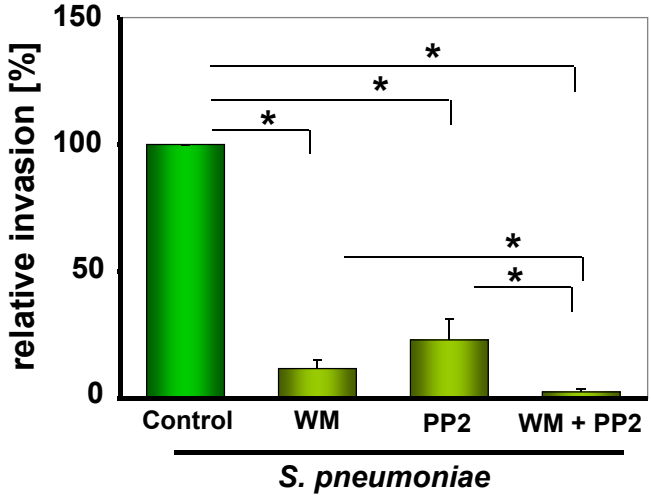


Figure 28 PI3-kinase and Src kinase are independently activated during pneumococcal infections. Invasion and intracellular survival of *S. pneumoniae* serotype 35A in MDCK-hpIgR cells was determined in the absence (control), presence of PI3-kinase inhibitor wortmannin (WM, 50 nM), or Src kinase inhibitor (PP2, 5 µM) and in assay with a combination of both inhibitors by using the antibiotic protection assay. Pneumococcal invasion in the absence of inhibitor was set to 100 %. * $P< 0.02$ relative to infections carried out in the absence of inhibitors.

Inhibitor	**relative invasion by *S. p.* serotype 35A [%]**	
	MDCK-hpIgR	
		p value relative to control
control	100 ± 0	-
50 nM Wortmannin	11.92 ± 3.49	1.64×10^{-6}
5 µM PP2	23.07 ± 7.89	7.22×10^{-5}
Wortmannin +PP2	2.31 ± 1.43	3.02×10^{-8}
p value relative to Wortmannin alone	0.01	
p value relative to PP2 alone	0.01	

Table 15 Percentage internalization of pneumococci into MDCK-hpIgR in the absence or presence of PI3-kinase inhibitor wortmannin, Src family of protein-tyrosine kinase inhibitor PP2, or combination of both inhibitors, respectively. *P* value less than 0.05 was taken as statistically significant.

The antibiotic protection assay revealed that an individual inhibition of PI3-kinase and Src protein-tyrosine kinase pathways and the simultaneous inhibition of both pathways strongly impaired hpIgR-mediated pneumococcal uptake by MDCK-hpIgR cells (Figure 28). However, compared to individual inhibitors, the combination of the inhibitors PP2 and wortmannin significantly increased blockage of pneumococcal uptake by host cells (Figure 28). The suggested the absence of cross-talk between these two signalling pathways and consequently an independent activation of PI3-kinase and Src kinase pathways during pneumococcal infection of host epithelial cells via the PspC-hpIgR mechanism.

4.7. Role of calcium during PspC-hpIgR mediated internalization of *S. pneumoniae* by epithelial cells

Calcium ions are the most ubiquitous and pluripotent cellular signalling molecules that control a wide variety of cellular processes. Calcium signalling has been implicated in various steps of bacterial infections. Bacterial toxins can induce an increase in the free cytosolic calcium in host cells, or independent of toxins, bacteria can induce calcium responses that play a role in cytoskeleton rearrangements thus facilitating their cell association or even internalization into these host cells.

Studies investigating the mechanism involved in the intracellular pathway of pIgR revealed that polymeric immunoglobulin A (pIgA) binding stimulates rabbit-pIgR transcytosis, owing to phospholipase-Cγ1 activation, and increase intracellular calcium levels (Cardone *et al.* , 1996). However, this effect was not observed with human-pIgR (Giffroy *et al.* , 2001) although after pIgA induction both rabbit and human-pIgR were able to transduce similar intracellular signal.

Here, the role of calcium during PspC-hpIgR mediated internalization of pneumococci was assessed. The infection assays were performed in the presence of pharmacological inhibitors of calcium signalling, BAPTA/AM and thapsigargin. BAPTA/AM is a membrane permeable form of BAPTA which once hydrolyzed by cytosolic esterases is trapped intracellularly as the active calcium chelator. Thapsigargin inhibits endoplasmic reticular Ca^{2+}-ATPase that normally sequesters calcium into intracellular stores. Short-term treatment with thapsigargin is widely used to raise intracellular calcium level. Both MDCK-hpIgR and Calu-3 were pretreated with 10 µM BAPTA/AM and 1 µM thapsigargin prior to bacterial infections. After 1 h infection with pneumococci, the intracellular survival of bacteria was determined by the antibiotic protection assay. To ensure that inhibitors do not affect

pneumococcal adherence to host cells, immunofluorescence staining of attached pneumococci was performed.

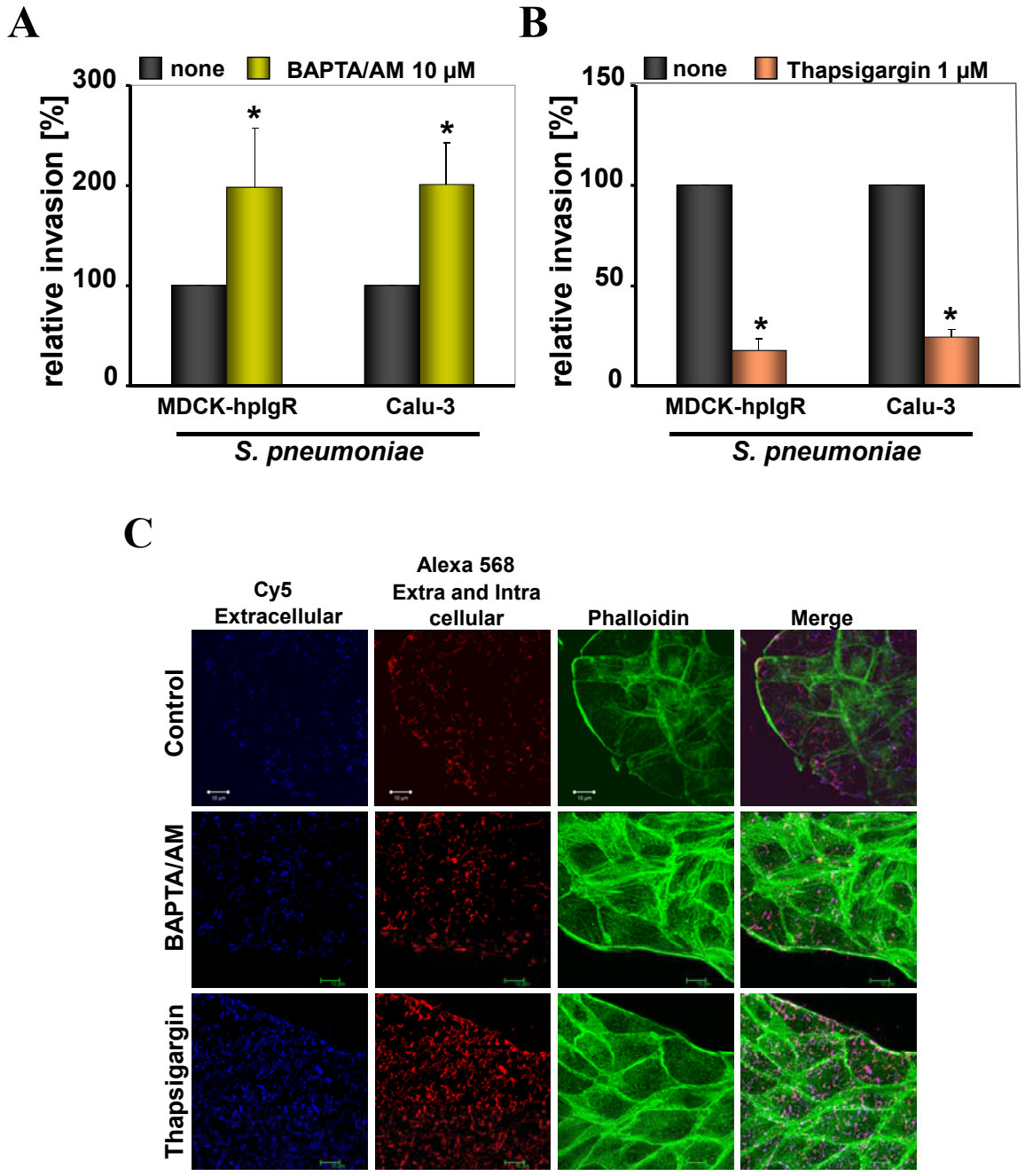


Figure 29 Role of calcium in PspC-hpIgR mediated pneumococcal host cell invasion. Invasion and intracellular survival of *S. pneumoniae* serotype 35A in MDCK-hpIgR (A) and Calu-3 (B) cells was monitored in the absence (none) or presence of BAPTA/AM (10 µM) and Thapsigargin (1 µM), respectively, by the antibiotic protection assay. Pneumococcal invasion in the absence of inhibitor was set to 100 %. * $P< 0.05$ relative to infections carried out in the absence of inhibitor. (C) Immunofluorescence microscopy of pneumococcal adherence to MDCK-hpIgR cells in the absence (control) or presence of inhibitors of calcium pathway.

Inhibitor	relative invasion by *S. p.* serotype 35A [%]			
	MDCK-hpIgR		Calu-3	
		p value relative to control		*p* value relative to control
control	100 ± 0	-	100 ± 0	-
10 µM BAPTA/AM	198.24 ± 58.69	0.015	200.49 ± 42.48	0.029
1 µM Thapsigargin	17.38 ± 5.92	1.73×10^{-5}	24.37 ± 3.48	6.64×10^{-6}

Table 16 Pneumococcal invasion of MDCK-hpIgR and Calu-3 cells in the absence or presence BAPTA/AM (10 µM) and Thapsigargin (1 µM), respectively. The results are demonstrated as percentage invasion by *S. p.* 35A compared to infection assay performed in absence of inhibitor. *P* value less than 0.05 was taken as statistically significant.

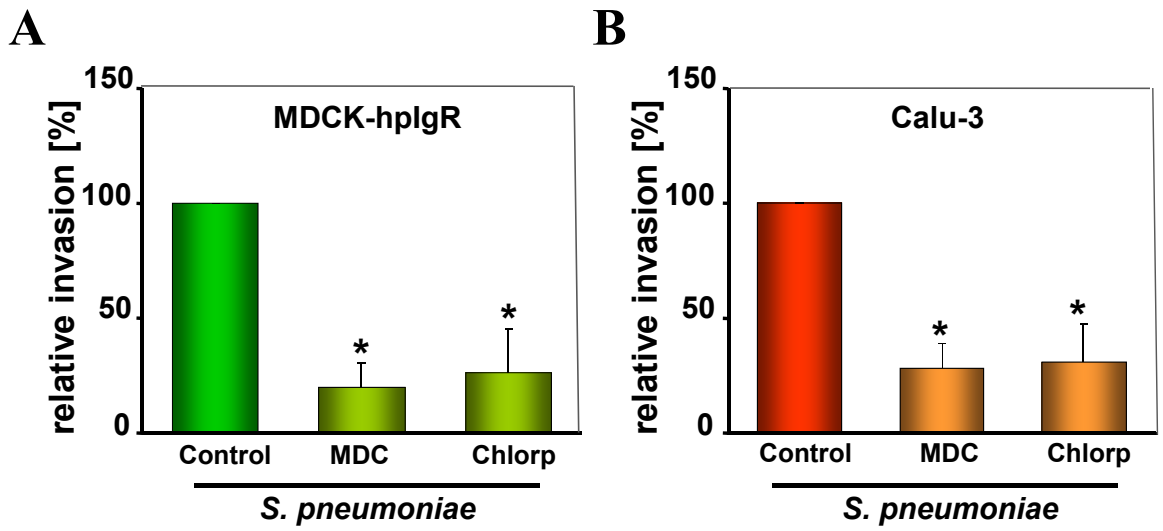
BAPTA/AM and thapsigargin had a differential effect on pneumococcal uptake by host cells. Pretreatment of cells with BAPTA/AM significantly increased the pneumococcal ingestion by pIgR expressing cells (Figure 29A). In contrast, increase in intracellular calcium upon treatment with thapsigargin significantly reduced pneumococcal uptake by host cells (Figure 29B). A 2-fold increase in the number of intracellular pneumococci was observed after calcium chelation by BAPTA/AM, whereas thapsigargin treatment caused an 80 % reduction of internalized bacteria. Taken together these data demonstrate the involvement of calcium signalling in uptake of pneumococci by pIgR expressing host epithelial cells. However, a detailed analysis is required to elucidate the role of calcium in PspC-hpIgR mediated pneumococcal infection.

4.8. Identification of the host endocytic machinery involved in the PspC-hpIgR mediated pneumococcal uptake by epithelial cells

Bacterial pathogens engage various strategies to promote their entry in non-phagocytic host cells. These endocytotic processes have utilized have been extensively investigated for other pathogens including viruses. However, the mechanism of how *S. pneumoniae* is taken up by host cells is not yet known. Radin *et al.* (2005) suggested that platelet activating factor receptor (PAFr) mediated pneumococcal uptake is clathrin dependent and have shown co-localization of vacuole containing pneumococci with Rab5, Rab7, Rab11 and Lamp-1. Nevertheless, the host endocytic machinery involved in the hpIgR mediated pneumococcal uptake is not known. It was shown that basolateral to apical transcytosis of rabbit-pIgR-pIgA complex involves the internalization through clathrin-coated pits, which is then delivered at the apical surface via various sorting endosomes (Hoppe *et al.*, 1985; Limet *et al.*, 1985).

4.8.1. Pneumococci co-opts clathrin and dynamin during invasion of epithelial cells

To investigate the role of clathrin during hpIgR mediated pneumococcal uptake by host epithelial cells, infection assays were performed in the presence of specific blocking reagents of the clathrin machinery. The clathrin machinery was blocked during pneumococcal infection by specific inhibitors, Monodansylcadaverine (MDC) and Chlorpromazine (Chlorp). The inhibitory activity of MDC is attributed to the stabilization of clathrin-coated pits. Chlorpromazine is a cationic amphipathic drug that causes loss of clathrin and the AP2 adaptor complex from the cell surface and in turn facilitates their artificial assembly on endosomal membranes. Pneumococcal uptake by pIgR expressing cells was determined by the antibiotic protection assay after 1 h of infection. Pneumococcal adherence to host cells was determined by immunofluorescence staining.



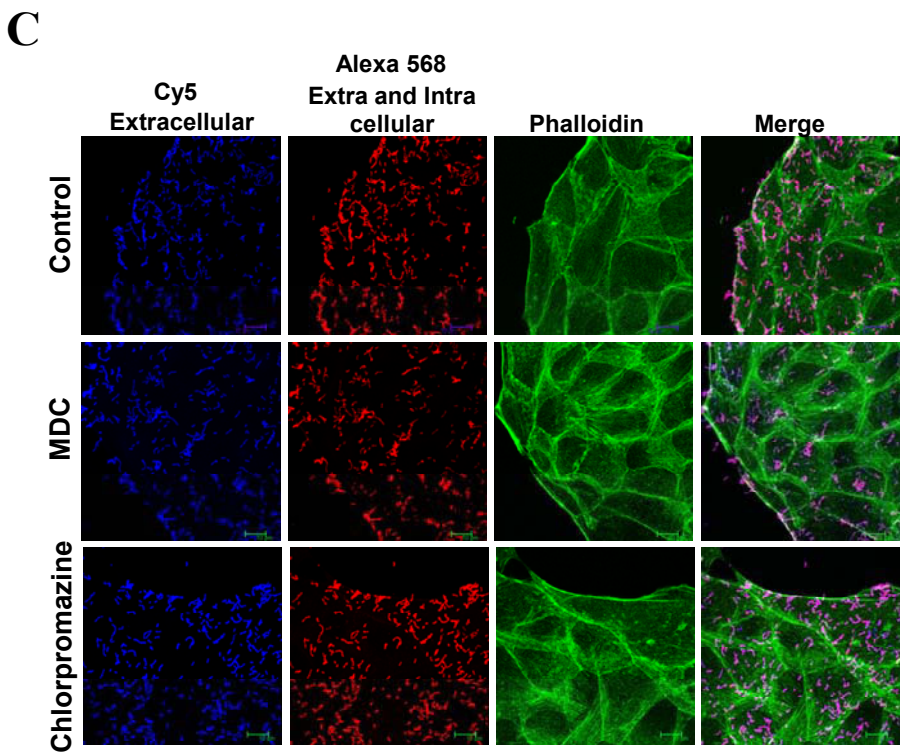


Figure 30 Clathrin mediated endocytosis facilitates PspC-hpIgR mediated pneumococcal uptake by epithelial cells. Invasion and intracellular survival of the bacteria in MDCK-hpIgR (A) and Calu-3 (B) cells was determined in the absence (control) or presence of monodansylcadaverine (MDC, 50 µM) or chlorpromazine (Chlorp, 10 µM) by the antibiotic protection assay. Invasion of pneumococci in the absence of inhibitor was set to 100 %. * $P<$ 0.005 relative to infections carried out in the absence of inhibitor. (C) Immunofluorescence microscopy of pneumococcal adherence to MDCK-hpIgR cells in the absence (control) or presence of inhibitors of clathrin mediated endocytosis pathway.

Inhibitor	**relative invasion by *S. p.* serotype 35A [%]**			
	MDCK-hpIgR		**Calu-3**	
		p value relative to control		*p* value relative to control
control	100 ± 0		100 ± 0	
50 µM MDC	19.89 ± 10.21	0.00017	28.16 ± 11.01	1.24×10^{-5}
10 µM Chlorpromazine	25.93 ± 18.93	0.0025	30.61 ± 16.74	0.00017

Table 17 Relative pneumococcal invasion (in %) of MDCK-hpIgR and Calu-3 cells in the absence or presence inhibitors of clathrin machinery. *P* value less than 0.05 was taken as statistically significant.

Inhibition of the clathrin machinery resulted in a significant reduction of pneumococcal uptake by pIgR expressing host epithelial cells (Figure 30A and 30B). However, no significant alteration in the adherence was monitored due to the use of MDC or chlorpromazine as inhibitor (Figure 30C). These results suggested a significant contribution of the clathrin machinery for pneumococcal uptake by pIgR expressing host epithelial cells.

The large GTPase dynamin is involved in the scission and subsequent formation of independent clathrin coated vesicles and has therefore an important role in the endocytosis. To elucidate the involvement of dynamin for pneumococcal ingestion by pIgR expressing host epithelial cells, pnemococcal uptake was determined after treatment of host cells with dynasore, a cell-permeable inhibitor of dynamin (Macia *et al.*, 2006). The host cells were preincubated with 80 µM dynasore for 2-3 min prior to the infection with pneumococci.

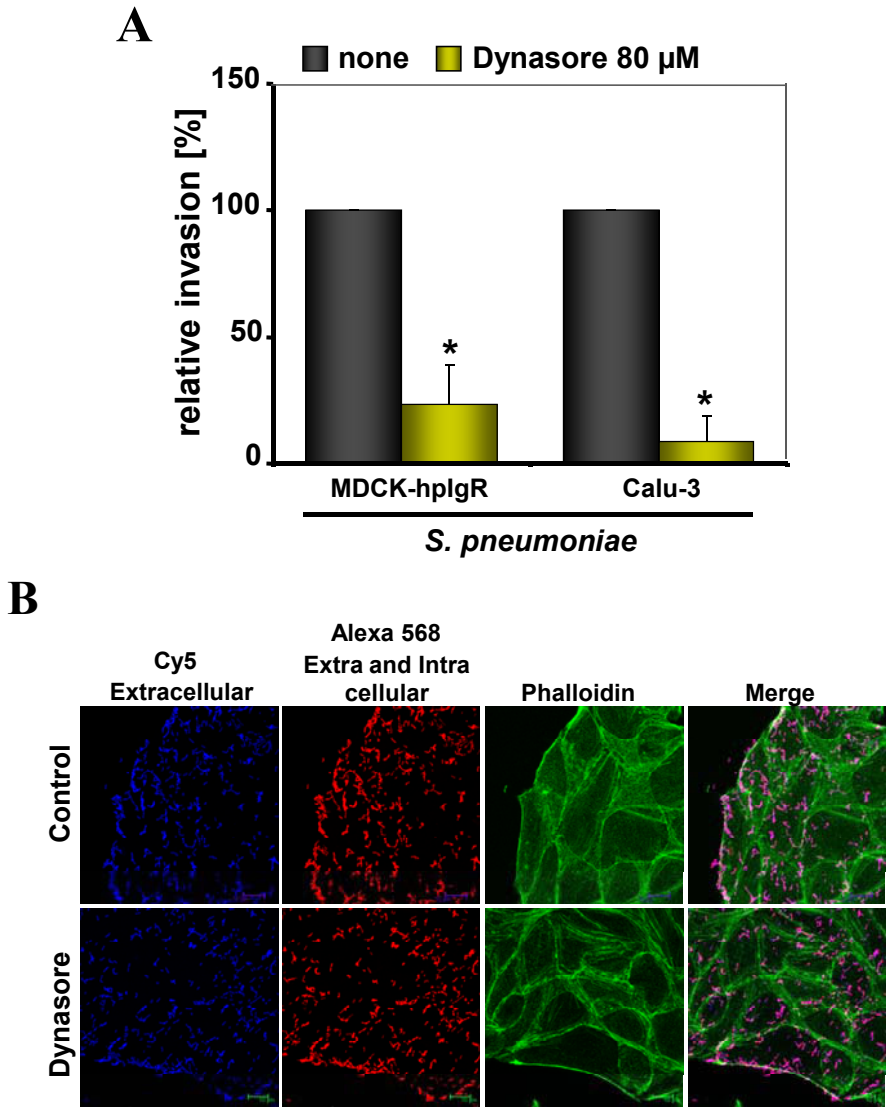


Figure 31 Dynamin in involved in pneumococcal uptake by pIgR expressing epithelial cells. (A) Pneumococcal invasion and intracellular survival in MDCK-hpIgR and Calu-3 was determined in the absence (none) or presence of Dynasore (80 µM) by the antibiotic protection assay. Pneumococcal invasion in the absence of inhibitor was set to 100 %. * $P<$ 0.05 relative to infections carried out in the absence of inhibitor. (C) Immunofluorescence microscopy of pneumococcal adherence to MDCK-hpIgR cells in the absence (control) or presence of dynasore.

Inhibitor	relative invasion by *S. p.* serotype 35A [%]			
	MDCK-hpIgR		Calu-3	
		p value relative to control		*p* value relative to control
control	100 ± 0	-	100 ± 0	-
80 µM Dynasore	23.29 ± 15.58	0.02	8.74 ± 9.88	0.0058

Table 18 Percentage pneumococcal uptake by MDCK-hpIgR and Calu-3 cells in the absence or presence Dynasore. *P* value less than 0.05 was taken as statistically significant.

Dynasore inhibited significantly the internalization of pneumococci tested by the gentamicin survival assay (Figure 31A).

The role of dynamin in the bacterial entry process was also analysed in infections, in which dynamin expression was knocked-down by using siRNA interference.

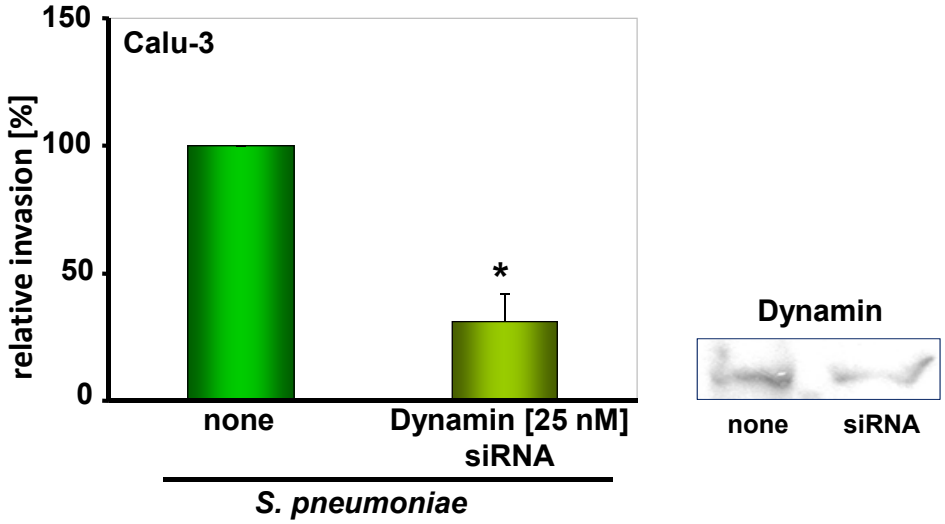


Figure 32 Dynamin-dependent pneumococcal uptake by pIgR expressing epithelial cells. Expression of dynamin was knocked-down by (25 nM) siRNA and the cells were infected for 1 h with *S. pneumoniae* serotype 35A. Bacterial invasion and intracellular survival was measured by cfu counts following the gentamicin protection assay. Pneumococcal invasion in the absence of dynamin knocked-down by siRNA (none) was set to 100 %. * $P< 0.001$ relative to infections carried out in the absence of dynamin knocked-down by siRNA.

siRNA	relative invasion by *S. p.* serotype 35A [%]	
	MDCK-hpIgR	
		p value relative to control
control	100 ± 0	-
25 nM Dynamin II siRNA	30.82 ± 10.95	0.0001

Table 19 Percentage invasion of dynamin was knocked-down Calu-3 cells by *S. p.* 35A. *P* value less than 0.05 was taken as statistically significant.

The genetic knocked-down of *dynamin* by siRNA in Calu-3 cells resulted in significant reduction of pneumococcal uptake as measured by the antibiotic protection assay (Figure 32). The number of internalized pneumococci was decreased by 70 % in *dynamin* knocked-down Calu-3 cells compared to host cells expressing dynamin. In conclusion, the result demonstrated that the pneumococcus engages the clathrin machinery for its entry into host epithelial cells.

4.8.2. Recruitment of clathrin during PspC-hpIgR mediated pneumococcal internalization of epithelial cells

Blocking of the clathrin machinery by pharmacological inhibitors or the genetic knocked-down of *dynamin* expression by siRNA demonstrated the key role of clathrin and dynamin dependent endocytotic machinery for pneumococcal uptake by pIgR expressing host epithelial cells. Recruitment of clathrin by pneumococci during host cell invasion was further analysed in MDCK-hpIgR cells which were transiently transfected with a plasmid expressing EGFP tagged clathrin light chain protein (LCa-EGFP). Transfected cells were infected for 3 h with pneumococci and a co-localization of pneumococci with clathrin was monitored by CLSM.

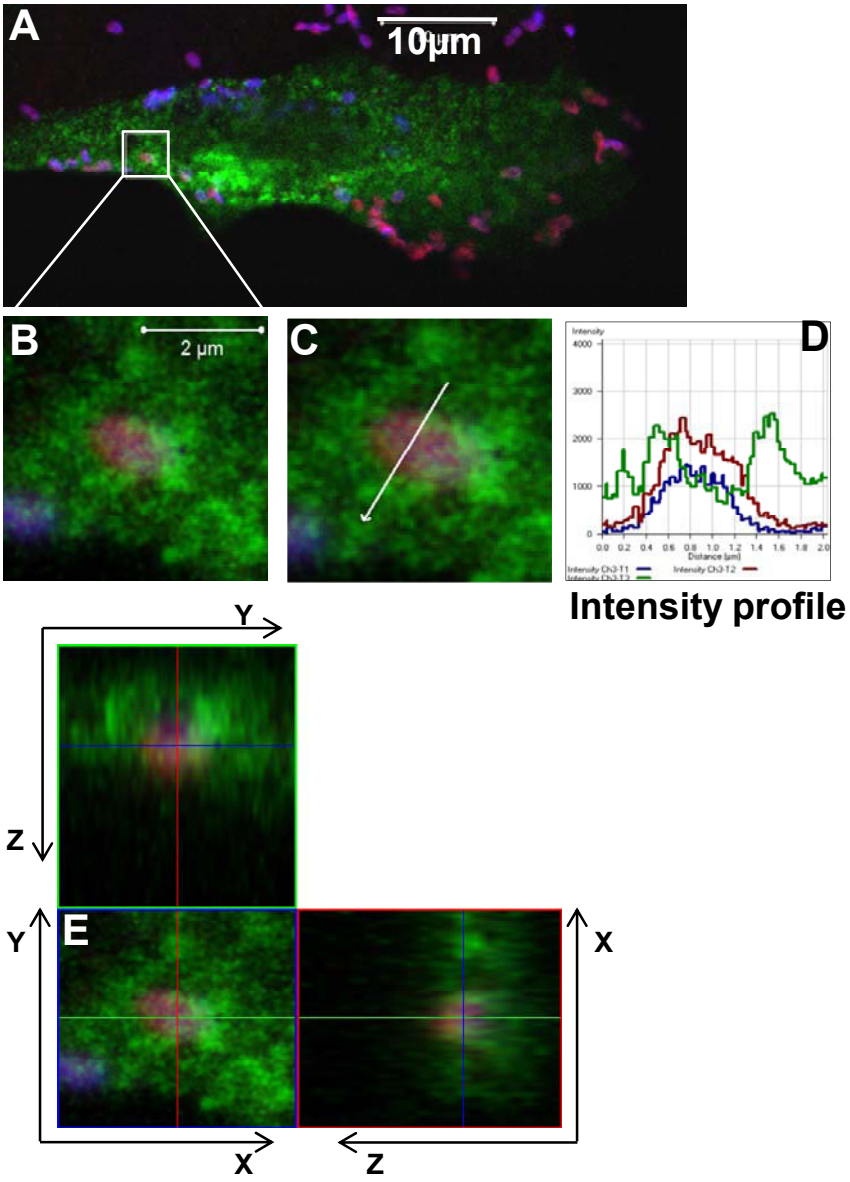


Figure 33 Co-localization of endogenous clathrin and pneumococci during PspC-hpIgR mediated ingestion by host epithelial cells. MDCK-hpIgR cells were transiently transfected to express the EGFP tagged clathrin light chain (LCa-EGFP) and were infected for 3 h with *S. pneumoniae* serotype 35A. (A) CSLM image illustrating pneumococci attached to or (B) in the process of invading MDCK-hpIgR cells expressing LCa-EGFP. (C and E) Pneumococci during internalization and colocalized with clathrin. (D) Fluorescene intensity profile depicting recruitment of clathrin by invading pneumococci. Bar equals 10 µm (A) and 2 µm (B).

The illustration by CLSM (Figure 33) revealed the clathrin co-localizes with pneumococci which are taken up by host cells (marked in box). The fluorescence intensity profile clearly demonstrated recruited clathrin (green) in the vicinity of invading pneumococci (red and blue). In conclusion, pneumococcal uptake by host epithelial cells, via the PspC-hpIgR mechanism, is clathrin and dynamin dependent.

4.9. Interaction of PspC with complement regulator Factor H

In addition to its role as an adhesin, PspC also mediates immune evasion by binding the host complement and innate immune regulator Factor H and C3 (Dave *et al.*, 2001; Smith and Hostetter, 2000). However, the two soluble host proteins Factor H and SC of hpIgR utilize two distinct epitopes on PspC for binding (Dave *et al.*, 2004). Moreover, binding of Factor H has been demonstrated for several pathogenic bacteria, thus conferring resistance against complement-mediated killing.

Although, earlier studies identified PspC binding sites within Factor H (Dave *et al.*, 2001; Duthy *et al.*, 2002), the results are inconsistent. Moreover, the activated form of Factor H binds via a RGD sequence in the SCR4 to eukaryotic host cell. Thus the interaction of complement regulator Factor H with the pneumococcal PspC protein was investigated in more detail. In addition, the impact of bacterial cell surface bound Factor H on pneumococcal adherence to host cells and the molecular mechanism that facilitates the uptake of Factor H bound pneumococci by epithelial cells were investigated. To elucidate the putative mechanism, pneumococci were preincubated with Factor H before being used in infection assays. Unless otherwise specified, 2 µg Factor H was used per 1 x 10^7 pneumococci and all the infection assays were performed for 3 h using *S. pneumoniae* serotype 35A (NCTC10319) with a MOI of 50 bacteria per host cell.

4.9.1. Recruitment of Factor H by *S. pneumoniae*

The ability of *S. pneumoniae* to recruit soluble Factor H from human plasma or serum was investigated. Pneumococci (1 x 10^9) were preincubated with 100 µl of human plasma or serum for 30 min at 37°C. After incubation in human plasma or serum, pneumococci were washed once with PBS. The samples and the elute fraction and the extract prepared from plasma or serum treated pneumococci were separated by 10 % SDS-PAGE and analyzed by Western blotting using a Factor H antiserum.

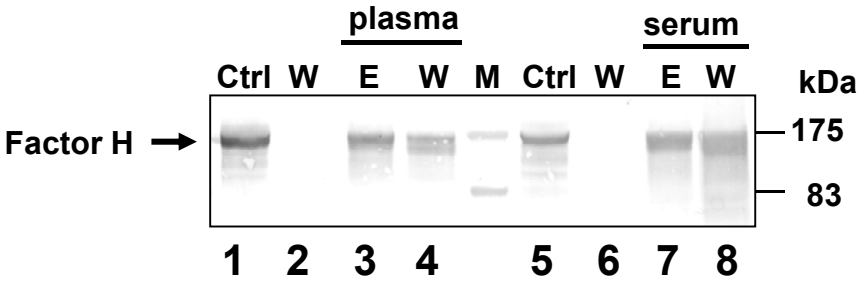


Figure 34 Binding of Factor H to pneumococci. Immunoblot analysis of Factor H binding to *S. pneumoniae* serotype 35A (NCTC10319) which were incubated with human plasma or serum. The samples were separated by SDS-PAGE, transferred to a PVDF membrane and

analyzed with Factor H antiserum. Lanes: 1, plasma control (Ctrl); 2 and 6, whole cell lysate of pneumococci (W) incubated in PBS; 3, proteins eluted from the pneumococcal cell surface (E) by treatment with 2 M NaCl after incubation in human plasma; 4 and 8, whole cell lysate (W) after incubation of pneumococci with human plasma or serum; 5, serum control (Ctrl); 7, proteins eluted from the pneumococcal cell surface after incubation in human serum. M: Protein Marker (from NEB Biolabs).

Immunoblot analysis detected Factor H in both elute and bacterial fraction (Figure 34, lanes 3 and 4), demonstrating that Factor H derived from human plasma binds to the surface of pneumococci. Similar results were observed when pneumococci were incubated in human serum (Figure 34, lanes 7 and 8).

In addition, recruitment of Factor H from human plasma was analyzed by flow cytometry. To verify PspC mediated pneumococcal recruitment of Factor H, binding of Factor H was quantitated using *S. pneumoniae* serotype 35A (NCTC10319) and its *pspC*-mutant (Δ*pspC*). Five times 10^7 pneumococci were incubated with different concentration (1 % to 100 %) of human plasma for 30 min at 37°C and binding of Factor H was detected by flow cytometry.

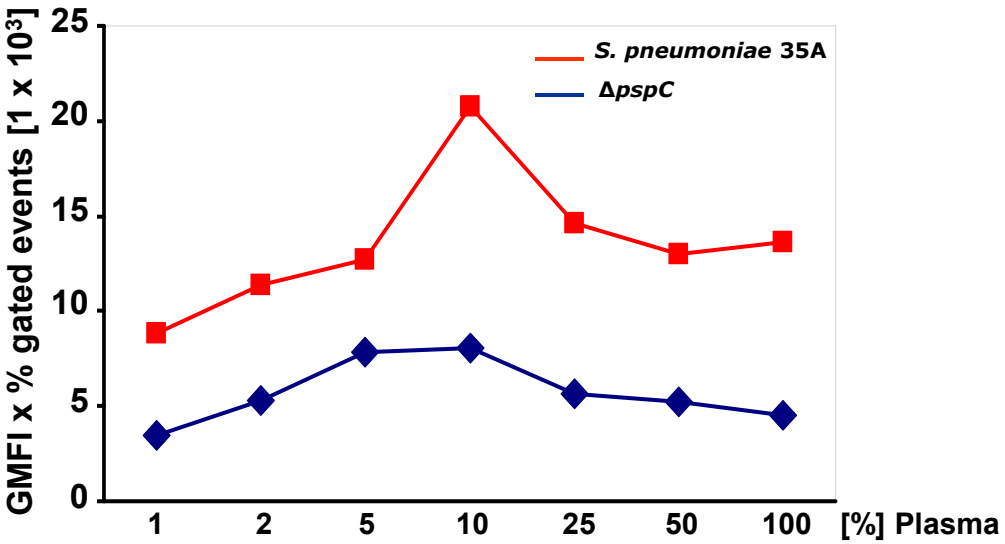


Figure 35 Recruitment of Factor H from human plasma by *S. pneumoniae* serotype 35A (NCTC10319) and isogenic PspC (Δ*pspC*) mutant was determined by flow cytometry and results were expressed as GMFI x % FITC labelled and gated bacteria.

Human plasma concentration [%]	GMFI x % FITC labeled and gated event	
	***S. p.* type 35A**	***S. p.* type 35A Δ*pspC***
1	8805.26	3455.67
2	11347.93	5267.72
5	12707.68	7814.57
10	20735.48	8027.56
25	14629.41	5649.22
50	12979.99	5221.42
100	13620.31	4518.08

Table 20 Quantification of Factor H recruited by *S. p.* type 35A and the *pspC*-mutant from human plasma, with the help of flow cytometry.

The results confirmed that *S. pneumoniae* serotype 35A was able to recruit Factor H from human plasma (Figure 35). However, binding of Factor H was also observed for the *pspC*-mutant, although it was about 2 to 2.5 fold lower as compared to the wild-type strain. The concentration of Factor H in human plasma is approximately 500 µg/ml and there might be the possibility of non-specific binding to the *pspC*-mutant. Moreover, recruitment of Factor H by the isogenic *pspC*-mutant can be mediated by other protein factors present in the plasma that may act as bridging molecules between other pneumococcal surface proteins and Factor H.

4.9.2. Species-specific interaction of Factor H with *S. pneumoniae*

The PspC-pIgR interaction is a human specific interaction (Hammerschmidt *et al.*, 2000), however it is not clear whether the PspC-Factor H interaction is also species-specific. The results clearly indicate that *S. pneumoniae* is able to recruit human Factor H. However, to investigate the binding of Factor H form other species, *S. pneumoniae* serotype 35A (NCTC10319) were incubated with 20 µl of 100 % mouse or rat serum for 30 min at 37°C. In control experiments pneumococci were incubated with same amount of human plasma. Following the incubations, pneumococci were washed once with PBS and binding of Factor H was analyzed by western blotting using species-specific Factor H antiserum.

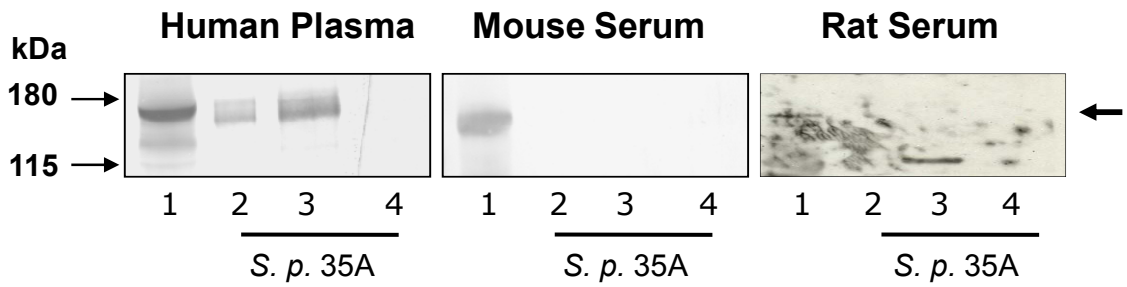


Figure 36 Species specific binding of Factor H to pneumococci. Immunoblot analysis of Factor H binding to *S. pneumoniae* serotype 35A (NCTC10319) which were incubated in human plasma, mouse serum or rat serum, respectively. The samples were separated by SDS-PAGE, transferred to a PVDF membrane and analyzed with species specific Factor H antiserums. Lane: 1, plasma or serum control; 2, proteins eluted from the pneumococcal cell surface by treatment with 2 M NaCl after incubation in respective plasma or serum; 3, whole cell lysate after incubation of pneumococci with respective plasma or serum; 4, whole cell lysate of pneumococci incubated in PBS.

Recruitment of Factor H by pneumococci was detected only for human plasma but not for mouse or rat serum (Figure 36). Although both anti-mouse Factor H and anti-rat Factor H detected the basal level of Factor H present in the serum, this approach was not sufficient to detect bound mouse or rat Factor H to pneumococci.

In order to corroborate the result, recruitment of Factor H was analyzed using flow cytometry. About 5 x10^7 pneumococci were incubated with 10 and 25 % human plasma, mouse serum and rat serum respectively for 30 min at 37°C. Binding of Factor H to pneumococci was detected after incubation with the human Factor H antiserum, anti mouse Factor H and anti rat Factor H (1:200) for 30 min at 37°C followed by FITC-conjugated secondary antibody. Bacteria were washed and fluorescence analyzed by flow cytometry using a FACS CantoI (Becton Dickinson).

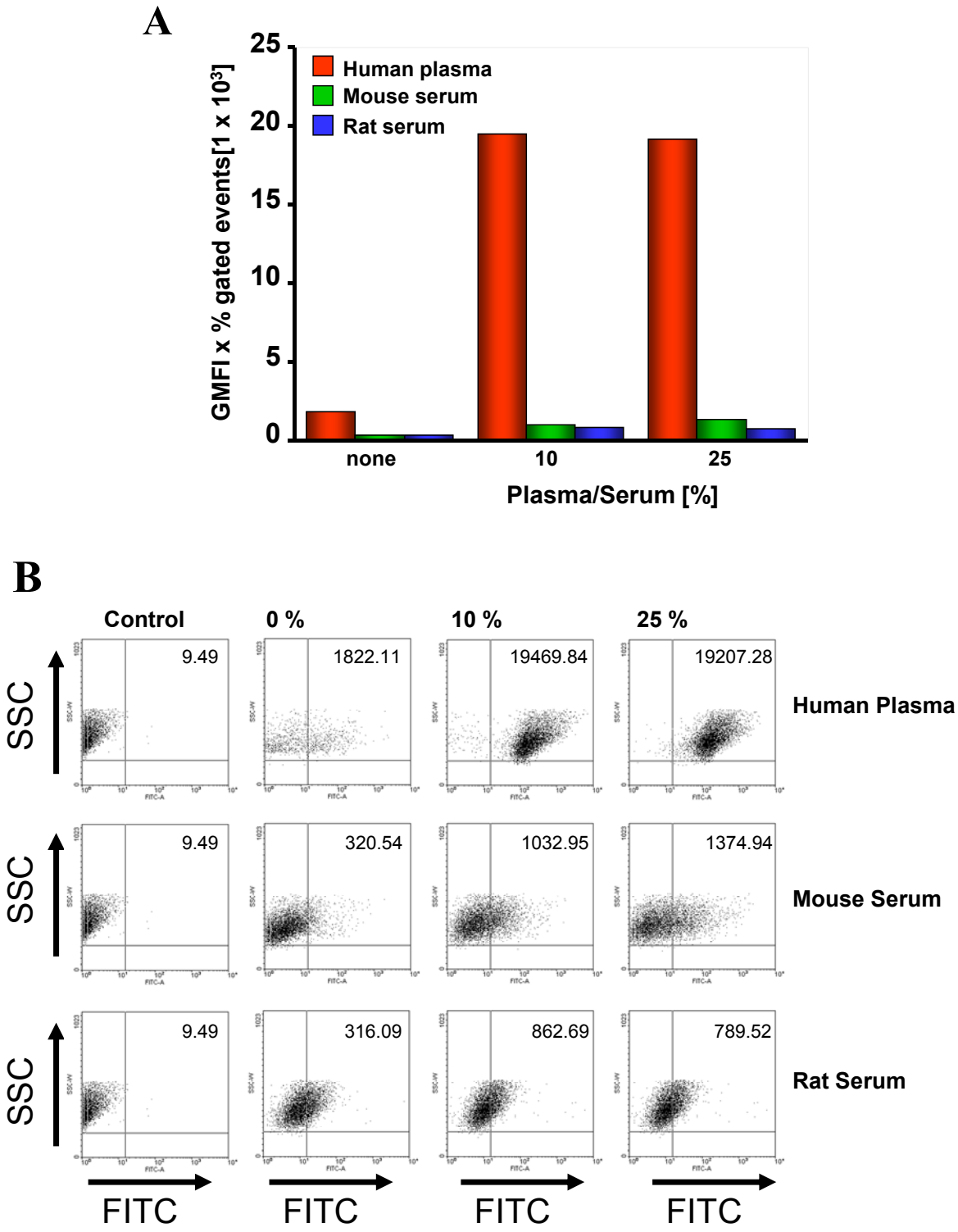


Figure 37 Pneumococcal recruitment of Factor H from different species. Bound Factor H from human plasma, mouse serum or rat serum, respectively, by *S. p* 35A was determined by flow cytometry. Pneumococci (5 x10^7) were incubated with 10 and 25 % human plasma, mouse serum and rat serum respectively and binding of Factor H was detected using species specific Factor H antiserum followed by FITC-conjugated secondary antibody. The results were expressed as GMFI x % FITC labelled and gated bacteria (A) or represented as dot plots (B), where the *x*-axis represents fluorescence (FITC) on a $\log_{10}$ scale and the *y*-axis represents the number of events (SSC).

Flow cytometry demonstrated that pneumococci preferentially recruit human Factor H as compared to mouse or rat Factor H (Figure 37). However, flow cytometry also showed binding of mouse and rat Factor H to pneumococci, albeit significantly lesser than human Factor H. The low binding of Factor H of mouse or rat origin could also be attributed to its low concentration in their respective serums. Further investigations are required in order to affirm the species-specificity of pneumococcal Factor H interactions and whether there is in addition to PspC another Factor H binding protein.

4.9.3. Association of purified Factor H with *S. pneumoniae*

The results have shown that *S. pneumoniae* recruits soluble Factor H from human plasma or serum. However, these data do not provide evidence that pneumococcal Factor H interaction is a direct interaction or is mediated by an unknown host molecule present in the plasma. In order to analyse the direct binding of Factor H, purified human Factor H was used and binding to *S. p.* Serotype 35A was determined by immunoblotting. Pneumococci (1 x 10^9) were preincubated with 1, 2 and 4 µg of purified human Factor H and binding of Factor H was determined by Western blotting using Factor H antiserum.

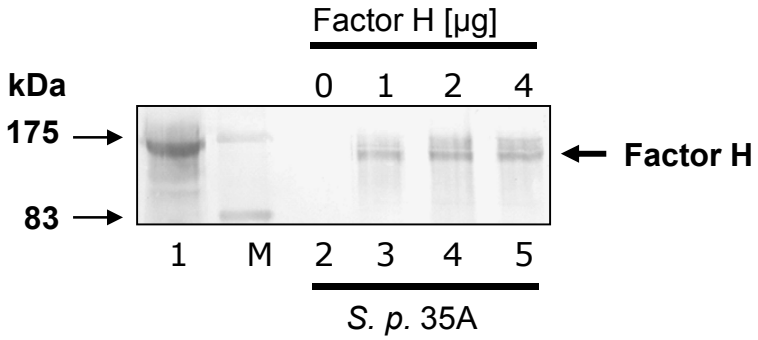


Figure 38 Binding of purified Factor H to pneumococci. The lysates of *S. pneumoniae* serotype 35A (NCTC10319) were incubated with increasing concentration of purified Factor H, separated by SDS-PAGE, transferred to a PVDF membrane and analyzed with Factor H antiserum. Lane: 1, plasma control; 2, whole cell lysate of pneumococci incubated in PBS; 3, 4 and 5, whole cell lysate after incubation of pneumococci with 1, 2 and 4 µg purified Factor H, respectively. M: Protein Marker (from NEB Biolabs).

The immunoblot in Figure 38 shows a dose-dependent binding of purified Factor H to *S. pneumoniae*. The result clearly showed that there is a direct interaction of Factor H with *S. pneumoniae*, most likely via the PspC protein.

Binding of purified Factor H to pneumococci was also analyzed by flow cytometry. Pneumococci (5 x10^7) were incubated with increasing concentration (0.1, 0.5, 2 and 5 µg) of purified human Factor H for 30 min at 37°C and binding of Factor H to pneumococci was analysed by flow cytometry using a FACS Calibur (Becton Dickinson).

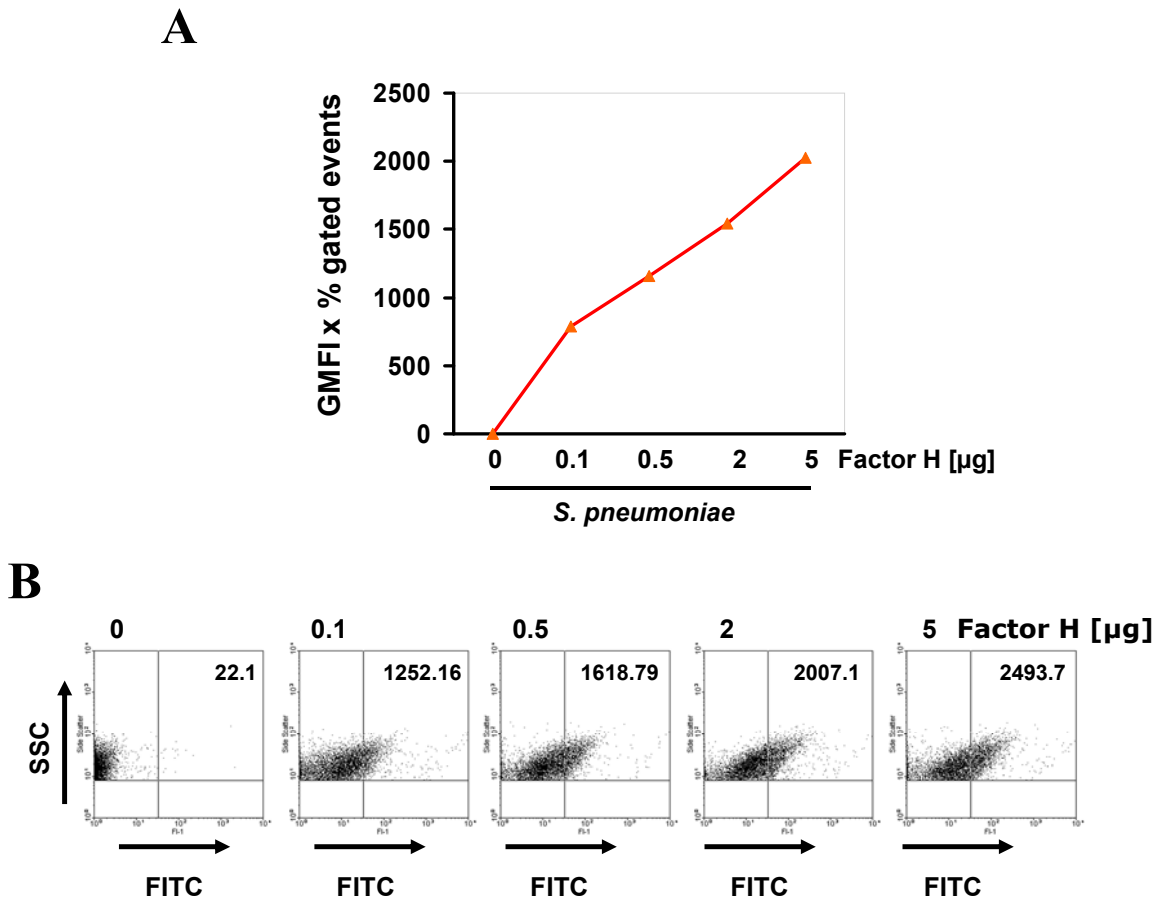


Figure 39 Binding of purified Factor H to *S. pneumoniae* serotype 35A (NCTC10319) was determined by flow cytometry. Pneumococci (5 x10^7) were incubated with 0, 0.1, 0.5, 2 and 5 µg of purified Factor H, respectively. The binding was detected using Factor H antiserum followed by FITC-conjugated secondary antibody. The results were expressed as GMFI x % FITC labelled and gated bacteria (A) or represented as dot plots (B), where the *x*-axis represents fluorescence (FITC) on a $\log_{10}$ scale and the *y*-axis represents the number of events (SSC).

Flow cytometry data revealed a dose-dependent increase in binding of purified Factor H to pneumococci (Figure 39 A and B). The data confirm the direct interaction between pneumococci via PspC and Factor H.

In addition, ^{125}I-radiolabeled Factor H was employed for blot overlay assays. Factor H was radiolabelled with ^{125}I by a standard chloramines-T method (Chhatwal *et al.* , 1987). Bacterial lysates of wild-type (WT) *S. pneumoniae* (NCTC10319) and its *pspC*-mutant were separated by SDS-PAGE and transferred to a PVDF membrane. After blocking with 10 % skim milk, the membrane was washed and incubated with ^{125}I-radiolabeled Factor H (300,000 cpm ml-1) in 5 ml of PBS-Tween 20 (0.05 %) for 4 h at room temperature. After extensive washing, bound Factor H was detected by autoradiography.

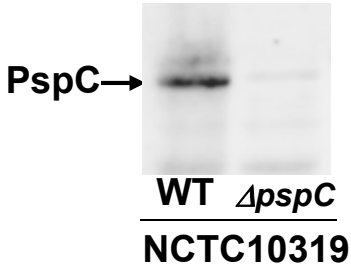


Figure 40 Binding of ^{125}I-radiolabeled Factor H wild-type (WT) NCTC10319 and its *pspC*-mutant. Bacterial lysates were separated by SDS-PAGE, transferred to a PVDF membrane, and used for an overlay assay with ^{125}I-radiolabeled Factor H.

The autoradiography data detected binding of ^{125}I-radiolabeled Factor H to the wild-type pneumococci of strain NCTC10319 but not to the pneumococci of the isogenic *pspC* knockout strain (Figure 40). The result indicated that PspC is the major and most likely the only surface protein of pneumococci that binds the host regulator Factor H.

4.9.4. Recruitment of Factor H by pneumococci is independent of the PspC subtypes

PspC is a highly variable surface protein with a modular organization (Iannelli *et al.*, 2002, Hammerschmidt *et al.*, 1997). In order to assess whether the PspC variability affects binding of Factor H, pneumococci producing different PspC subtypes including the serotype 3 strain A66, which expresses Hic (PspC11.4), were used in binding experiments. Pneumococci (5×10^7) were incubated with 2 µg of purified human Factor H for 30 min at 37°C and binding was analyzed by flow cytometry using a FACS Canto I (Becton Dickinson).

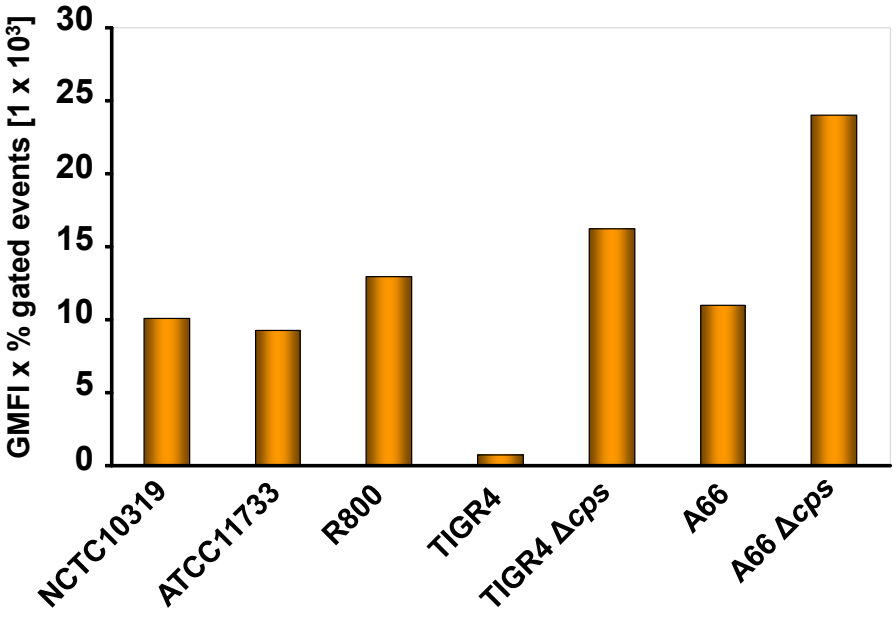


Figure 41 Recruitment of Factor H to encapsulated and nonencapsulated pneumococci producing different PspC subtypes. Binding of Factor H (2 µg) was determined by flow cytometry and results were expressed as GMFI x percentage of FITC-labeled and gated bacteria.

S. pneumoniae strain	GMFI x % FITC labeled and gated event
NCTC10319	10104.2
ATCC11733	9238.58
R800	12927.18
TIGR4	754.33
TIGR4Δ*cps*	16189.75
A66	10969.34
A66Δ*cps*	23985.26

Table 21 Quantification of Factor H recruitment to encapsulated and nonencapsulated pneumococcal strains producing different PspC subtypes using flow cytometry.

As demonstrated by flow cytometric analysis (Figure 41), all pneumococcal strains recruited Factor H to the bacterial cell surface independent of the PspC subtype. Factor H binding efficiency increased significantly when non-encapsulated pneumococcal strains were used (Figure 41), indicating that the CPS interferes with Factor H binding.

In addition, ^{125}I radiolabeled Factor H was employed in binding assay with encapsulated wild-type strain NCTC10319 (Cps^{+}) and nonencapsulated R6x (Cps^{-}) and their respective *pspC*-mutants.

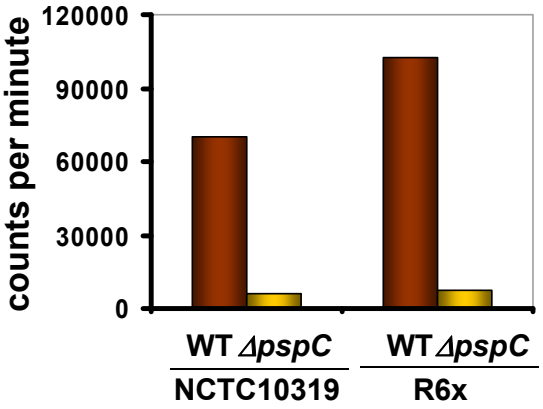


Figure 42 Binding of soluble ^{125}I-radiolabeled Factor H to viable pneumococcal wild-type (WT) strain NCTC10319 (Cps^{+}) and R6x (Cps^{-}) and their *pspC*-mutants.

^{125}I radiolabeled Factor H bound to the wild-type strain but not to the isogenic pspC-mutant strains representing the encapsulated strain NCTC10319 or the nonencapsulated R6x (Figure 42).

4.10. The role of Factor H on host cellular adherence and invasion by *S. pneumoniae*

Several pathogenic bacteria have been shown to recruit Factor H, thus providing them with another mechanism for evading the host innate immunity. Factor H has been shown to mediate complement control at the surface of pneumococci (Neeleman *et al* ., 1999).

However, to identify additional biological relevant functions for the bacterial-bound host complement regulator, the role of Factor H for adhesion of pneumococci to human cells was assessed.

4.10.1. Factor H facilitates adherence of *S. pneumoniae* to host cells

To investigate the role of Factor H in pneumococcal colonization, adhesion of pneumococci (NCTC10319), which were preincubated with Factor H, was studied to human epithelial and endothelial cells. Human nasopharyngeal epithelial cells, Detroit 562, human lung alveolar epithelial cells, A549 and human brain-derived microvascular endothelial cells, HBMEC were infected with pneumococci that were pre-incubated purified human Factor H. The host cells infected with pneumococci, not pre-incubated with Factor H, were taken as control. Pneumococcal adherence of eukaryotic host cells was estimated by counting approximately 50 host cells using immunofluorescence microscope.

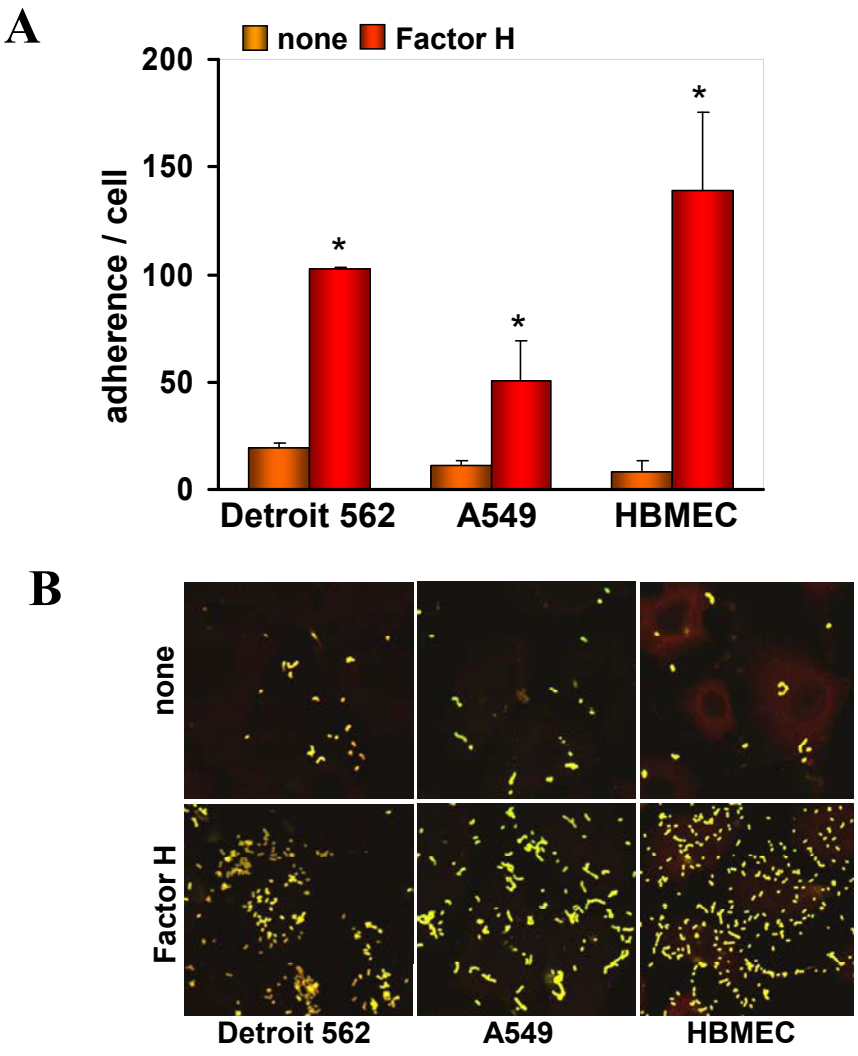


Figure 43 Pneumococcal surface-bound Factor H mediates bacterial adherence to host cells. A, Attachment of pneumococcal strain NCTC10319 (Cps^+, serotype 35A) was counted by immunofluorescence microscopy after infection of the epithelial cells Detroit 562 and A549, respectively, or the endothelial cell line HBMEC. The infection assays were conducted with or without the preincubation of pneumococci with 3 µg of Factor H. B, Immunofluorescence microscopy of adherent pneumococci. * $P< 0.005$ relative to infections conducted in the absence of Factor H.

Cell lines	**adherent bacteria *S. p.* serotype 35A per cell**		
	none	**Factor H [3 µg]**	*p* value
Detroit 562	18.98 ± 2.93	102.67 ± 0.87	2.98×10^{-7}
A549	10.85 ± 2.74	50.83 ± 18.39	0.0051
HBMEC	8.44. ± 5.14	138.69 ± 36.43	0.0036

Table 22 Number of adhered bacteria *S. p.* 35A per cell. Pneumococcal attachment of the epithelial cells Detroit 562 and A549 or the endothelial cell line HBMEC after 3 h infections was counted by immunofluorescence microscopy. The infection assays were conducted with or without the preincubation of pneumococci with 3 µg of Factor H. *P* value less than 0.05 was taken as statistically significant.

The infection assay revealed that Factor H significantly increased attachment of pneumococci to host cells. Apparently Factor H mediated adherence is a general mechanism as this effect was observed for several human cell lines including epithelial and endothelial cells (Figure 43A and 43B). Approximately 6 fold increase in pneumococcal adherence to Detroit 562 cells was observed in the presence of bacteria-bound Factor H. Similar results were obtained for A549 cells, where about 5 fold increase in adherence was observed. A 16 fold increase, the maximum increase in Factor H mediated adherence was observed for HBMEC cells.

The confocal laser scanning microscopic images of the infection assays (Figure 43B) clearly demonstrate the increase in pneumococcal adherence to host epithelial and endothelial cells upon preincubation of pneumococci with human Factor H. In conclusion, cell culture infection assays demonstrated a significant role of bacteria-bound Factor H in pneumococcal adherence independent of the cell type.

4.10.2. Factor H facilitates invasion by *S. pneumoniae* of host cells

Pneumococcal preincubation with Factor H facilitates pneumococcal adherence to host epithelial and endothelial cells. However, the role of increase in adherence upon pneumococcal internalization was still not clear. Therefore, internalization of *S. pneumoniae* (NCTC10319) to human cells was quantitated by employing the antibiotic protection assay.

Briefly, attached bacteria were killed by antibiotics and internalized bacteria were recovered and plated on blood agar plates.

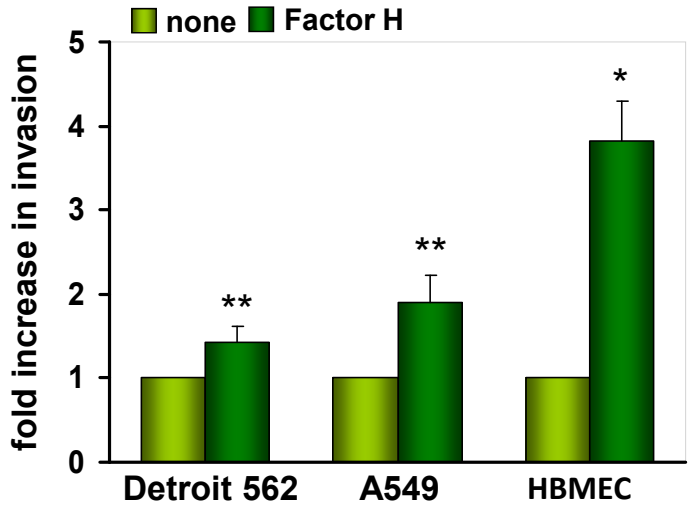


Figure 44 Pneumococcal surface-bound Factor H mediates bacterial invasion of host cells. Invasion and intracellular survival of *S. pneumoniae* NCTC10319 in host cells was determined by the antibiotic protection assay. Results are shown as the fold increase in the invasion of pneumococci that were pretreated with Factor H relative to untreated pneumococci. * $P< 0.005$ and ** $P< 0.02$ relative to infections conducted in the absence of Factor H.

Cell lines	fold invasion by *S. p.* serotype 35A		
	none	Factor H [3 µg]	*p* value
Detroit 562	1	1.42 ± 0.21	0.022
A549	1	1.91 ± 0.31	0.027
HBMEC	1	3.82 ± 0.47	0.00014

Table 23 Fold increase in Factor H mediated pneumococcal invasion of host cells. Invasion and intracellular survival of *S. p.* 35A in host cells was determined by the antibiotic protection assay. Results are shown as the fold increase in the invasion of pneumococci that were pretreated with Factor H relative to untreated pneumococci. *P* value less than 0.05 was taken as statistically significant.

The result revealed that bacterial pretreatment with Factor H significantly increased the number of internalized pneumococci (Figure 44). However, the increase in pneumococcal uptake was lower as compared to the increase in adherence. Although pretreatment with Factor H resulted in about 6 fold increase in adherence, bacterial uptake increased by only about 1.4 fold for Detroit 562 cells. For A549, bacterial uptake in host cells was 1.9 fold higher compared to approximately 5 fold increase in adherence. However, similar to increase in adherence, HBMEC showed the maximum increase with a 3.82 fold number of internalized pneumococci upon pretreatment with Factor H compared to untreated bacteria. These results

suggest that bacterial bound Factor H plays a pivotal role for adhesion and influences internalization.

4.10.3. Interference of the capsular polysaccharide on Factor H-mediated adherence to host cells

The capsular polysaccharide (CPS) of pneumococci has been shown to interfere with bacterial adherence to host cells (Hammerschmidt *et al.*, 2005). In order to elucidate whether the CPS affects the Factor H-mediated adherence of pneumococci to host cells, adherence of wild-type TIGR4 was compared with that of the CPS-deficient mutant TIGR4Δ*cps*. Infection assays were performed after preincubating 1 x 10^7 pneumococci with 2 µg of Factor H. Detroit 562 cells were infected with pneumococci using a MOI of 50 per host cell for 3 h at 37°C under 5 % CO_2. Following the infection, cells were washed and fixed for immunofluorescence staining. The adherence of pneumococci was estimated by counting about 50 cells under fluorescence microscope.

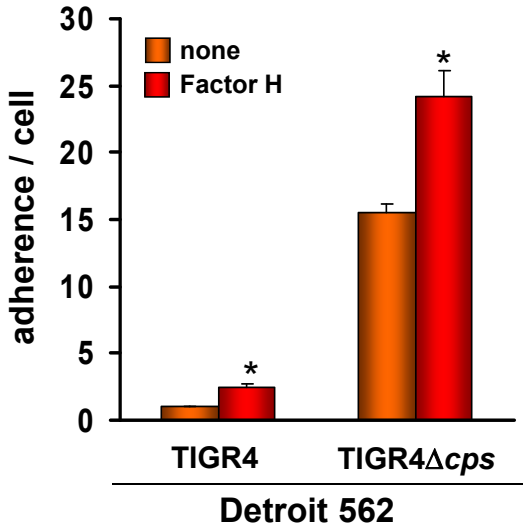


Figure 45 Role of capsular polysaccharide on Factor H mediated bacterial adherence to host cells. Factor H mediated adherence of wild-type pneumococcal strain TIGR4 and its non-encapsulated mutant TIGR4Δ*cps* to Detroit 562 nasopharyngeal epithelial cells as determined by immunofluorescence microscopy.

***S. pneumoniae* strain**	**adherent bacteria per cell (Detroit 562)**		
	none	**Factor H [3 µg]**	*p* value
TIGR4	0.97 ± 0.015	2.4 ± 0.28	0.00089
TIGR4 Δ*cps*	15.52 ± 0.63	24.18 ± 1.89	0.0017

Table 24 Number of adhered bacteria per cell. Attachment of wild-type pneumococcal strain TIGR4 and its non-encapsulated mutant TIGR4Δ*cps* to Detroit 562 after 3 h infections was determined by immunofluorescence microscopy. The infection assays were conducted with or without the preincubation of pneumococci with 3 µg of Factor H. *P* value less than 0.05 was taken as statistically significant.

As observed earlier for other pneumococcal strains (Hammerschmidt *et al.*, 2005) , genetic removal of the CPS increased the number of host-cell attached TIGR4Δ*cps* when compared to the encapsulated wild-type TIGR4 (Figure 45). Similar to the results with NCTC10319, pre-treatment of the non-encapsulated TIGR4Δ*cps* with Factor H increased significantly adherence (Figure 45). Although pre-treatment of wild-type TIGR4 with Factor H slightly enhanced adherence, the absolute values of host cell bound pneumococci remained low (Figure 45). These data demonstrate that the Factor H binding protein PspC is at least partially concealed below the CPS and that bacteria-bound Factor H plays a significant role in pneumococcal virulence.

4.11. Inhibition of Factor H-mediated pneumococcal adherence to host epithelial cells via N-terminal PspC fragments.

Pneumococcal PspC uses two different epitopes for binding the soluble host complement regulator Factor H and the secretory component of pIgR (Dave *et al.*, 2004). The Factor H binding residues of the subgroup II PspC11.4 protein (Hic) were mapped to residue 29-269 (Janulczyk *et al.*, 2000). Whereas, Hammerschmidt *et al.* (2007) mapped the Factor H binding site within the pneumococcal subgroup I PspC protein to a 121-aa-long stretch positioned in the N-terminus (residues 38-158).

To confirm the role of surface-attached Factor H in pneumococcal adhesion via binding to the very N-terminal part of PspC, blocking experiments were performed. PspC deletion products, PspC SH3 and PspC SM1 were constructed and employed in inhibition studies. Binding of Factor H to viable pneumococci in competitive inhibition experiments was tested using flow cytometry. Pneumococci (5×10^7) in 100 µl PBS were incubated with Factor H, for 30 min at 37°C, in the absence or presence of PspC proteins which were used as competitors. Binding of Factor H to pneumococci in the presence of SH3 domain of PspC or PspC SM1 was detected by flow cytometry using a FACSCalibur (Becton Dickinson).

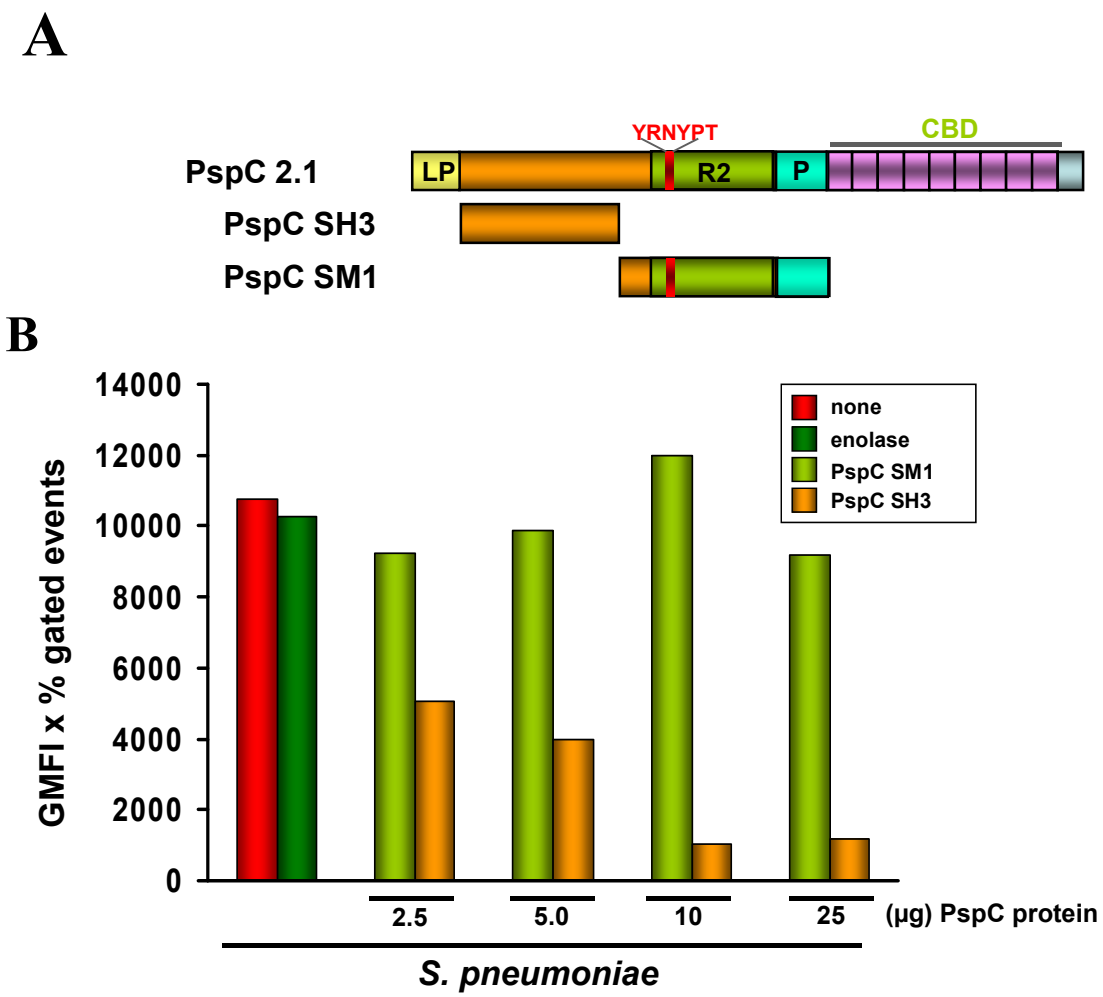


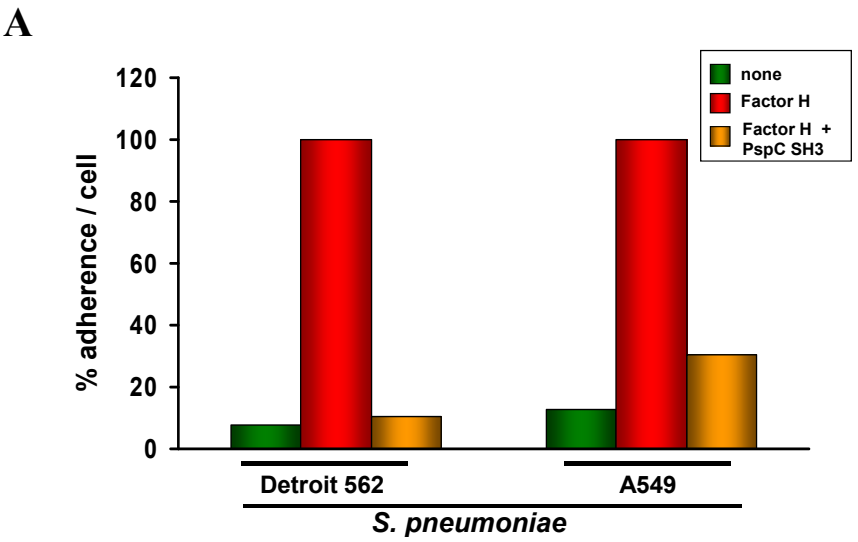
Figure 46 Blocking of Factor H binding to pneumococci by PspC. (A) Schematic models of PspC deletion constructs. PspC SH3 contains the Factor H-binding epitope, whereas, PspC SM1 represents the SC-binding R domain of PspC. LP: leader peptide; CBP: choline-binding domain; P: proline-rich sequence; R: R domain. (B) Competitive inhibition experiments. Factor H (2 µg) binding to pneumococci (NCTC10319) was measured in the absence of exogenous added PspC proteins, in the presence of PspC derivatives as indicated (Figure 42A) and also in the presence of His_6-enolase (10µg) of *S. pneumoniae* which was used as control protein. Binding of Factor H was determined by flow cytometry and results were expressed as GMFI x percentage of FITC-labeled and gated bacteria.

Protein concentration [µg]	GMFI x % FITC labeled and gated event			
	Factor H [2 µg]			
	none	PspC SM1	Pspc SH3	Enolase
0	10780.11	-	-	-
2.5	-	9212.54	5035.19	-
5	-	9852.5	3958.74	-
10	-	12004.19	9179.82	10278.16
25	-	1027.98	1196.09	-

Table 25 Quantification of Factor H binding to pneumococci in the absence of exogenous added PspC proteins or presence of PspC derivatives and also in the presence of His_6-enolase (10µg) of *S. pneumoniae* by flow cytometry

The flow cytometric analysis demonstrated competitive inhibition of Factor H binding to pneumococci by PspC protein SH3, which contains the Factor H-binding epitope (Figure 46B). In contrast, the PspC derivative SM1, which represents the SC-binding R domain of PspC and lacks the Factor H binding region, showed no inhibitory effect (Figure 46B)

In addition, the role of PspC derivative SH3 was assessed for its ability to inhibit Factor H-mediated adherence of pneumococci in cell culture infection assays. Host cells were infected for 3 h with pneumococci that had been pretreated with a mixture of Factor H and the PspC derivative SH3. The effect of PspC derivative SH3 on pneumococcal adherence to eukaryotic host cells was estimated by counting approximately 50 host cells using immunofluorescence microscope.



B

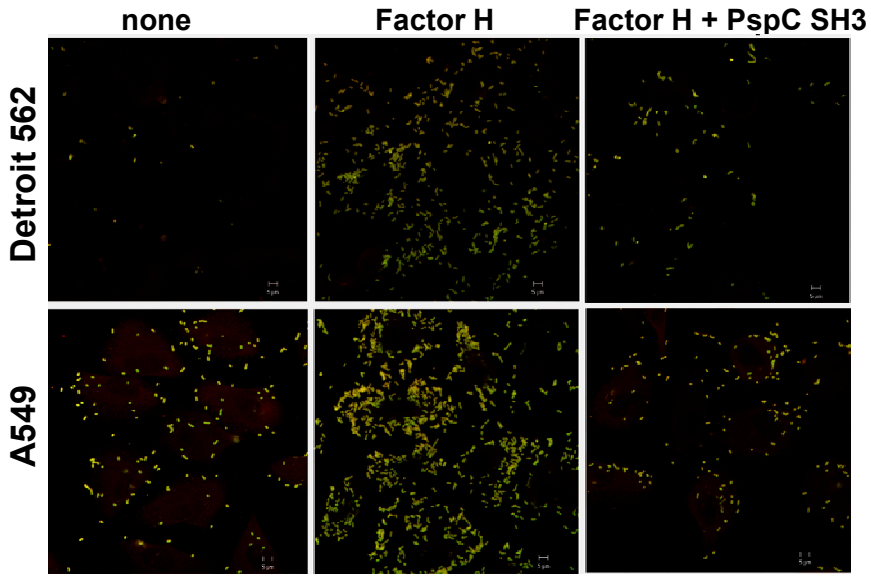


Figure 47 Blocking of Factor H-mediated adherence to host cells by PspC. (A) In cell culture blocking experiments PspC SH3 (2.5µg) was used. Adherence of *S. pneumoniae* in the presence of Factor H was set to 100%. The results revealed an inhibitory effect of PspC SH3 on Factor H-mediated pneumococcal attachment to host cells. Results are from a representative experiment. Inhibition studies were performed at least three times with similar inhibition patterns. (B) Immunofluorescence microscopy of pneumococci attached to host cells when PspC SH3 was used to block Factor H-mediated adherence.

Preincubation with Factor H [µg]	**adherent bacteria *S. p.* serotype 35A per cell [%]**	
	Detroit 562	**A549**
0	7.72	12.94
2	100	100
2 + PspC SH3 (2.5 µg)	10.68	30.41

Table 26 Percentage adherence of *S. p.* 35A to epithelial cells. Blocking of Factor H-mediated pneumococcal attachment to host epithelial cells by PspC-SH3 derivative as determined by immunofluorescence microscopy.

The result showed that the Factor H-binding domain SH3 of PspC inhibited Factor H-mediated pneumococcal adhesion to host cells (Figure 47A and 47B). In conclusion, inhibition experiment confirmed the specific interaction of N-terminal residues of PspC for Factor H-mediated pneumococcal adherence to host cells.

4.12. Characterization of the host cellular receptor for Factor H mediated pneumococcal adherence

Factor H binds to cell surfaces of host cells via polyanionic cell surface such as proteoglycans, sialic acids, heparansulfate chains or glycosaminoglycans (Meri and Pangburn, 1990; Jokiranta *et al*., 2006; Manuelian *et al.*, 2003). Moreover, Factor H splicing variant Factor H-like protein 1 (FHL-1), which consists of the first seven SCR, bind via an RGD sequence of SCR4 to host cells thus showing cell adhesion activity. Factor H binding was demonstrated to interfere with fibronectin binding, suggesting that both molecules may utilize identical cellular receptors (Hellwage *et a l.*, 1997). Similarly, human PMNs bind to immobilized Factor H via integrin CD11b/CD18, also termed CR3 (DiScipio *et al.*, 1998). In contrast, binding to human endothelial cells is mediated via the heparin/glycosaminoglycan-binding site within SCR20 of Factor H (Jozsi *et al.*, 2006; Jokiranta *et al.* 2006, Cheng *et al.*, 2006).

Factor H interacts with the pneumococcal PspC protein via two contact sites, which were localized to SCR8-11 and SCR19-20 (Hammerschmidt *et a l.*, 2007). This interaction improves survival by inhibiting complement mediated lysis of the bacteria, and promotes pneumococcal adherence to and invasion of host cells. However, the molecular mechanism that facilitates uptake of Factor H loaded pneumococci by epithelial cells is still unresolved. Moreover the potential receptors modulating the interaction of bacteria-bound Factor H with the host cell surface have not been characterized yet.

4.12.1. Role of pneumococcal surface bound Factor H on association with PMNs

Avery and Gordon (1993) have shown that the 38 kDa N-terminal tryptic fragment of Factor H binds to PMNs via an association with integrin $\alpha_M\beta_2$ also referred as CD11b/CD18, Mac-1, and CR3. In accordance, DiScipio RG et al. (1998) suggested that PMNs can bind to immobilized Factor H via integrin CD11b/CD18 (α_M/β_2). Moreover, the recruitment of Factor H to the surface of pneumococci was shown to efficiently prevent activation of C3b and complement mediated opsonophagocytosis of pneumococci (Jarva *et al.* , 2004). Therefore, the role of Factor H in pneumococcal association with PMNs was investigated in more details.

The role of integrin CD11b/CD18 as possible receptor for bacterial-bound Factor H was investigated in inhibition studies. The pH-regulated Antigen 1 (Pra1p) that is released by *Candida albicans* and is a major ligand for leukocyte integrin CD11b/CD18 (α_M/β_2) (Soloviev *et al.*, 2007) was employed as a competitive inhibitor during PMNs infection by Factor H

coated pneumococci. In addition, integrin CD11b and CD18 specific mAbs, anti-CD11b and anti-CD18, were employed as blocking molecules. PMNs were preincubated with 2 µg/ml Pra1p or mAbs anti-CD18 or anti-CD11b for 30 min and then infected with pneumococci pretreated with or without Factor H. Pneumococcal association with PMNs was monitored by flow cytometry.

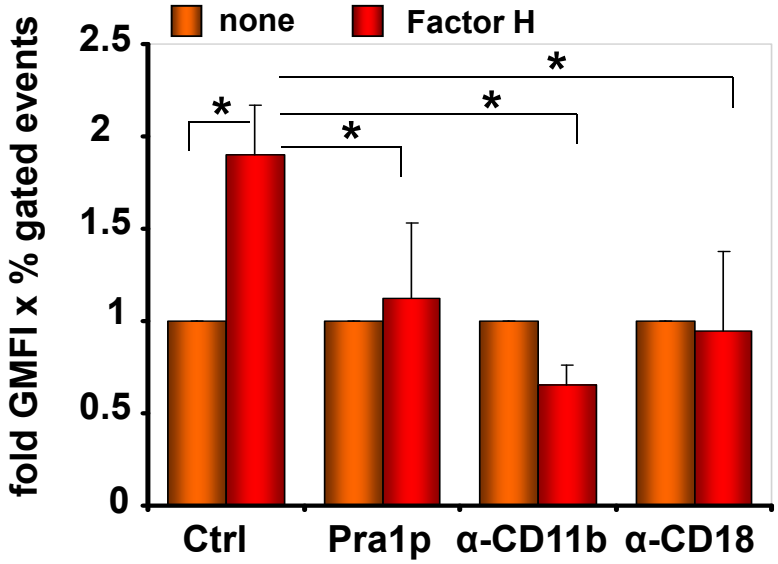


Figure 48 Factor H promotes pneumococcal association with PMNs via integrin CD11b/CD18. Pneumococci were incubated with PMNs for 30 min, in the absence (control, Ctrl) or presence of Pra1p (2 µg), anti-CD11b (2 µg) or anti-CD18 (2 µg). Pneumococcal association with PMNs was investigated in the absence (none) or presence of bacteria-bound Factor H by flow cytometry. The results are expressed as fold GMFI x percentage of FITC-labeled and gated bacteria, relative to pneumococci not pretreated with Factor H.

Treatment of PMNs	**fold GMFI x % FITC labeled and gated event**		
	none	**Factor H**	*p* value
control	1	1.9 ± 0.27	0.0012
Pra1p (2 µg)	1	1.13 ± 0.4	0.49
p value relative to control		0.017	
anti-CD11b (2 µg)	1	0.66 ± 0.11	0.0013
p value relative to control		0.00045	
anti-CD18 (2 µg)	1	0.95 ± 0.42	0.79
p value relative to control		0.0098	

Table 27 Association of Factor H coated pneumococci with PMNs in the absence or presence of Pra1p, anti CD11b or anti-CD18, as determined by flow cytometry

Pretreatment of pneumococci with Factor H significantly increased association of the bacteria with PMNs (Figure 48). A 2 fold increase in pneumococcal association with PMNs was determined in the presence of Factor H compared to untreated pneumococci. Inhibition

experiments using Pra1p, anti CD11b or anti CD18 antibodies as blocking substances demonstrated inhibition of Factor H-mediated association of *S. pneumoniae* with PMNs (Figure 48). Although presence of Pra1p blocked association of Factor H-coated pneumococci with PMNs, inhibition was more prominent in presence of blocking antibodies, suggesting the presence of additional cell surface receptor(s) of Pra1-p protein. In conclusion, PMNs assay demonstrates the role of integrin CD11b/CD18 as a receptor for Factor H bound to pneumococci.

4.12.2. Role of integrin CD11b/CD18 as a host cell surface receptor for bacteria-bound Factor H

The integrin CD11b/CD18 also referred to as Mac-1 or CR3, belongs to the β_2 integrin subfamily and is a heterodimer composed of an alpha (α_M or CD11b) and a beta (β_2 or CD18) subunit. This integrin recognizes a variety of molecules thereby inducing various functions within eukaryotic cells. Moreover, PMNs bind to immobilized Factor H via integrin CD11b/CD18 (α_M/β_2) i.e. CR3 (DiScipio *et al.*, 1998).

To investigate the role of integrins as a potential host cell receptor for Factor H mediated invasion of *S. pneumoniae*, CHO cell stably transfected with cDNA for full length human CD11b and CD18 (Ingalls *et al.*, 1997) were employed in infection assays. As a control non-transfected CHO-K1 cells were engaged in the infection assays. Pneumococcal adherence was followed by immunofluorescence microscopy.

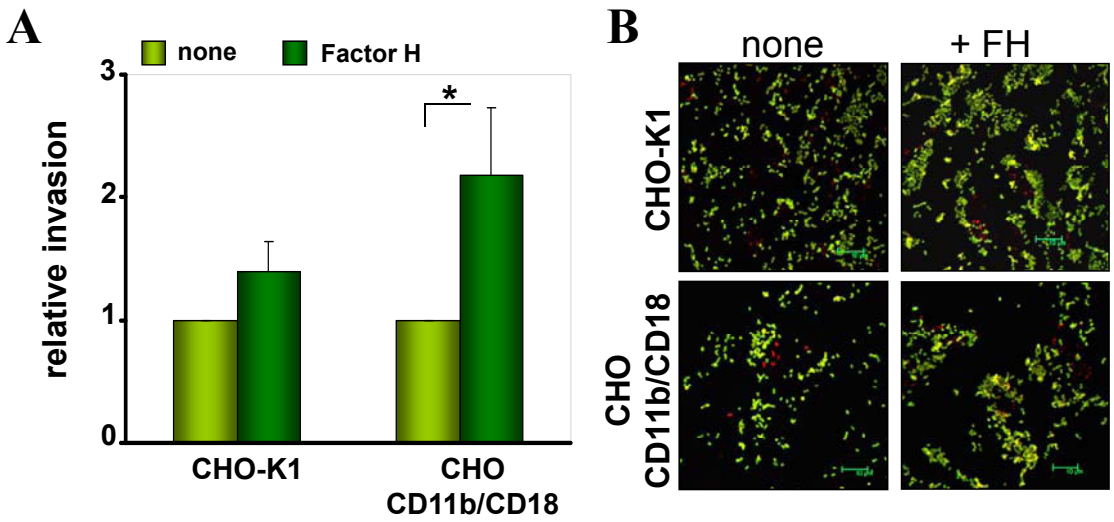


Figure 49 Interaction of pneumococcal bound Factor H with integrin CD11b/CD18 promotes pneumococcal invasion of epithelial cells. A, invasion and intracellular survival of pneumococci in CHO-K1 and CHO cells stably expressing CD11b/CD18, was determined by the antibiotic protection assay. The results are shown relative to pneumococci not pretreated with Factor H. B, Immunofluorescence microscopy of adherent pneumococci. * $P< 0.02$

Cell Line	fold invasion by *S. p.* serotype 35A		
	none	**Factor H**	*p* value
CHO-K1	1	1.39 ± 0.25	0.051
CHO CD11b/CD18	1	2.18 ± 0.55	0.019

Table 28 Relative invasion of epithelial cells by *S. p*. 35A. Pneumococcal uptake by CHO-K1 and CHO cells stably expressing CD11b/CD18 as determined by the antibiotic protection assay. The infection assays were conducted with or without the preincubation of pneumococci with Factor H. *P* value less than 0.05 was taken as statistically significant.

The result showed that pretreatment with Factor H significantly increased the number of internalized bacteria in CHO cells stably transfected for integrin CD11b/CD18 (Figure 49A); while only a minor increase of internalized bacteria was observed for CHO-K1 cells. In the presence of integrins CD11b/CD18 and bacteria bound Factor H a 2.18 ± 0.55 fold increase of internalized bacteria was measured, whereas CHO-K1 cells showed only a 1.39 ± 0.25 fold increase of pneumococcal invasion as compared to infection carried out with untreated *S. pneumoniae*. CSLM images depict the effect of Factor H on pneumococcal adherence to both CHO-K1 and CHO CD11b/CD18 cell line (Figure 49B).

The results demonstrated that absence of integrin CD11b/CD18 could not completely block Factor H-mediated pneumococcal invasion of host cells, suggesting the existence of additional host cell surface receptor(s) for bacterial bound Factor H.

The integrins CD11b/CD18 are primarily thought to be expressed on polymorphonuclear leukocytes (PMNs), on activated leukocytes, on monocytes and macrophages and are involved in various functions (Wagner *et al.* , 2001). In contrast, Sandilands and Whaley, (1985), Edwards *et al.* (2001) and Hussain *et al.* (1995) demonstrated the expression of CR3 in renal glomerular, human cervical, and rectal epithelial cells, respectively. However, there are no published reports demonstrating the expression of CR3 in lung epithelial cells A549.

PMNs infection assays using Pra1p protein as an inhibitor, suggested that Pra1p could also interact with other host cell surface receptor(s) in addition to CD11b/CD18. Therefore, the inhibitory role of Pra1p was investigated in cell culture infection assays using lung epithelial cells A549 and *S. pneumoniae*. The ability of Pra1p to block Factor H-mediated pneumococcal adherence to or ingestion by host epithelial cells, A549 cells were preincubated with 2 µg/ml Pra1p (30 min) and Factor H pretreated pneumococci were used to infect A549 cells after removal of free or unbound Pra1p protein. The total number of adherent plus intracellular pneumococci was estimated after 3 h infection, after plating the bacteria on blood

agar plates and counting the bacterial cfu obtained per well. To quantify internalized pneumococci, the antibiotic protection assay was performed. Simultaneously, infection samples were also prepared for immunofluorescence microscopy to visualize differences in pneumococcal adherence to host cells.

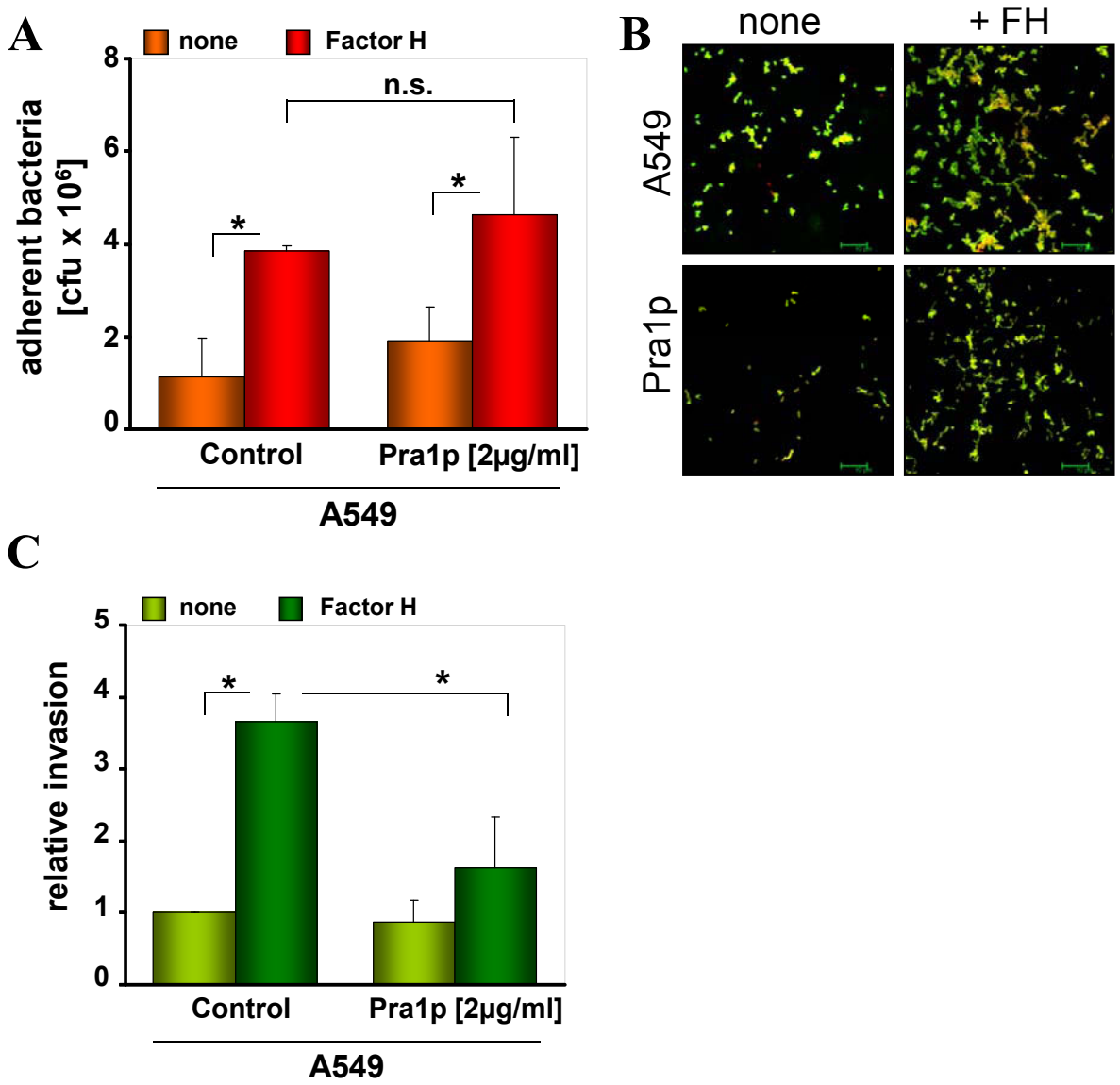


Figure 50 Blocking of Factor H mediated pneumococcal invasion of epithelial cells using integrin CD11b/CD18 interacting protein Pra1p. A, adherence of *S. pneumon iae* strain NCTC10319 (serotype 35A) to A549 cells was determined in the presence of Pra1p (2 µg/ml) or absence of Pra1p. The infection assays were conducted with or without pretreatment of pneumococci with Factor H. The inhibitory effect of Pra1p was assessed after 3 h of infection by counting the cfu per well obtained from plating onto blood agar plates. B, Immunofluorescence microscopy of adherent pneumococci. C, invasion and intracellular survival of pneumococci in presence of Pra1p was determined by the antibiotic protection assay. The results are shown relative to infections conducted by Factor H untreated pneumococci (control). * $P< 0.03$

Treatment of A549 cells	adherent bacteria [cfu x 10^6] per 1x 10^5 cells		
	none	Factor H	*p* value
control	1.13 ± 0.83	3.86 ± 0.11	0.0013
Pra1p (2 µg/ml)	1.91 ± 0.74	4.62 ± 1.69	0.023
p value relative to control	0.16	0.33	

Table 29 Number of pneumococci attached to A549 cells was determined in the presence or absence of Pra1p (2 µg/ml) after 3 h of infection. The infection assays were conducted with or without pretreatment of pneumococci with Factor H. *P* value less than 0.05 was taken as statistically significant.

Treatment of A549 cells	fold invasion by *S. p.* serotype 35A		
	none	Factor H	*p* value
control	1	3.65 ± 0.39	0.00031
Pra1p (2 µg/ml)	0.87 ± 0.3	1.62 ± 0.71	
p value relative to control	0.51	0.012	

Table 30 Relative invasion of Pra1p pretreated A549 cells by Factor H-coated pneumococci. Invasion of A549 cells by *S. p.* 35A as determined in the presence of Pra1p by the antibiotic protection assay. Prior to infections, pneumococci were pretreated with or without Factor H. The results are shown relative to Factor H untreated pneumococcal infections (control). *P* value less than 0.05 was taken as statistically significant.

The competitive inhibition experiments with Pra1p showed no inhibition of Factor H mediated pneumococcal adherence to A549 cells (Figure 50A). In contrast, preincubation of A549 epithelial cells with Pra1p significantly reduced the Factor H-mediated pneumococcal invasion of host cells (Figure 50C).

The pretreatment of pneumococci with Factor H significantly increased the number of adhered bacteria. Similarly a significant increase in pneumococcal adherence was observed when host cells were pretreated with Pra1p and Factor H-coated pneumococci were used to infect the cells. These results were confirmed by immunofluorescence microscopy, as demonstrated in the images (Figure 50B). In contrast, figure 50C showed that the increase in Factor H-mediated pneumococcal invasion significantly reduced from 3.65 ± 0.39 fold to 1.62 ± 0.71 fold in presence of Pra1p, which has been demonstrated to interact with integrin CD11b/CD18 (Soloviev *et al.* , 2007). Although, presence of Pra1p significantly blocked Factor H-mediated pneumococcal ingestion by host cells, it showed no inhibitory effect on Factor H-coated pneumococcal adherence to host epithelial cells. Thus the results once again confirmed the existence of additional host cell surface receptor(s) that may interacts with Pra1p of *Candida albicans* . Further investigations are required to characterize the new receptor(s) for Pra1p in order to understand the pathogenesis of *Candida albicans*.

4.12.3 Effect of glycosaminoglycans on Factor H mediated pneumococcal adherence to and invasion of host cells

4.12.3.1. Heparin inhibits Factor H mediated pneumococcal adherence to host epithelial cells

Factor H is a heparin binding protein with three distinct binding sites present in SCR7, SCR13 and SCR19-20 (Pangburn *et al.*, 1991; Blackmore *et al.*, 1996, 1998). In order to investigate whether bacteria-bound Factor H engages host cell surface glycosaminoglycans such as heparin, to promote adherence of bacteria, competitive inhibition experiments were performed. Infection assays were carried out in the presence of soluble heparin (50 U/ml) or after pretreatment of the cells with (10 mU/ml) heparinase III, an enzyme that cleaves only heparin sulfate and not low molecular weight heparin. Prior to the infection with pneumococci, bacteria (1 x 10^7) were preincubated with 2 µg of Factor H for 30 min at 37°C. A549 cell were infected for 3 h with pneumococci with a MOI of 50. The number of bacteria attached to the host cell was quantified by plating onto blood agar plates. Simultaneously samples were also prepared for immunofluorescence microscopy.

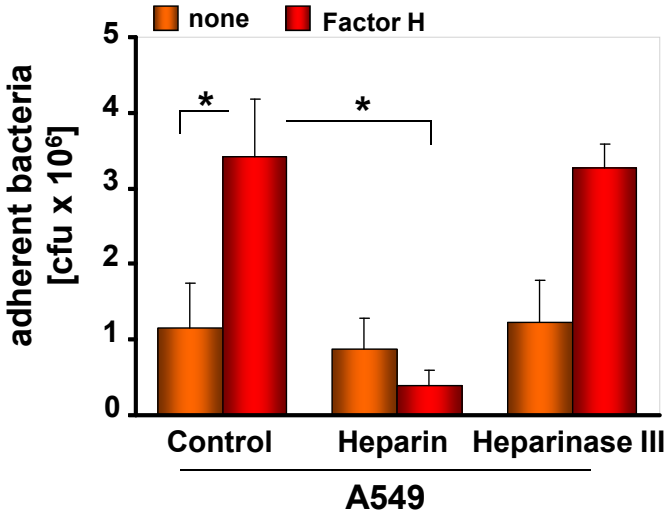


Figure 51 Blocking of Factor H mediated pneumococcal adherence to host cells by heparin. Adherence of Factor H bound pneumococcal strain NCTC10319 (serotype 35A) to A549 cells in the absence (control) or presence of heparin (50 U/ml) or after pretreatment with heparinase III (10 mU/ml) was estimated by quantifying the cfu per well obtained from plating onto blood agar plates. The infection assays were conducted with or without (none) pretreatment of pneumococci with Factor H. * $P< 0.02$

Treatment of A549 cells	adherent bacteria [cfu x 10^6] per 1x 10^5 cells		
	none	Factor H	*p* value
control	1.16 ± 0.59	3.42 ± 0.76	0.015
Heparin (50 U/ml)	0.87 ± 0.42	0.39 ± 0.21	0.14
p value relative to control	0.52	0.0026	
Heparinase III (10 mU/ml)	1.22 ± 0.57	3.27 ± 0.32	0.0055
p value relative to control	0.904	0.77	

Table 31 Inhibition of Factor H mediated *S. p.* 35A adherence to A549 cells. Number of Factor H-coated pneumococci attached to A549 cells estimated after quantifying the cfu per well obtained from plating onto blood agar plates. The assays were performed in the absence (control) or presence of heparin (50 U/ml) or after pretreatment of epithelial cells with heparinase III (10 mU/ml) and with or without (none) pretreatment of pneumococci with Factor H. *P* value less than 0.05 was taken as statistically significant.

Host cell infection performed in the presence of heparin significantly reduced the Factor H mediated adherence of pneumococci to epithelial cells (Figure 51). However, there was no significant effect on the basal level of pneumococcal attachment to epithelial cells. The results indicate that the heparin binding sites of Factor H, which is bound to bacteria, are involved in pneumococcal attachment to host cells via Factor H. In contrast, heparinise III pretreatment of host cells had no effect on Factor H-mediated adherence of pneumococci.

To evaluate the effect of heparin and heparinase III on internalization of pneumococci, antibiotic protection assays were performed. The increase or decrease in invasion was calculated relative to the basal level of pneumococcal invasion which was monitored when infection were carried out without preincubation of bacteria with Factor H and in the absence of inhibitors or enzymes.

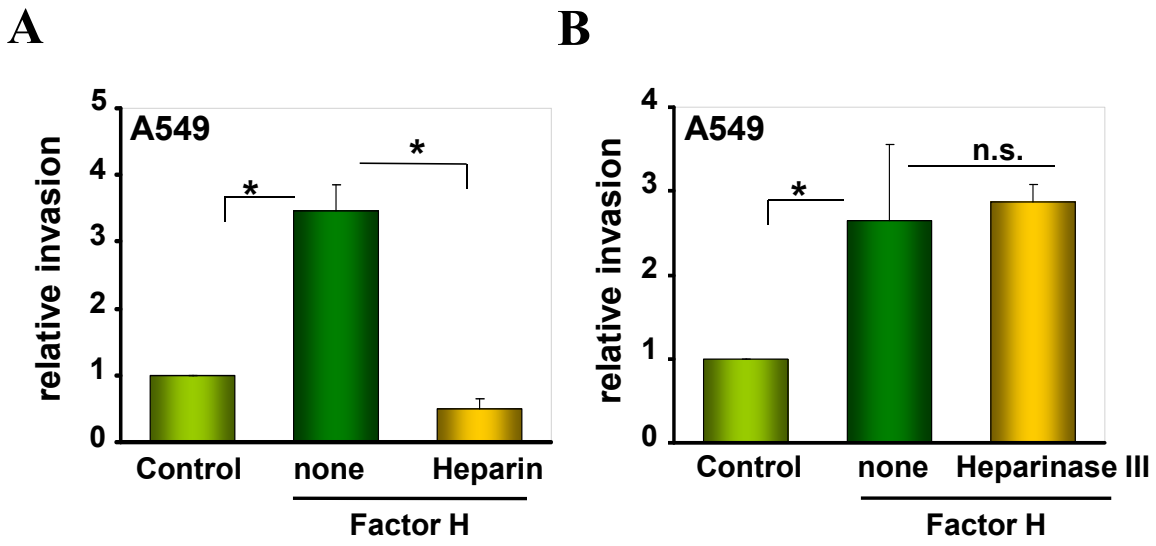


Figure 52 Inhibition of Factor H mediated pneumococcal invasion of host cells by heparin. Invasion and intracellular survival of Factor H bound pneumococcal strain NCTC10319 (serotype

35A) in A549 cells in the absence (none) or presence of heparin (50 U/ml) (A) or after pretreatment with heparinase III (10 mU/ml) (B) was determined by employing the antibiotic protection assay. The infection assays were conducted with or without pretreatment of pneumococci with Factor H for 3 h at 37°C under 5 % CO_2. The results are shown as fold increase or decrease in invasion of pneumococci, relative to untreated pneumococci (control). * $P< 0.02$

Treatment of A549 cells	fold invasion by *S. p.* serotype 35A		
	without	Factor H	*p* value
control	1	3.47 ± 0.39	0.00041
Heparin (50 U/ml)		0.49 ± 0.16	
p value		0.00027	
control	1	2.64 ± 0.91	0.011
Heparinase III (10 mU/ml)		2.87 ± 0.21	
p value		0.58	

Table 32 Blocking of Factor H-coated pneumococcal uptake by host epithelial cells in the presence of heparin. Number of ingested pneumococci pretreated with or without Factor H as determined by the antibiotic protection assay. The infection assays were performed in the absence (control) or presence of heparin (50 U/ml) or after pretreatment of A549 cells with heparinase III (10 mU/ml). The results are shown as fold increase or decrease in invasion of pneumococci, relative to pneumococci not pretreated with Factor H (control). *P* value less than 0.05 was taken as statistically significant.

Heparin significantly reduced the number of internalized pneumococci (Figure 52 A). In presence of Factor H a 3.47 ± 0.39 fold increase in number of invasive bacteria relative to control infected cells was calculated, which however decreased to 0.49 ± 0.16 fold in presence of heparin. The decrease in number of internalized bacteria was attributed to decrease in adherence of pneumococci in presence of heparin. In contrast, no inhibitory effect was measured for host cells pretreated with heparinase III (Figure 52 B).

4.12.3.2. Heparin interacts with Factor H but do not influence its recruitment by pneumococci

The results of cell culture infection experiments, figure 52, showed that the presence of heparin reduced significantly Factor H mediated adherence of pneumococci to epithelial cells. There exists also the possibility that this decrease in adherence is a result of lower amount of bacteria recruited Factor H. Therefore, the effect of heparin on the binding of Factor H to pneumococci was investigated. First the interaction of bacteria-bound Factor H with heparin was analyzed by flow cytometry. One times 10^7 pneumococci were preincubated with 1 µg FH for 30 min at 37°C, washed and incubated with 2 µg heparin-FITC (Invitrogen) for 30 min at 37°C. Bacteria incubated with heparin-FITC were taken as control and the

association of heparin was analyzed by flow cytometry using a FACS CantoI (Becton Dickinson).

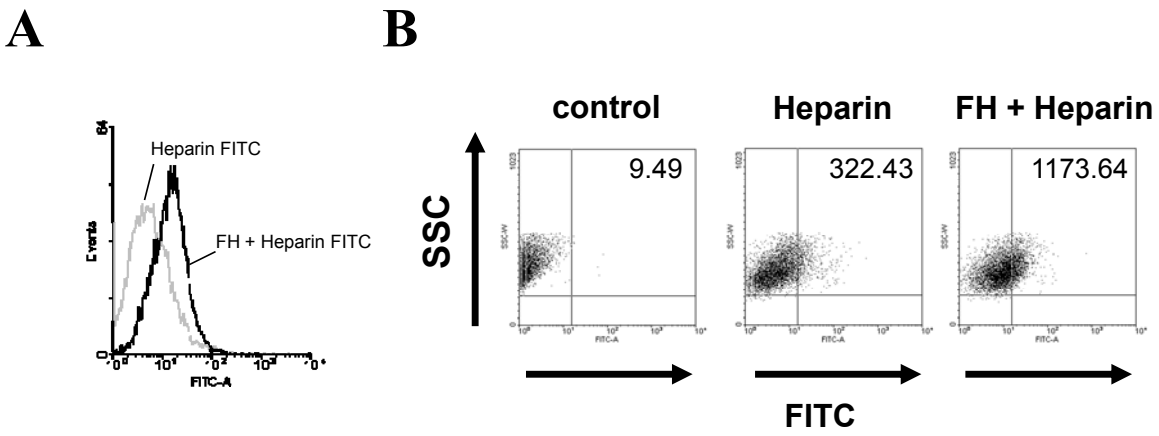


Figure 53 Interaction of pneumococcal surface bound Factor H with heparin. Association of heparin -FITC (2 µg) with pneumococcal surface bound Factor H was determined by flow cytometry and results were expressed as GMFI x percentage of FITC-labeled and gated bacteria bacteria (A) or represented as dot plots (B), where the *x*-axis represents fluorescence of associated heparin (FITC) on a $\log_{10}$ scale and the *y*-axis represents the number of events (SSC).

The flow cytometric analysis confirmed the interaction of heparin with Factor H that was bound to the surface of pneumococci (Figure 53 A and B). Thus confirming that Factor H bound to the surface of pneumococci utilizes its heparin binding site for interaction with the host cell surface heparin.

Secondly binding of Factor H to pneumococci was investigated in presence of heparin and analysed by flow cytometry. Factor H (2 µg) was pre incubated with increasing amounts of heparin and this protein solution was used for binding assays with pneumococci. Bacteria were incubated for 30 min at 37°C and bound of Factor H was detected by flow cytometry using a FACS CantoI (Becton Dickinson).

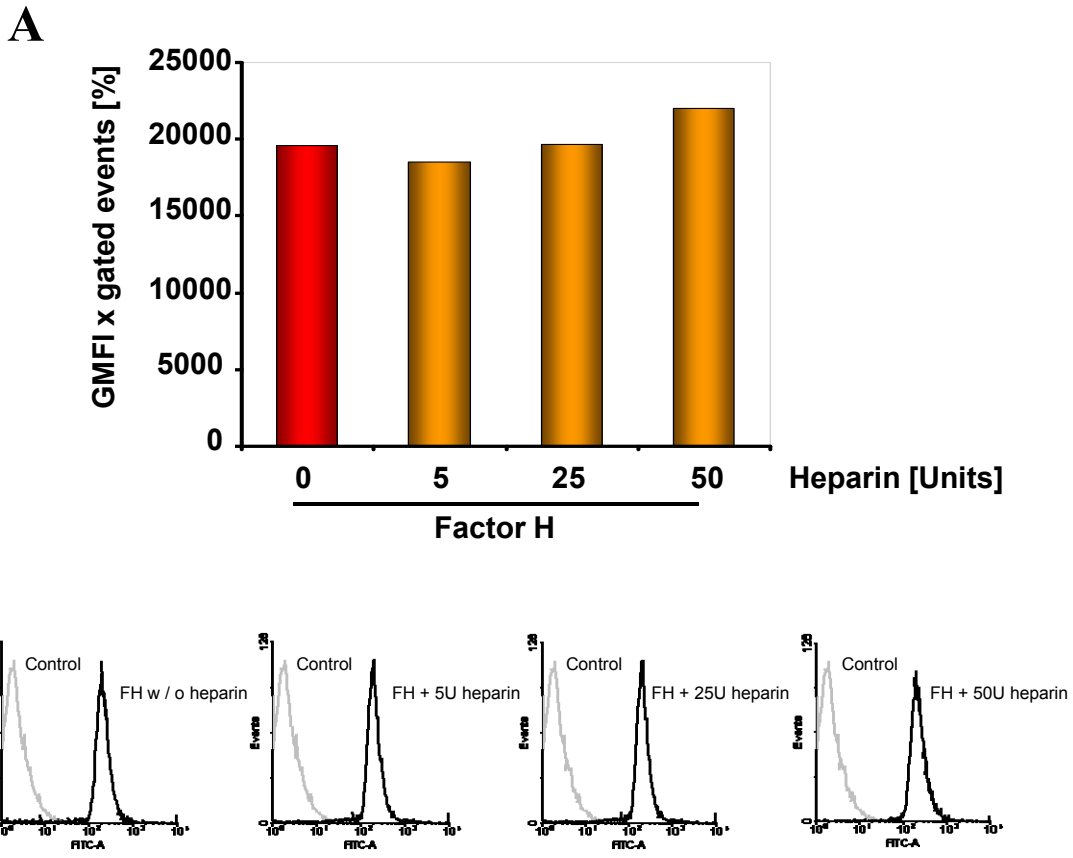


Figure 54 Binding of heparin treated Factor H to pneumococci. The effect of heparin on Factor H binding to pneumococci was investigated by preincubating 2 µg purified Factor H with indicated amounts of heparin followed by incubation with pneumococci. The binding of Factor H was determined by flow cytometry and results were expressed as GMFI x percentage of FITC-labeled and gated bacteria bacteria (A) or represented as graphs (B), where the *x*-axis represents fluorescence of associated Factor H on a $\log_{10}$ scale and the *y*-axis represents the number of events.

Heparin [U]	GMFI x % FITC labeled and gated event Factor H [2 µg]
0	19556.11
5	18521.11
25	19634.43
50	21992.83

Table 33 Quantification of Factor H binding to *S. p.* 35A in presence of heparin by flow cytometry

The data demonstrated that pretreatment of Factor H with heparin does not affect the interaction between pneumococci and Factor H (Figure 54). In conclusion, the decrease in adherence of pneumococci to epithelial cells via the Factor H mechanism in the presence of heparin as observed in cell culture infection assays is due to blocking of the heparin binding sites in Factor H by heparin. Heparin does not influence recruitment of Factor H by

pneumococci hence indicating that the Factor H-pneumococcal interaction is mediated via other binding sites.

4.12.3.3. Dermatan sulphate inhibits Factor H mediated pneumococcal adherence to and invasion of epithelial cell

Dermatan sulphate (DS) is a sulphated glycosaminoglycan that is a constituent of various proteoglycans present on the cell surface and in the extracellular matrix (Kjellen and Lindahl, 1991; Iozzo and Murdoch 1996; Iozzo 1998). Recently it has been shown that Factor H is also a DS binding protein (Saito and Munakata, 2005). The role of DS on Factor H mediated pneumococcal adherence and invasion of epithelial cells was investigated. Therefore, infection assays were carried out in the presence of increasing concentrations (0, 50, 100, 250 µg/ml) of soluble DS. Prior to host cells infection approximately 1 x 10^7 *S. pneumoniae* serotype 35A were preincubated with 2 µg of Factor H for 30 min at 37°C. A549 host cells were then infected with pneumococci using a MOI of 50. After 3 h of infection, the total adherence was estimated by counting the cfu per well obtained from plating the sample aliquots onto blood agar plates. The internalization of pneumococci to human cells was quantitated by the antibiotic protection assay. Attached bacteria were killed by antibiotic treatment and internalized bacteria were recovered and quantitated by determination of the cfu per well after plating. The samples were also prepared for immunofluorescence microscopy.

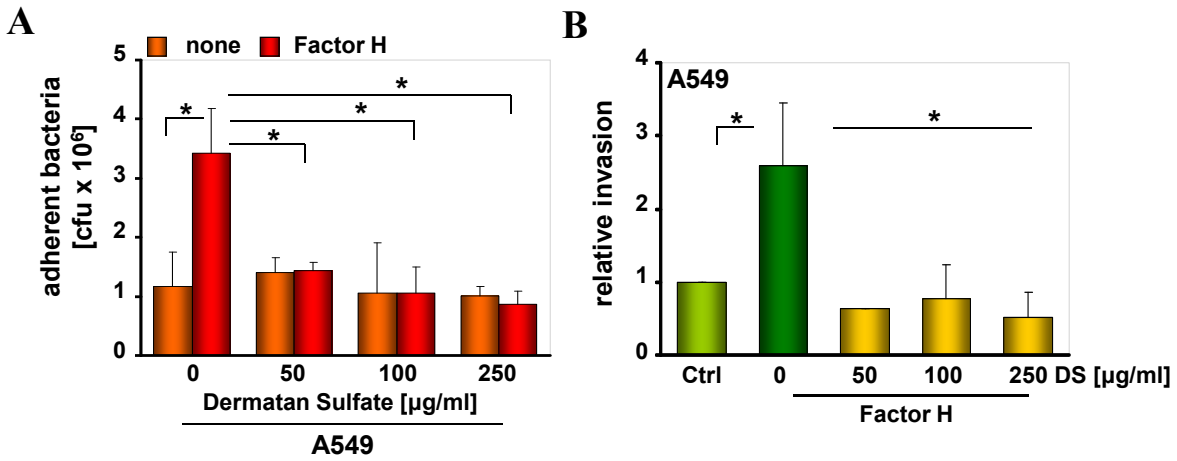


Figure 55 Blocking of Factor H-mediated pneumococcal adherence to and invasion of pneumococcal strain NCTC10319 (serotype 35A) in A549 cells by dermatan sulphate (DS). A, adherence of Factor H bound pneumococci to A549 cells in presence of indicated concentrations of DS was estimated by counting the cfu per well obtained from plating onto blood agar plates. The infection assays were conducted with or without the pretreatment of pneumococci with Factor H for 3 h at 37°C under 5 % CO_2. B, invasion

and intracellular survival of Factor H-bound pneumococci in the presence of DS was determined by the antibiotic protection assay. The results are shown as fold increase or decrease of bacterial invasion relative to untreated pneumococci (control). * $P< 0.02$

Dermatan sulphate [µg/ml]	adherent bacteria [cfu x 10^6] per 1x 10^5 A549 cells		
	none	Factor H	*p* value
0 (control)	1.16 ± 0.59	3.42 ± 0.76	0.015
50	1.41 ± 0.25	1.43 ± 0.15	0.91
p value relative to control	0.84	0.0078	
100	1.05 ± 0.86	1.06 ± 0.44	0.99
p value relative to control	0.86	0.0094	
250	1.01 ± 0.15	0.86 ± 0.23	0.39
p value relative to control	0.69	0.005	

Table 34 Inhibition of Factor H-mediated *S. p.* serotype 35A adherence to A549 cells. Number of attached pneumococci in presence of DS as estimated by quantifying the cfu per well obtained from plating onto blood agar plates. The infection assays were conducted with or without (none) pretreatment of pneumococci with Factor H. *P* value less than 0.05 was taken as statistically significant.

Dermatan sulphate [µg/ml]	fold invasion by *S. p.* serotype 35A		
	without	Factor H	*p* value
control	1	2.6 ± 0.85	0.0099
50	0.64		
100	0.78 ± 0.45		
p value relative to control	0.0096		
250	0.51 ± 0.35		
p value relative to control	0.005		

Table 35 Blocking of Factor H-coated pneumococcal invasion by DS. Invasion of Factor H-bound pneumococci in the presence of DS as determined by the intracellular survival assay. The results are shown as fold increase or decrease of bacterial invasion relative to pneumococci not pretreated with Factor H (control). *P* value less than 0.05 was taken as statistically significant.

The results revealed a significant decrease for the Factor H-mediated adherence of pneumococci to A549 cells in the presence of DS (Figure 55 A). The adherence of Factor H bound pneumococci reduced in a dose dependent manner in presence of 50, 100 and 250 µg/ml of DS. However no significant change in the basal level of pneumococcal adherence was observed in the presence of DS. The results suggest a role of host cell surface DS as a potential receptor for the bacteria-bound Factor H. Similar to adherence, Factor H mediated invasion was significantly inhibited in a dose dependent manner (Figure 55 B).

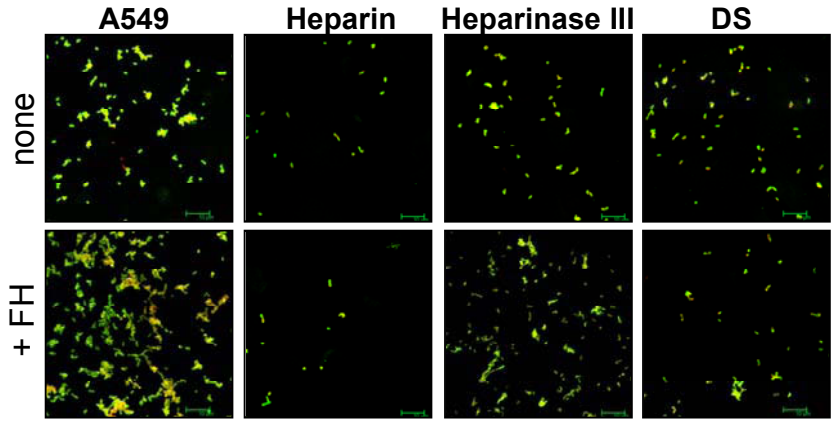


Figure 56 Illustration of effect of glycosaminoglycans on Factor H mediated pneumococcal adherence to A549 cells.

The confocal laser scanning microscope images of the infection assay (Figure 56) clearly demonstrated the inhibition of Factor H-mediated pneumococcal adherence to A549 cells in presence of heparin and DS. However, pretreatment of cell with heparinase III showed no significant decrease in the adherence. In conclusion, the cell culture infection assays demonstrated the role of heparin and DS as potential receptors for bacteria-bound Factor H in pneumococcal adherence to epithelial cell surface.

4.12.4. Pneumococcal surface bound Factor H interacts via SCR 19-20 with the host epithelial cells

Factor H is a single chain plasma glycoprotein comprising of 20 domains, referred to as short-consensus repeats (SCR), Each SCR consists of ~60 amino acids. The four N-terminal SCRs (SCR 1-4) are required for full cofactor and decay acceleration activities in fluid phase (Gordon *et al.* , 1995; Kuhn *et al.* , 1995). Moreover, the self/non-self discrimination by Factor H occurs predominantly through glycosaminoglycan binding to SCR 7 and/or 19-20 (Pangburg, 2000). Apart from this, an endothelial cells surface heparin/glycosaminoglycan-binding site has been mapped within SCR20 of Factor H (Jozsi *et al.*, 2006; Jokiranta *et al.*, 2005; Cheng *et al.*, 2006).

In order to characterize and identify the potential host cellular receptor(s) for Factor H-mediated pneumococcal adherence, inhibition experiments were performed. Monoclonal antibodies M14, M16 and CO2 were used as inhibitors in cell culture infection assays. These antibodies recognize the middle region of Factor H (M14), SCR14-18 (M16) or C-terminal of SCR19 (CO2). Prior to the infections 1 x 10^7 pneumococci were preincubated with 2 µg of Factor H for 30 min at 37°C. Lung epithelial cells A549 were infected with Factor H treated

pneumococci and infections were conducted for 3 h in the presence of the various monoclonal antibodies (2 µg/ml). The total number of adherent and invasive pneumococci was determined by counting the cfu per well obtained from plating the bacteria onto blood agar plates. In addition, internalization of pneumococci was quantitated by antibiotic protection assay. The increase or decrease in invasion was calculated relative to the basal level of pneumococcal invasion without preincubation with Factor H or mAbs.

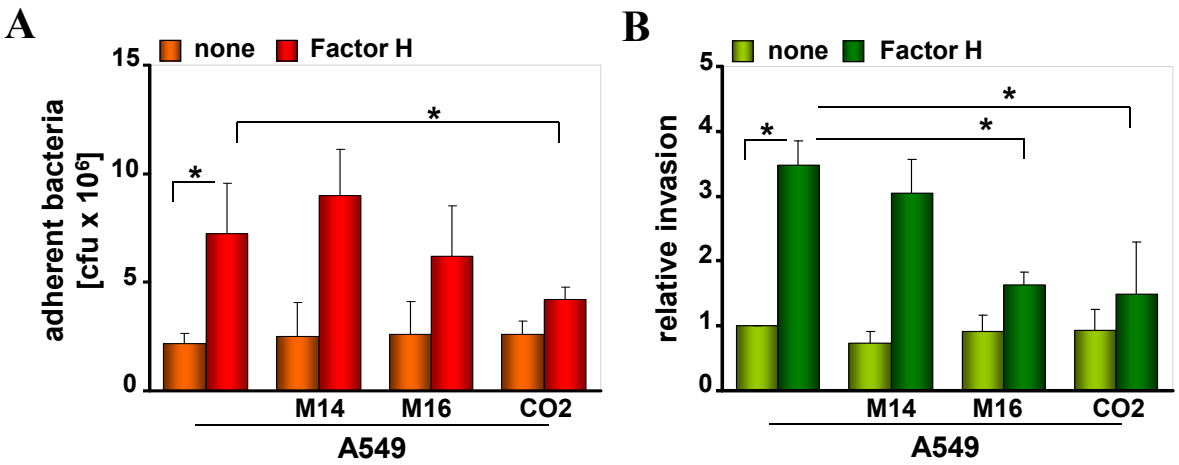


Figure 57 Identification of SCR(s) of Factor H involved in Factor H-mediated pneumococcal adherence to host cells. A, adherence of Factor H-bound pneumococcal strain NCTC10319 (serotype 35A) to A549 cells was determined in the presence of mAbs M14, M16 and CO2 (2 µg/ml). The infection assays were for 3 h at 37°C under 5 % CO_2 and the inhibitory effect of the mAbs was assessed by counting the cfu per well obtained. B, invasion and intracellular survival of Factor H-bound pneumococci in the presence of mAbs was determined by the antibiotic protection assay. The results are shown relative to Factor H untreated pneumococci. * $P< 0.02$

Blocking antibodies [2µg/ml]	**adherent bacteria [cfu x 10^6] per 1x 10^5 A549 cells**		
	none	**Factor H**	*p* value
control	2.19 ± 0.48	7.22 ± 2.33	0.0056
M14	2.51 ± 1.56	8.98 ± 2.12	0.0027
p value relative to control	0.72	0.31	
M16	2.59 ± 1.53	6.21 ± 2.31	0.039
p value relative to control	0.64	0.56	
CO2	2.58 ± 0.64	4.22 ± 0.54	0.008
p value relative to control	0.38	0.046	

Table 36 Number of pneumococci attached to A549 as estimated by quantifying the cfu per well obtained from plating onto blood agar plates. The infection assays were conducted with or without (none) pretreatment of pneumococci with Factor H and in the presence of mAbs M14, M16 and CO2. *P* value less than 0.05 was taken as statistically significant.

Blocking antibodies [2 µg/ml]	fold invasion by *S. p.* serotype 35A		
	without	**Factor H**	*p* value
control	1	3.47 ± 0.39	0.0004
M14	0.74 ± 0.18	3.04 ± 0.52	
p value relative to control	0.07	0.32	
M16	0.91 ± 0.25	1.63 ± 0.19	
p value relative to control	0.58	0.002	
CO2	0.94 ± 0.31	1.48 ± 0.81	
p value relative to control	0.76	0.018	

Table 37 Inhibition of Factor H-mediated pneumococcal uptake by A549 cells by SCR specific mAbs. Number of invasive pneumococci as quantified by the antibiotic protection assay. The results are shown as fold increase or decrease of bacterial invasion relative to Factor H untreated pneumococci (control). *P* value less than 0.05 was taken as statistically significant.

The results revealed that mAb CO2, which interacts with the C-terminal of SCR19, reduced significantly Factor H mediated pneumococcal adherence to epithelial cells (Figure 57A). In contrast, mAbs M14 and M16 were not able to block the Factor H effect on bacterial adherence (Figure 57A). No significant change in the basal level of adherence was observed due to the presence of the mAbs in the infection experiments.

Blocking of the C-terminal SCR19 with mAb CO2 significantly reduced Factor H mediated pneumococcal invasion (Figure 57B). A similar reduction in bacterial invasion was also observed for mAb M16 that interacts with SCR14-18. In contrast mAb M14 showed no inhibitory effect (Figure 57B). The results suggest that the carboxy terminal part of Factor H bound to pneumococcal surface is essential of interaction with host epithelial cells surface receptors.

In order to confirm the role of C-terminal SCRs in Factor H- mediated interaction, mAb C18 was employed as inhibitor in infection assays. The binding site for mAb C18 has been mapped to SCR19-20 of Factor H (Oppermann *et al.* , 2006). Infection assays were performed in the presence of 2 µg/ml antibody and the effect on total adherence and invasion of pneumococci was investigated. Subsets of cells were prepared for immunofluorescence microscopy.

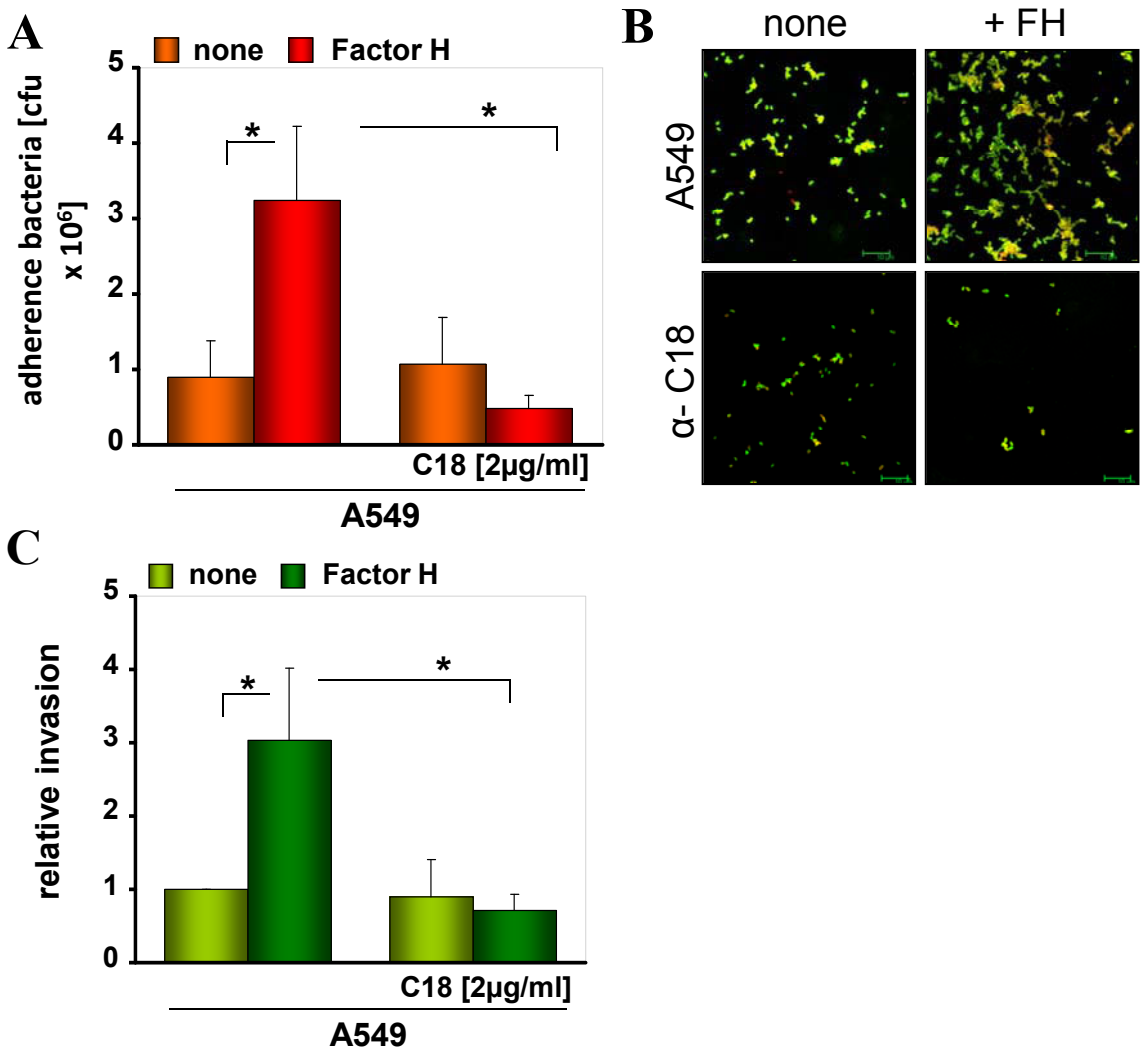


Figure 58 Factor H mediated pneumococcal adherence to host cells depends on SCR 19-20 of Factor H. A, adherence of pneumococcal strain NCTC10319 (serotype 35A) to A549 cells was determined after 3 h of infection in the presence of mAbs C18 (2 µg/ml). The infection assays were conducted with or without pretreatment of pneumococci with Factor H and the effect was assessed by platting the total amount of host cell associated bacteria and cfu determination B, Immunofluorescence microscopy of adherent pneumococci. C, invasion and intracellular survival of pneumococci in the presence of mAb was determined using the antibiotic protection assay. The results are shown relative to infections by untreated pneumococci in the absence of inhibitor. * $P< 0.03$

Treatment of A549 cells	**adherent bacteria [cfu x 10^6] per 1x 10^5 cells**		
	none	**Factor H**	*p* value
control	0.89 ± 0.49	3.24 ± 0.98	0.006
α-C18 (2 µg/ml)	1.07 ± 0.62	0.48 ± 0.17	0.089
p value relative to control	0.6	0.002	

Table 38 Number of pneumococci adhered to A549 cells as quantified after 3 h of infection, in the presence or absence of α-C18 (2 µg/ml). The infection assays were conducted with or without pretreatment of pneumococci with Factor H. *P* value less than 0.05 was taken as statistically significant.

Treatment of A549 cells	fold invasion by *S. p.* serotype 35A		
	none	Factor H	*p* value
control	1	3.04 ± 0.97	0.022
α-C18 (2 µg/ml)	0.89 ± 0.51	0.71 ± 0.23	
p value relative to control	0.74	0.015	

Table 39 Relative invasion of A549 cells by *S. p.* 35A, pretreated with or without Factor H, as determined by the antibiotic protection assay.The infection assays were performed in the presence of α-C18 antibody (2 µg/ml). The results are shown relative to untreated pneumococcal infections conducted in the absence of α-C18 (control). *P* value less than 0.05 was taken as statistically significant.

Blocking of the C-terminal SCRs19-20 with mAb C18 significantly blocked Factor H mediated adherence and invasion. Factor H mediated adherence of pneumococci reduced significantly from 3.24 ± 0.98 x 10^6 cfu/well to 0.48 ± 0.17 x 10^6 cfu/well in the presence of mAb C18 (Figure 58A and 58B). Similarly, the invasion reduced significantly from 3.04 ± 0.97 fold relative to control infected cells, to 0.71 ± 0.23 fold (Figure 58C). In conclusion, SCR 19-20 of Factor H bound to *S. pneumoniae* mediates the bacterial interaction with host epithelial cells.

4.13. Role of the host cell cytoskeleton dynamics on Factor H mediated internalization of *S. pneumoniae* by epithelial cells

The impact of the actin cytoskeleton and microtubules on pneumococcal internalization by host cells during Factor H mediated adherence and invasion was investigated in the presence of pharmacological inhibitors cytochalasin D and nocodazole. A549 cells were preincubated with varying amounts of these inhibitors and the antibiotic protection assay was performed in order to ascertain the potential effect of the inhibitors on pneumococcal uptake.

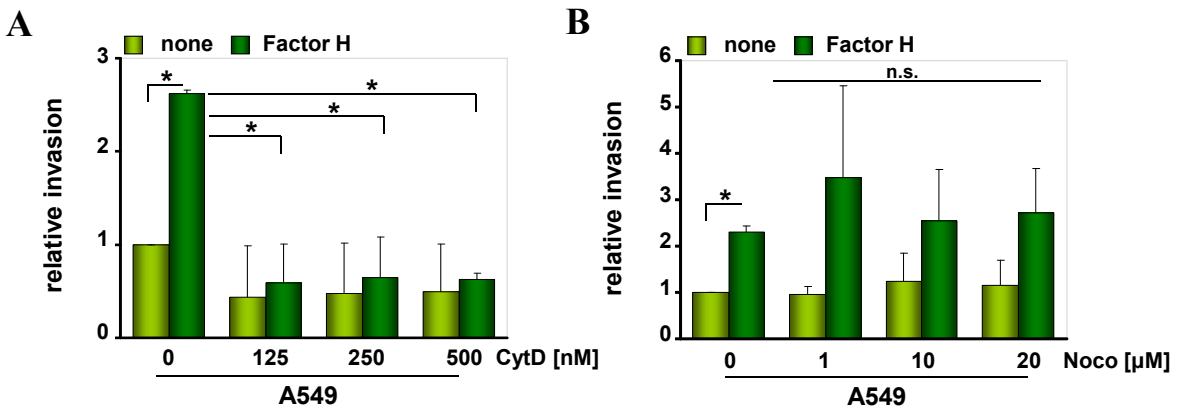


Figure 59 Impact of actin cytoskeleton on Factor H mediated pneumococcal invasion of epithelial cells. The invasion and intracellular survival of *S. pneumoni ae* strain NCTC10319

(serotype 35A) with in A549 cells was determined in the presence of (A) cytochalasin D (CytD) and (B) nocodazole (Noco) by the antibiotic protection assay. The results are shown relative to Factor H untreated pneumococci. * $P < 0.001$

Inhibitor	**fold invasion by *S. p.* serotype 35A**		
	none	**Factor H**	*p* value
control	1	2.62 ± 0.04	7.83×10^{-8}
Cytochalasin D (125 nM)	0.44 ± 0.55	0.59 ± 0.42	
p value relative to control	0.68	0.0003	
Cytochalasin D (250 nM)	0.47 ± 0.55	0.65 ± 0.43	
p value relative to control	0.078	0.0003	
Cytochalasin D (500 nM)	0.49 ± 0.52	0.63 ± 0.06	
p value relative to control	0.071	0.0001	

Table 40 Relative invasion of A549 cells by *S. p.* 35A in presence of actin cytoskeleton inhibitor cytochalasin D. The number of invasive bacteria was determined by the antibiotic protection assay. Pneumococci were pretreated with or without Factor H prior to infections. The results are shown relative to Factor H untreated pneumococcal infections (control). *P* value less than 0.05 was taken as statistically significant.

Inhibitor	**fold invasion by *S. p.* serotype 35A**		
	none	**Factor H**	*p* value
control	1	2.3 ± 0.14	2.3×10^{-5}
Nocodazole (1 µM)	0.95 ± 0.17	3.48 ± 0.97	
p value relative to control	0.51	0.22	
Nocodazole (1 µM)	1.25 ± 0.6	2.55 ± 1.11	
p value relative to control	0.37	0.62	
Nocodazole (1 µM)	1.16 ± 0.53	2.72 ± 0.96	
p value relative to control	0.51	0.35	

Table 41 Factor H-mediated pneumococcal invasion of A549 cells in the presence of microtubule polymerization inhibitor nocodazole. Pneumococci were pretreated with or without Factor H prior to epithelial cells infection for 3 h and the number of invasive bacteria were determined by the antibiotic protection assay. The results are shown relative to infections conducted by Factor H untreated pneumococci (control). *P* value less than 0.05 was taken as statistically significant.

The presence of cytochalasin D significantly inhibited Factor H mediated pneumococcal invasion of A549 cells (Figure 59A). A dose-dependent decrease in the number of invasive bacteria was observed. In contrast, inhibition of microtubules polymerization by nocodazole was not able to interfere with the Factor H mediated pneumococcal uptake by host cells (Figure 59B). The results demonstrated the role of host cells actin cytoskeleton dynamics during Factor H mediated pneumococcal ingestion by host epithelial cells.

4.14. Role of protein tyrosine kinases and PI3-kinase on Factor H mediated pneumococcal ingestion by host cells

To investigate the role of protein tyrosine kinases and PI3-kinase during Factor H mediated pneumococcal ingestion by epithelial cells, pharmacological inhibitors genistein and wortmannin were used. A549 cells were preincubated with different concentrations of genistein and wortmannin for 30 min, prior to bacterial infections. After 3 h of infection, number of internalized pneumococci was determined by antibiotic protection assay wherein the attached bacteria were killed by the antibiotic treatment and intracellular survived bacteria were recovered.

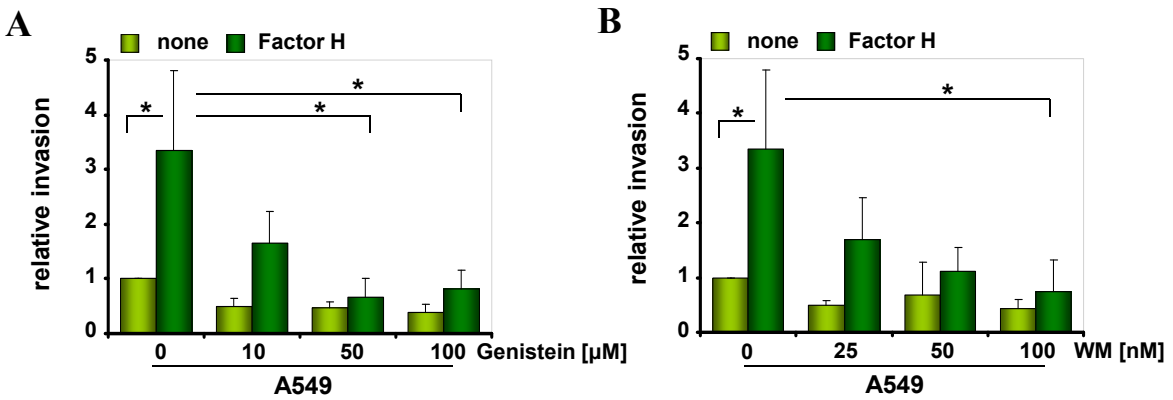


Figure 60 Activities of protein tyrosine kinases and PI3 kinase are essential for Factor H mediated pneumococcal invasion of epithelial cells. The invasion and intracellular survival of pneumococci in A549 cells was determined in the presence (A) genistein, a phospho tyrosine kinase inhibitor and (B) PI3 kinase inhibitor wortmannin (WM) by the antibiotic protection assay. The results are shown relative to pneumococci not pretreated with Factor H. * $P< 0.05$

Inhibitors	fold invasion by *S. p.* serotype 35A		
	none	Factor H	*p* value
control	1	3.34 ± 1.46	0.049
Genistein (10 µM)	0.49 ± 0.15	1.65 ± 0.58	
p value relative to control	0.003	0.14	
Genistein (50 µM)	0.47 ± 0.12	0.67 ± 0.33	
p value relative to control	0.0001	0.036	
Genistein (100 µM)	0.39 ± 0.15	0.81 ± 0.35	
p value relative to control	0.002	0.043	
Wortmannin (25 nM)	0.49 ± 0.08	1.7 ± 0.76	
p value relative to control	0.0003	0.16	
Wortmannin (50 nM)	0.68 ± 0.61	1.12 ± 0.43	
p value relative to control	0.41	0.06	
Wortmannin (100 nM)	0.43 ± 0.17	0.75 ± 0.57	
p value relative to control	0.004	0.046	

Table 42 Inhibition of Factor H-mediated pneumococcal uptake by host epithelial cells in the presence of protein kinase inhibitor. Number of invasive pneumococci was determined in the presence of genistein, a phospho tyrosine kinase inhibitor or PI3 kinase inhibitor wortmannin by the antibiotic protection assay. The infection assays were conducted with or without pretreatment of pneumococci with Factor H. The results are shown relative to Factor H untreated pneumococci (control). *P* value less than 0.05 was taken as statistically significant.

Pretreatment of A549 epithelial cells with genistein and wortmannin blocked in dose-dependent manner, Factor H mediated pneumococcal internalization (Figure 60A and 60B). The results revealed the essential role of host cell protein tyrosine kinases and PI3-kinases in Factor H mediated pneumococcal invasion. In conclusion, Factor H mediated pneumococcal infection is a highly complex process requiring a concerted role of host epithelial cell surface glycosaminoglycans, integrins and series of host cell signalling pathways. Moreover the C-terminal SCR19-20 plays a dual role of interacting with bacterial and host cell surface receptors.

5. Discussion

In humans *Streptococcus pneumoniae* asymptomatically colonizes the nasopharynx, whereas the host innate and adaptive immune system prevents the colonization from progressing into disease. However, an alteration in the host-pathogen homeostasis results in acute local infections like otitis media, sinusitis or life-threatening invasive diseases, such as pneumonia, sepsis and meningitis (Siber, 1994; Tuomanen *et al.,* 1995; Cartwright, 2002). *Streptococcus pneumoniae* utilizes various strategies for colonization of the respiratory tract, transcytosis through host cells and transmigration of the blood-brain-barrier. Several virulence factors of *S. pneumoniae* have been identified that are involved in the progression of pneumococcal diseases (Kadioglu *et al.* , 2008; Bergmann and Hammerschmidt 2006; Hammerschmidt 2006). The burden of diseases is highest in the youngest and elderly population and in patients with immunodeficiencies. The pneumococcus is the prime cause of community-acquired pneumoniae in adults and accounts for 50-75 % cases (Brown and Lerner, 1998). Moreover, each year 1 million children younger than 5 years die from pneumonia and invasive disease (Obaro and Adegbola, 2002). However, despite the use of antibiotics and availability of vaccines the mortality rate remains high. The carrier protein of the current available heptavalent vaccine is not derived from pneumococci therefore it is thought to substitute this carrier by a highly conserved and immunogenic pneumococcal-specific protein. Therefore, a detailed characterization of pneumococcal virulence factors and potential vaccine antigens is required. One such virulence factor and vaccine candidate is pneumococcal surface protein C (PspC, also referred to as CbpA or SpsA). The *pspC* gene was found in almost all the defined strains, from culture collection and clinical isolates analysed, but shows a high level of gene diversity (Brooks-Walter *et al.*, 1999; Iannelli *et al.*, 2002). PspC sequences share a common organization; however based on the anchor encoding sequences two major sub-groups have been distinguished. The first sub-group of proteins have a typical choline binding regions whereas the second group have a C-terminal LPXTG anchoring domain (Iannelli *et al.*, 2002).

PspC is a multifunctional protein that plays an important role in virulence and pathogenesis of this versatile pathogen. The functions attributed to PspC include binding of the free secretory component (SC) or to SC as part of the secretory IgA (SIgA) or polymeric immunoglobulin receptor (pIgR) (Hammerschmidt *et al.*, 1997; Zhang *et al.*, 2000; Elm *et al.*, 2004). In addition, PspC contributes to pneumococcal binding to epithelial cells (Rosenow

et al., 1997), is suggested to bind complement component C3 (Cheng *et al.*, 2000; Smith and Hostetter, 2000) and was shown to interacts specifically with the complement regulator Factor H (Dave *et al.*, 2001; Durthy *et al.*, 2002).

PspC functions as an adhesin and interacts in a human specific manner with the SC of pIgR, thereby mediating the adherence to and transmigration of pneumococci through human epithelial cells (Hammerschmidt *et al.* , 2000; Zhang *et al.* , 2000; Elm *et al.* , 2004). Additionally, PspC mediates immune evasion by binding the host complement and innate immune regulator Factor H (Dave *et al.*, 2001). Apparently PspC uses two different epitopes for binding the soluble host protein Factor H and SC of pIgR (Dave *et al.*, 2004). However, the mechanism by which these independent interactions facilitate pneumococcal infections under physiological and host specific conditions are not completely known.

The aim of this study was to investigate the impact of the PspC interaction with human pIgR (hpIgR) or complement regulator Factor H on pneumococcal virulence. Likewise, the cellular and molecular basis of PspC-mediated adherence to and invasion of host epithelial and endothelial cells was determined. In the present study, roles of various signal transduction pathways initiated via hpIgR-mediated pneumococcal infection have been demonstrated. Additionally, the impact of pneumococcal cell surface bound Factor H on adherence to host cells and the molecular mechanism facilitating the uptake of Factor H bound pneumococci by epithelial cells was illustrated.

5.1. Role of PspC-hpIgR interaction in host cell induced signal transduction cascades

The polymeric immunoglobulin receptor, which is broadly expressed by mucosal epithelium, is involved in the transport of immunoglobulins (IgA and IgM) across the mucosal epithelial barriers from the basolateral to apical surface, (Mostov and Kaetzel 1999, Johansen *et al.*, 1999, Shimada *et al.*, 1999). Here, the immunoglobulins protect the mucous membrane from inhaled or ingested pathogens such as bacteria, viruses, parasite and toxins (Fubara and Freter, 1973; Outlaw and Dimmock, 1990; Mazanec *et al.* , 1993, Enriquez and Riggs, 1998). At the apical cell surface, pIgR is proteolytically cleaved off and the extracellular binding domain of the receptor, known as secretory component (SC), bound to dimeric-IgA (dIgA) is released into the mucosal secretions as SIgA or alternatively as free SC. The association of SC to dIgA has been shown to protect SIgA antibodies from proteolytic degradation. SIgA prevents colonization and invasion of pathogens into mucosal surfaces by interfering with their motility and by competing with pathogens for adhesion sites

on the apical surface of the epithelial cells (Giugliano *et al.*, 1995; Wold *et al.*, 1990; Dallas and Rolfe, 1998; Williams and Gibbons, 1972). In addition, pIgR transports immune complexes, microorganisms and antigens coated with IgA from the basolateral surface of mucosal epithelial to the apical surface, thereby providing a mechanism for a safe disposal of potential pathogens and harmful antigens (Mazanec *et al.*, 1992; Kaetzel *et al.*, 2001). Finally, luminal SIgA can neutralize the toxic activity of pathogen products such as bacterial toxins (Vaerman *et al.*, 1985). However, despite its role in host defence, some pathogens and viruses have developed strategies to exploit pIgR for their invasion into the epithelium (Sixbey *et.al.*, 1992, Gan *et.al.*, 1997, Lin *et.al.*, 1997; Zhang *et al.*, 2000).

The interaction of PspC with pIgR is critical for pneumococcal translocation from nasopharynx and spread to normally sterile parts of the respiratory tracts such as lungs or the blood stream during infections. The cell culture infections assays, where bacteria were centrifuged to host cells, demonstrated a significant reduction of *pspC*-mutant pneumococci to adhere to and invade Calu-3 cells, which naturally expresses pIgR, or in MDCK-cells which were stably transfected and produce human pIgR (MDCK-hpIgR) cell compared to wild-type strain. In addition, blocking of pIgR using anti-secretory component (α-SC) antibodies significantly reduced pneumococcal ingestion by pIgR expressing host epithelial cells. The result confirmed that PspC mediated pneumococcal internalization of mucosal host cells occurs in a hpIgR-dependent manner. Moreover, the specificity of PspC-hpIgR interaction was not altered after synchronizing the infections.

Hammerschmidt *et al.* (2000) demonstrated that PspC interacts exclusively with human SC or SIgA but not with SC or SIgA derived from animals, specifically those from bovine, canine, equine, guinea pig, hamster, rabbit, rat and mouse. Likewise, Zhang *et al.* (2000) demonstrated that human-pIgR but not rabbit-pIgR expressed by MDCK cells enhances pneumococcal invasion. The study of Zhang *et al.* (2000) and colleagues also hypothesized that *Streptococcus pneumoniae* may utilize the apical recycling pathway of hpIgR i.e. the transport in the retrograde fashion to basolateral surface, for bacterial translocation across human epithelial barriers. However, whether this apical to basolateral pneumococcal translocation occurs by utilizing the hpIgR-transcytosis machinery in reverse or by other mechanisms is still not clear.

The intracellular pathway for pIgR transcytosis after binding of it ligand pIgA have been clearly documented. The basolateral to apical cell surface and corresponding retrograde transport across epithelium was extensively investigated using the polarized monolayer of

rabbit (rb)-pIgR transfected MDCK cells as model cell line (Song *et al.*, 1994, Cardone *et al.*, 1996). The vast knowledge regarding the pIgR-dIgA traffic has provided important insight into receptor sorting, intracellular compartments involved and the modulating receptor signal transduction pathways (Rojas and Apodaca, 2002). Although unloaded pIgR undergoes constitutive transcytosis, binding of dIgA stimulates the receptor transcytosis both *in vitro* and *in vivo* (Song *et al.*, 1994, Giffroy *et al.*, 1998). The dIgA stimulated pIgR transcytosis requires the production of secondary messengers, including inositol-1, 4, 5-triphosphate and free intracellular calcium (Cardone *et al.*, 1996; Luton *et al.*, 1998; Luton and Mostov, 1999). In addition, Luton *et al.* (1999) demonstrated that the generation of these signalling molecules depends on the *Src* family protein tyrosine kinase $p62^{yes}$ and involves concerted role of phospholipase Cγ, diacylglycerol (DAG), protein kinase C epsilon (PKCε). In contrast, this process might not be true for human pIgR, as in human Calu-3 cells, and hpIgR transfected MDCK cells, pIgA binding fails to induce transcytosis, even though induced intracellular signalling pathways are similar to rb-pIgR (Giffroy *et al.* 2001). In part inconsistent and contradictious data raised the question on extrapolation of animal based studies to humans. Therefore, the cellular and molecular basis of PspC-hpIgR mediated pneumococcal infections of host epithelial cells which includes analysis of initiated signal transduction pathways was determined.

Bacterial pathogens have a variety of cell-surface adhesins that enable them to attach to host cells. Some of these adhesins can bind to host cell receptors on non-phagocytic cells, thereby allowing the uptake of bound bacteria into the host cells. Striking examples are the invasin of *Yersinia* spp. and the internalins (Internalin A and B) of *Listeria* spp. Although pathogen internalization mechanisms differ amongst pathogens, they share common features such as the ability to engage and modulate host intracellular-signalling pathways. Commonly described and extensively investigated cellular target of pathogens is the host cytoskeleton. Various intracellular microorganisms exploit cytoskeleton components, including actin filaments, microtubules and intermediate filaments, to gain entry into host cells (Bhavsar *et al.*, 2007). Instead interacting directly with actin filaments, pathogens control polymerization of actin filaments by modulating cellular regulators of this process, such as small Rho family of GTPases (Finlay, 2005). The type-3 secretion system (T3SS) effector proteins SopE and SopE2 of *Salmonella enterica*, which function as guanine-nucleotide-exchange factors, activates small GTPases Cdc42 and Rac in the target cells thus inducing the generation of actin-rich membrane ruffles to facilitate engulfment and internalization of the

bacteria (Hardt *et al.*, 1998; Stender *et al.*, 2000; Zhou *et al.*, 2001). In addition, IcsA from *Shigella flexneri* or ActA from *Listeria* spp. manipulate host cell actin-filament dynamics in order to facilitate intracellular motility of these bacteria (Egile *et al.*, 1999; Chakraborty *et al.*, 1995; Welch *et al.*, 1997). Moreover, extracellular pathogens such as enterohaemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) hijack host actin cytoskeleton during their attachment and ingestion. Here, the bacterial effector protein Tir mediates extensive modification of host cell actin filaments beneath the adherent microorganism (Gruenheid *et al.*, 2001). Likewise, microtubules are also targeted by microorganisms. For example, the VirA protein of *Shigella* spp. or EspG of EPEC destabilizes the host cell microtubules through interaction with heterodimers of α-tubulin and β-tubulin (Yoshida *et al.*, 2002; Hardwidge *et al.*, 2005). In contrast, a strain of *Campylobacter jejuni* has been shown to use microtubules and their associated molecular motors to support host cell invasion (Hu and Kopecko, 1999).

Here, the complexity of pneumococcal ingestion by host epithelial cells via the PspC-hpIgR mechanism and induced signal cascades has been demonstrated for the first time. During uptake by host cells pneumococci exploit proteins of the host epithelial cell cytoskeleton signalling molecules for its own benefit. In the presence of pharmacological inhibitors cytochalasin D and latrunculin B, both inhibiting actin polymerization, pneumococcal uptake by pIgR expressing MDCK-hpIgR and Calu-3 cells was significantly reduced. Similar, pretreatment of cell lines with jasplakinolide, a potent inducer of actin polymerization also reduced uptake of pneumococci. In addition, inhibition of microtubules formation by using nocodazole, that inhibits polymerization of microtubules, significantly blocked PspC-hpIgR mediated pneumococcal ingestion by host epithelial cells. Taken together, the inhibition experiments suggested that the host cell cytoskeleton dynamics plays a key role during pneumococcal ingestion by host epithelial cells via the PspC-hpIgR mechanism.

The members of small Rho family GTPases including RhoA, Rac1 and Cdc42, are small GTP-binding proteins and are critical regulators of the actin cytoskeleton that participate in several signalling events (Bishop *et al.*, 2000, Kaibuchi *et al.*, 1999). These Rho family GTPases serve as guanine nucleotide-regulated switches that transduces external stimuli to modulate various cellular functions (Nobes and Hall, 1995; Caron and Hall, 1998; Tran Van Nhieu *et al.*, 1999; Cossart and Sansonetti, 2004). The Rho GTPases cycle between an active GTP-bound state and an inactive GDP-bound state. Notably, the activity of one or

more members of the small Rho family GTPases are required for host cell invasion by pathogenic bacteria (Cossart and Sansonetti, 2004; Rottner *et al.*, 2004). For example, it has been demonstrated that RhoA is important for uptake of *Mycobacterium avium* and *Pseudomonas areuginosa* (Sangari *et al.,* 2000; Kazimierczak *et al.*, 2001), whilst Rac1 and Cdc42 play a crucial role in host cell invasion of *Salmonella enterica*, *Shigella flexneri* and *Campylobacter jejuni* (Hardt *et al.*, 1998; Tran Van Nhieu *et al.*, 1999; Krause-Gruszczynska *et al.* , 2007). In addition, Rho-family GTPases RhoA, Rac1 and Cdc42 are required for efficient invasion of HeLa cells by group B streptococci (Burham *et al.* , 2007). The Rho family GTPases have also been shown to be involved in the regulation of rabbit-pIgR-dimeric IgA transcytosis across mucosal epithelium (Leung *et al.*, 1999; Jou *et al.*, 2000; Rojas *et al.*, 2001). However, involvement of Rho family GTPases in pIgR mediated pneumococcal infection of host epithelial cells has not been addressed so far. In this study Cdc42 was identified as a key GTPase regulating PspC-hpIgR mediated pneumococcal invasion of epithelial cells. During uptake of pneumococci by pIgR expressing host epithelial cells Cdc42 was significantly active. Inhibition of endogeneous Rho family members by *Clostridium difficile* toxin TcdB-10463 or TcdB-1470 or inhibition of Cdc42 using a specific pharmacological inhibitor (Secramine A) significantly reduced pneumococcal ingestion by pIgR expressing epithelial cells. The genetic approach to interfere with Cdc42 function by transient over expression of dominant-negative (dn) Cdc42 (Cdc42-T17N) inhibited pneumococcal invasion of MDCK-hpIgR cells, confirming the involvement of Cdc42 in PspC-hpIgR mediated pneumococcal internalization process. Finally, precipitation of active GTP bound Cdc42 following pneumococcal infection of pIgR expressing epithelial cells demonstrated activation of Cdc42 and indicates the essential role of Cdc42 during pneumococci host cell infections. In contrast, specific inhibition of Rac1 using NSC23766 or blocking of Rho-associated protein kinase using the inhibitory substance Y27632 had no effect on pneumococcal uptake by pIgR expressing host epithelial cells. Similar results were demonstrated when the genetic approach to interfere with Rac1 and RhoA function was employed. Here, transient transfection of epithelial cells with the dn alleles of Rac1 (Rac1-T17N) or RhoA (Rho-T19N) showed no inhibition of pneumococcal internalization into MDCK-hpIgR cells. Pull-down assays revealed no change in the level of RhoA activation following pneumococcal infection of host epithelial cells. In contrast, activation of Rac1 demonstrated a gradual decrease, which reduced to an undetectable level between 60 and 120 min post infection of pIgR expressing cells with pneumococci.

It has been proposed that Rho proteins are involved in the formation of stress fibers and focal adhesion complexes, while Cdc42 triggers formation of filopodia (microspikes), and Rac1 is essential for the formation of lamellipodia and membrane ruffles (Kozma *et al.* 1995, Nobes & Hall 1995, Ridley *et al.* 1992). Recently it was shown that the pneumococcal pneumolysin has the ability to activate small GTPases, which leads in neuronal cells to a rapid formation of filopodia, stress fibers and lamellipodia (Iliev *et al.*, 2007). These morphological alterations of the cellular phenotype are consistent with activation of RhoA and Rac1. PspC-hpIgR-mediated invasion induced microspikes like structure formation which was blocked with secramine A, indicative of a crucial role of Cdc42 for microspikes formation.

The Phosphoinositide 3-kinase (PI3-kinase) signaling pathway is implicated in a variety of cellular functions including regulation of the actin cytoskeleton, vesicle trafficking (Stokoe, 2005). PI3-kinase activation is specifically implicated in phagocytosis, pseudopod formation and membrane ruffling, cell survival pathways, gene regulations and cell metabolism (Cox *et al.*, 1999; Pizarro-Cerda & Cossart, 2004; Stokoe, 2005). One of the downstream effector of PI3-kinase is the serine-threonine kinase Akt (protein kinase B) which regulates the activity of a number of targets including kinases, transcription factors and other regulatory molecules (Scheid & Woodgett, 2003). Akt is phosphorylated at Thr308 through the 3-phosphoinositide-dependent kinase (PDK1) while phosphorylation at Ser473 was shown to depend on PI3-kinase activity and mTOR. The key role of PI3-kinase/Akt pathway is indicated for several pathogenic microorganisms such as group B streptococcus (Burnham *et al.*, 2007), group A streptococcus (Purushothaman *et al.*, 2003), *Pseudomonas aeruginosa* (Kierbel *et al.*, 2005), *Helicobacter pylori* (Kwok *et al.*, 2002), *Chlamydia pneumoniae* (Coombes & Mahony 2002), *Escherichia coli* K1 (Reddy *et al.*, 2000) and *Listeria monocytogenes* (Ireton *et al.*, 1999). Strikingly, vitronectin-$\alpha_V\beta_3$ integrin mediated *S. pneumoniae* invasion of host epithelial cells was demonstrated to be PI3-kinase/Akt pathway dependent (Bergmann *et al.*, in press). Apparently, PI3-kinase/Akt pathway was also shown to be essential for PspC-hpIgR mediated pneumococcal uptake by pIgR expressing cell. Inhibition of PI3-kinase in MDCK-hpIgR and Calu-3 cells or inhibition of Akt caused a significant reduction of pneumococcal invasiveness. In addition, kinetic infections demonstrated phosphorylation of PI3-kinase p85α subunit and Akt when hpIgR expressing host cells were incubated with pneumococci. Taken together, these results indicate a key role of PI3-kinase and Akt during pneumococcal infections via the PspC-hpIgR mechanism.

Activation of PI3-kinase in turn triggers activation of downstream signalling molecules and hence, given the complexity of signal transduction pathways, other signalling molecules are probably involved in pneumococcal uptake processes. To assess the role of protein tyrosine kinases (PTKs), especially of *Src* family of protein tyrosine kinases and mitogen-activated protein kinases (MAPKs), during PspC-hpIgR mediated pneumococcal uptake by pIgR expressing host cells their activity was investigated. Src tyrosine kinase is a critical signal transducer that modulates a wide variety of cellular functions. Activities of *Src* family of protein tyrosine kinases play a critical role in various bacterial and viral infections. Activation of *Src* PTKs is important for infections with *Staphylococcus aureus* , *Listeria monocytogenes*, *Helicobacter p ylori* or *Neisseria menin gitidis* and pathogenic fungus *Paracoccidioides brasiliensis* (Agerer *et al.*, 2003; Sousa *et al.*, 2007; Kwok *et al.*, 2007; Hoffman *et al.*, 2001; Maza *et al.*, 2008). In addition, *Src* family kinase Lck and Lyn contributes to HIV type 1 and Epstein-Barr virus pathogenesis, respectively (Strasner *et al*., 2008; Rovedo & Longnecker, 2008).

Other important PTKs are the MAPKs which includes, in addition to others, the extracellular regulated kinases 1 and 2 (ERK1 [p44 MAPK] and ERK2 [p42 MAPK]), c-Jun NH2 terminal kinases (JNK1/2) and p38 MAPK. The MAPKs phosphorylate specific serines and threonines of other protein kinases, phospholipases, and cytoskeletal proteins, thereby regulating various cellular processes (Krishna and Narang, 2008). Furthermore, they influence gene expression and affect the amount and activity of a number of nuclear transcription factors including activator protein (AP)-1 factor c-Jun (for JNK, p38 MAPK or ERK pathways depending on the stimulus) (Krishna and Narang, 2008). MAPKs were demonstrated to be involved in host cell invasion and cytokine release induced by different pathogenic bacteria. Activation of several MAPKs were found in response to epithelial cell infection with *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium and EPEC (Hobbie *et al.*, 1997; Tang *et al.*, 1998; Czerucka *et al.*, 2001). In particular JNK activation was described to be associated with the invasive process of *Porphyromonas gingivalis* in gingival cells, *Neisseria gonorrhoeae* in epithelial cells, and *Neisseria meningitidis* infection of HBMEC cells (Watanabe *et al.*, 2001; Ellington *et al.*, 2001; Naumann *et al.*, 1998; Sokolova *et al.*, 2004).

The role of PTKs in pneumococcal pathogenesis has not been addressed so far. *S. pneumoniae* was demonstrated to induce JNK MAPK and AP-1 dependent IL-8 release by lung epithelial BEAS-2B cells (Schmeck *et al.*, 2006). In addition, nonencapsulated

pneumococci R6x induced p38MAPK and JNK-mediated caspase-dependent apoptosis in human endothelial cells (N´Guessan *et al.*, 2005). Here, it is demonstrated that PspC-hpIgR mediated pneumococcal infection of epithelial cells requires Src kinase activity to activate ERK 1/2 and JNK. Inhibition studies with the pharmacological inhibitors genistein, which is a general inhibitor of protein-tyrosine kinases, or PP2, a *Src* family of protein-tyrosine kinase inhibitor, demonstrated blockage of pneumococcal uptake by pIgR expressing cells. Similar, inhibitions of MAPKs including MAP kinase kinase (MEK), JNK, or p38 MAPK, demonstrated a significant reduction in pneumococcal uptake. Immunoblot analysis indicated activation of ERK1/2 and revealed the important role played by ERK1/2 during pneumococcal infection of pIgR expressing cell line. Phosphorylation of ERK 1/2 was prevented by infecting PD98059 pretreated epithelial cells, thus suggesting that the PspC-hpIgR mediated pneumococcal infection of epithelial cells induces MEK-dependent phosphorylation and activation of ERK 1/2. In the case of JNK MAPK, phosphorylation of isoforms p54 and p46 was detected, but the activation was quite low as compared to ERK1/2. However, activation of transcription factor c-Jun, which is one of the downstream effector of JNK MAPK, suggested that JNK plays an essential role during pneumococcal infection of pIgR expressing cells. In contrast, p38 MAPK was not activated during pneumococcal infections although inhibition studies with the inhibitor SB202190 showed a significant reduction in pneumococcal uptake. The contradictory results regarding the role of p38 MAPK in pneumococcal infections probably results from the non-specificity of the pharmacological inhibitor (Bain *et al*., 2007; Davies *et al.*, 2000). The eukaryotic cell signalling pathways involves plethora of host molecules coordinating various cellular events. There are some 500 protein kinases encoded by the human genome, most of which are members of the same superfamily, and are capable of phosphorylating two or more proteins, therefore the issue of selectivity is highly critical. The p38 MAPK inhibitor SB202190 has been demonstrated to be a more potent inhibitor of α or β isoforms compared to the γ or δ isoforms of p38 (Bain *et al.*, 2007). Moreover, Bain and coworkers reported that SB202190 at a concentration of 1 μM reduces, in addition to other known kinases, activity of JNK2 by approximately 60 %. Therefore, additional investigations are critical in order to confirm pharmacological inhibitors based results. The transient over expression of plasmids encoding the wild-type C-terminal Src kinase (Csk WT), a negative regulator of Src protein-tyrosine kinase, kinase-inactive form of Csk (Csk KM), or dominant-negative kinase-inactive version of Src (Src K297M)

demonstrated once again the crucial role of Src protein-tyrosine kinase activity for hpIgR mediated pneumococcal uptake by host cells.

A significant reduction in the number of invasive bacteria, after treatment of the cells with various pharmacological inhibitors of signalling molecules suggest that *S. pneumoniae* triggers signal transduction pathways in order to facilitate their uptake by host cells. Signal transduction cascades are highly complex and tightly regulated pathways in which activation of one signalling molecule leads to downstream activation or deactivation of various effector proteins or stimulation of other signalling pathways. Activation of ERK1/2 in pIgR expressing host cells after infections with pneumococci was significantly reduced in the presence of PP2, which is thought to inhibit specifically *Src* family of tyrosine kinases. Notably, inhibition of ERK1/2 activation during pneumococcal infections was demonstrated when dominant-negative, kinase-inactive versions of Src (Src K297M) were over-expressed in pIgR expressing cells. The result indicated that Src kinase facilitates ERK activation during hpIgR mediated pneumococcal infection. Inhibition experiments performed in the presence of individual inhibitors or with a combination of inhibitors suggested interplay between the Src kinase and the JNK pathway. In addition, the results also revealed that Src kinase is activated upstream of JNK pathway in the signal transduction cascade induced during PspC-hpIgR mediated pneumococcal infection. Importantly, PI3-kinase and Src kinase are independently activated during pneumococcal infection of hpIgR expressing cells and consequently simultaneous inactivation of these signal molecules has synergistic effect on bacterial internalization by host cells. In conclusion the result revealed the complexity of PspC induced signalling events in epithelial cells via its interaction with hpIgR.

The processes in which bacteria or other pathogens invade mammalian host cells have been extensively investigated. Most microorganism or toxins penetrates into the cells through an existing entry mechanism, for example, clathrin-mediated endocytosis, phagocytosis and macropinocytosis. Only few of these ingested microorganisms can replicate and moves within the vacuolar compartments or escape the killing within the host cells. Phagocytosis is restricted to “professional phagocytes” such as neutrophils, macrophages or dendritic cells, whilst other type of ingestion occurs in almost all cell types. Bacterial pathogens are known to employ diverse strategies to induce their entry in non-phagocytic cells. Based on the mechanism used, they have been classified into two well differentiated groups, namely “zippering” and “triggering” mechanism (Cossart and Sansonetti, 2004; Veiga and Cossart, 2006). Bacteria which uses the zipper mechanism for host cell entry include *Listeria*

monocytogenes, *Yersinia pseudotuberculosis*, and *Staphylococcus aureus*, while *Salmonella typhimurium* and *Shigella flexneri* employ the trigger mechanism (Cossart and Sansonetti, 2004; Pizarro-Cerda and Cossart, 2006). Bacteria inducing the "zippering" phenotype use a calthrin and dynamin-dependent mechanism for entry into host cells (Veiga *et al.*, 2007). Clathrin-mediated endocytosis is the main process by which many transmembrane proteins are internalized from the plasma membrane (McNiven and Thompson, 2006). These transmembrane proteins recruit intracellular adaptor proteins that together with clathrin forms an endocytic coated pit at the plasma membrane. These coated pits finally pinch off the membrane and forms the clathrin-coated vesicles. Dynamin is a GTPase principally involved in the scission of newly formed vesicles from the membrane (Henley *et al.*, 1999).

S. pneumoniae interacts via its surface protein PspC with the hpIgR and this interaction facilitates bacterial uptake into host cells. However, the mechanism of pneumococcal endocytosis by host cells has not been addressed. It has been hypothesized that platelet activating factor receptor (PAFr) mediated pneumococcal uptake depends on clathrin (Radin *et al.*, 2005). After ingestion via PAFr pneumococci were found in vacuoles and were co-localized with Rab5, Rab7, Rab11 and Lamp-1 (Radin *et al.*, 2005). Moreover, studies have shown the involvement of clathrin mediated pathway for both apical and basolateral internalization of rabbit-pIgR (Hoppe *et al.*, 1985; Limet *et al.*, 1985). To assess the endocytosis mechanism involved in hpIgR-mediated pneumococcal invasion of host cells, the clathrin-dependent endocytotic machinery was inhibited using specific pharmacological inhibitor monodansylcadaverine or chlorpromazine. The results revealed that clathrin significantly blocked the pneumococcal invasion, as determined by bacterial plating experiment after infection assay. The invasion rate of pneumococci in presence of dynasore, a cell-permeable specific inhibitor of dynamin, or was also significantly impeded. In addition, the genetic knocked-down of dynamin by siRNA in hpIgR expressing Calu-3 cells caused reduction of pneumococcal ingestion by these cells. Illustration by confocal laser scanning microscopy clearly demonstrated colocalization of pneumococci with clathrin which was over-expressed in cells. These results provided experimental evidence that pneumococcal uptake by host cells via hpIgR is a clathrin and dynamin-dependent mechanism.

In conclusion, *S. pneumoniae* interacts via its PspC protein with human polymeric immunoglobulin receptor and this interaction mediates bacterial invasion of host epithelial cells. PspC-hpIgR mediated pneumococcal infection is a highly complex process that involves the concerted role of host cytoskeleton and various host cell signalling molecules. The

dynamics of host cell actin microfilaments and microtubules is essential for pneumococcal uptake by mucosal epithelial cells. Moreover, this study demonstrates for the first time that the activity of small GTPase Cdc42 is critical for the infection of host cells and consequently, probably also for invasive diseases caused by *S. pneumoniae*. In addition, several signalling pathways and molecules including PI3-kinase/Akt and PTKs are implicated during pneumococcal infections of epithelial cells via the PspC-hpIgR mechanism. Amongst PTKs, the Src kinase pathway, ERK1/2 and JNK pathways have been shown to be essential for pneumococcal ingestion by hpIgR expressing cells. The activation of Src kinase has been demonstrated to be upstream of both ERK1/2 and JNK pathways. In contrast, the results revealed independent activation of PI3-kinase/Akt and Src kinase pathways during pneumococcal infection of pIgR expressing cells. Finally it was demonstrated that pneumococcal uptake by host epithelial cells, via PspC-hpIgR mechanism, is clathrin and dynamin dependent process.

Although in the present study I tried to investigate the process of PspC-hpIgR mediated pneumococcal infections, there are still number of unresolved questions. Investigations are required to identify the host cell endosomal compartments involved in pneumococcal transcytosis across the epithelial cell barrier. In addition, activation of signalling molecules especially Akt, ERK1/2 and/or JNK mediated host cell response, including cytokines release, during pneumococcal infections have to be resolved. Apart from this, *in vi vo* studies using a transgenic mouse model expressing human pIgR should be performed in order to elucidate the importance of PspC-hpIgR interaction in pneumococcal pathogenesis under *in vivo* relevant conditions.

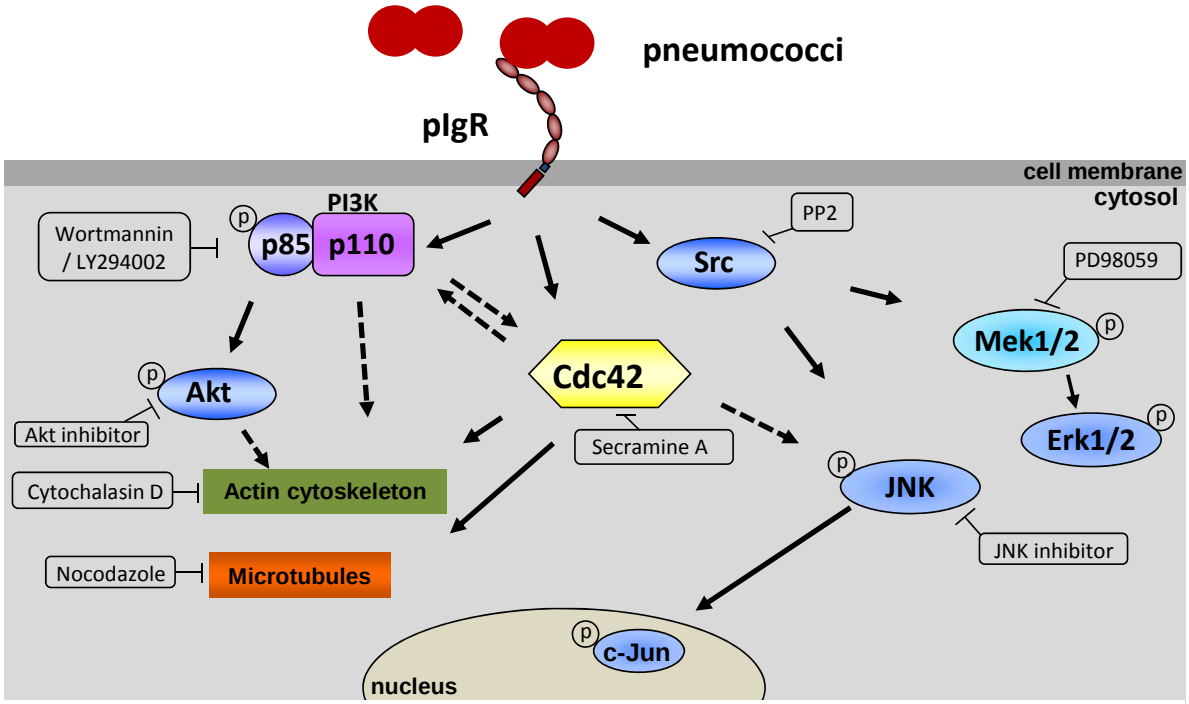


Figure 58 Model of pneumococci-induced signaling cascades during invasion of hpIgR-producing host epithelial cells. The model is based on the results obtained by inhibition of signaling molecules or using host cells in which the signaling molecules have been genetically knocked down. Solid arrows, signaling events characterized in the present study; dashed arrows, already known signaling events and/or interactions.

5.2. Role of PspC-Factor H interaction

Infectious diseases represent a major health, social and economic problem worldwide. Although advances in medical sciences have improved to understand and combat microbial infections, the increase in antimicrobial resistance, emergence of new pathogens and re-emergence of known pathogens still form a major threat for our health systems (Fauci, 2006). Therefore, new strategies are required to fight infectious diseases. One important aspect to identify new virulence factors and develop new antimicrobial substances is an improved understanding of the host-pathogen interaction.

The complement system is a central component of the innate immune response and represents one of the first lines of defence which is immediately and directly activated upon entry of pathogens. It fulfils numerous functions including recognition of foreign cells, communication with and activation of adaptive immunity and finally removal of the cellular debris. (Walport, 2001). The complement system consists of a well balanced network of circulating and cell surface bound proteins, which generates after its activation a highly regulated and very potent antimicrobial response. Based on the activation mechanism, which differ considerably, the complement system has been classified as the classical, lectin and alternative pathways. The classical pathway is stimulated by the recognition of antigen-antibody complexes on foreign cells surfaces by the hexameric complement component C1q. The structurally similar pattern-recognition receptors, mannose-binding lectin and ficolins, bind to carbohydrate ligand on microbial intruders and initiate the lectin pathway. Conversely, the alternative pathway is stimulated by the spontaneous hydrolysis of native C3 on the foreign surfaces. The activation of complement pathway involves the formation of unstable protease complexes, namely C3-convertase (C3bBb for alternative pathway; and C2a4b for classical or lectin pathways) and the cleavage of C3 which generates C3b. Then C3b exponentially amplifies the activation of the alternative pathway by forming more C3-convertase. C3b interacts with C3-convertase to generate C5-convertase, which binds and cleaves C5 and initiates the formation of the lytic membrane attack complex (MAC) (Morgan 1999).

The human immune system and microorganisms share a highly complex relationship. On the one hand the host immune system seek to eliminate the foreign intruder and whilst the pathogen attempts to survive within the host. All microbes and pathogens including Gram-positive and Gram-negative bacteria, pathogenic fungi, multi-cellular organisms, as well as parasites and viruses are attacked and targeted by the complement system of their host. While

non-pathogenic microbes are normally recognized and eliminated by the activated complement system, pathogenic microorganisms employ a broad range of evasion strategies to interfere with and to inactivate the highly efficient attack of the complement. There are multiple strategies which microorganism execute to evade recognition or eradication including, recruitment or mimicking of complement regulators, modulation or inhibition of complement proteins by direct interactions, and inactivation by enzymatic degradation as well (Lambris *et al.* , 2008; Zipfel *et al.* , 2007; Rooijakers and van Strijp, 2007; Kraicy and Würzner, 2006; Zipfel *et al.*, 2002).

The complement system is part of the innate immune system and critical for host defence mechanisms. In order to establish an infection the pathogen must overcome this first line of defence mechanism. Therefore, pathogens have developed immune evasion strategies to counteract complement attacks. The recent studies in the field of complement escape mechanism by pathogens have shown diversity in the strategies as well as molecules employed by pathogens to evade the complement attack. One strategy of pathogens that has attracted particular interest is the ability to acquire fluid phase soluble complement regulators to the pathogen cell surface (Lambris *et al.*, 2008; Zipfel *et al.*, 2007; Kraicy and Würzner, 2006)

The complement system is highly regulated but excessive or uncontrolled complement activation on self-tissues has severe effects and can cause various diseases (de Córdoba and de Jorge, 2008; Markiewski and Lambris, 2007). In order to differentiate between self and non-self and to avoid unnecessary consumption of components, complement is under the control of multiple regulatory glycoproteins. These self-control limits complement activation, either by inactivating C3b or C4b, by dissociating the C3/C5-convertase, or by inhibiting the MAC formation. The glycoproteins involved in regulation include fluid phase regulators such as Factor H, Factor H-like protein1 (FHL-1), C4b binding protein (C4BP), C1 inhibitor, and cell membrane bound regulators like CR1/CD35, CR2/CD21, MCP/CD46, DAF/CD55, and protectin/CD59 as well (Morgan and Harris, 2003). These glycoproteins are evolutionary tuned as natural regulators of the complement; therefore, they are synthesized in relatively high amounts by the host. Moreover, all these regulators share common structural features and are composed of so called short consensus repeat (SCR) domains, thus allowing recruitment of multiple regulators by the same pathogen. The ability to recruit complement regulators is the most widely disseminated strategy amongst pathogens for avoiding complement activation and complement-mediated phagocytosis. Recruitment of membrane bound CD59 protects

against terminal complement attack and lysis has been reported for *Escherichia coli* (Rautemaa *et al.* , 1998) and *Helicobacter pylori* (Rautemaa *et al.* , 2001). However, recruitment of fluid phase complement regulators to subvert complement function was not only reported for bacteria, including *Borrelia burgdorferi, Neisseria* spp. and streptococci, (Zipfel *et al.*, 2007); but was also shown for viruses, including HIV-1 (Bernet *et al.*, 2003; Stoiber *et al.* , 1996); fungi, like *Candida albicans* (Meri *et al.* , 2002, 2004), *Aspergillus fumigatus* (Behnsen *et al.*, 2008) and parasites, such as *Echinococcus* spp. (Inal, 2004; Diaz *et al.*, 1997).

Factor H, a 150 kDa soluble glycoprotein, is a central fluid phase regulator of the alternative complement pathway. Factor H is abundant in plasma and can associate with host cell membranes and other self-surfaces via recognition of polyanionic components such as glycoaminoglycans (GAGs) and sialic acid (Meri and Pangburn, 1990; Jokiranta *et al.*, 2006). Factor H is structurally composed of 20 individually folded SCRs (Nilsson and Müller-Eberhard, 1965; Ripoche *et al.* , 1988) and so far, seven structurally and immunologically related members of the human Factor H protein family are identified (Zipfel *et al.* , 2002). FHL-1 (reconectin), a 42 kDa plasma protein, is derived from the human Factor H gene by an alternative splicing of the mRNA and the protein is identical with the first seven SCRs of Factor H. Both Factor H and FHL-1 show complement regulatory properties, by competing with factor B for binding to C3b; by accelerating the decay of the C3-convertase, C3bBb (decay accelerating activity), and by acting as cofactor for factor I-mediated degradation of C3b (Pangburn *et al.*, 1977; Kühn *et al.*, 1995; Zipfel and Skerka, 1999; Zipfel *et al.*, 2002).

Microbial Factor H binding proteins that contribute to pathogenicity have been identified in several organisms including group A streptococci (Horstmann *et al.* , 1988), group B streptococci (Areschoug *et al.* , 2002; Jarva *et al* ., 2004), *Yersinia enterocolitica* (China *et al.* , 1993), HIV-1 (Pinter *et al.* , 1995a, 1995b; Sadlon *et al.* , 1994), *Onchocerca volvulus* (Meri *et al.* , 2002), *Borrelia burgdorferi* (Hellwage *et al.* , 2001; Kraiczy *et al.* , 2004), *Borrelia afzelii* (Wallich *et al.* , 2005), and *Borrelia hermsii* (Hovis *et al.*, 2006). Recently Factor H binding proteins have been identified in *Neisseria gonorrhoeae* (Ngampasutadol *et al.* , 2008), *Borrelia spielmanii* sp. nov. (Herzberger *et al.*, 2007), *Aspergillus fumigatus* (Behnsen *et al.* , 2008), *Leptospira interrogans* (Verma *et al.* , 2006), West Nile virus (Chung *et al.* , 2006), *Neisseria meningitides* (Madico *et al.* , 2006), *Pseudomonas aeruginosa* (Kunert *et al.*, 2007).

Pneumococci are major cause of upper respiratory tract infections, and may cause life-threatening diseases such as pneumonia, meningitis, septicemia (Tuomanen *et al.* , 1995; McDaniel and Swiatlo, 2004). Pneumococci can overcome complement-mediated killing either by direct inhibition of complement activation via surface protein PspA (Tu *et al.*, 1999; Ren *et al.* , 2003, 2004), or by toxin pneumolysin (Paton *et al.* , 1984). Deletion of PspA attenuates virulence and increases complement-receptor mediated clearance of pneumococci (Ren *et al.*, 2004; Quin *et al.*, 2007). It was suggested, that PspA functions as an inhibitor of C3b deposition by controlling Factor B-mediated alternative complement pathway activation (Ren *et al.* , 2003; Tu *et al.* , 1999). Pneumolysin which is a 52kDa soluble protein oligomerizes in the membrane of the target cells to form a large ring shape transmembrane pore. In addition to its cell-modulatory activity, pneumolysin activates the classical complement pathways thus mediating the complement mediated clearance of pneumococci. Moreover, pneumococci can acquire Factor H, fluid phase regulator of alternative pathways, to PspC and Hic (Dave *et al.*, 2001, 2004a, b; Duthy *et al.* , 2002; Neeleman *et al.* , 1999, Janulczyk *et al.*, 2000, Jarva *et al.*, 2002, 2004).

Pneumococcal surface protein C (PspC) is a major pneumococcal virulence factor and based on their different anchorages in the bacterial cell wall, PspC-like protein were distinguished and two sub-groups were proposed. The classical PspC of subgroup I possess a choline binding domain (CBD) that mediates pneumococcal adherence by interacting with the secretory component of polymeric Ig receptor (pIgR) (Zhang *et al.*, 2000; Elm *et al.*, 2004). In addition to its role as an adhesin, PspC also mediates immune evasion by binding the C3 or the host complement and innate immune regulator Factor H. Apparently; PspC uses two different epitopes for binding the soluble host protein Factor H and SC (Dave *et al.*, 2004). Hic (PspC sub-type 11.4) protein of subgroup II is membrane anchored via a C-terminal LPXTG motif and interacts with Factor H. Hic shows considerable sequence homology with the N-terminal sequence of the subgroup I PspC proteins (Janulczyk *et al.*, 2000; Iannelli *et al.*, 2002). Recruitment of Factor H to the surface of pneumococci efficiently prevents activation of C3b and complement mediated opsonophagocytosis of pneumococci (Jarva *et al.*, 2004).

In addition to its known function in complement regulation, Factor H acts as an adhesion ligand for neutrophils and platelets and may also participate in immune adherence of various host tissues (Alexander *et al.*, 2001; Discipio *et al.*, 1998). Therefore, the interaction of the host complement regulator Factor H and pneumococcal PspC protein was investigated.

Briefly, the impact of pneumococcal cell surface bound Factor H on adherence to host cells and the molecular mechanism facilitating the uptake of Factor H bound pneumococci by epithelial cells was characterized.

S. pneumoniae evolved the ability to recruits complement regulator Factor H from human plasma and serum. However, flow cytometric analysis demonstrated that the pneumococcal *pspC*-mutant was also able to recruit Factor H from human plasma, although 2 to 2.5 fold lower compared to the wild-type strain. The interaction of PspC-deficient pneumococci with Factor H was not expected, since earlier data suggested that PspC is the sole FH-binding protein. However, it might be possible that a non-specific binding was measured as the Factor H concentration (~500 µg/ml) in human plasma is high. Moreover, recruitment of Factor H by the PspC-deficient pneumococcal strain can be mediated by other protein factors present in the plasma that may act as bridging or carrier molecules between other pneumococcal surface proteins and Factor H. Importantly, it was demonstrated that all pneumococcal strains recruited Factor H to the bacterial cell surface independent of the PspC subtype.

The PspC-pIgR interaction is a human specific interaction (Hammerschmidt *et al.*, 2000); however, it was and still not clear whether the PspC-Factor H interaction is also a species-specific trait. Immunoblot analysis and flow cytometry, respectively demonstrated that pneumococci preferentially recruit human Factor H compared to weak interactions if any detected for mouse or rat Factor H. Flow cytometry showed a low binding of mouse and rat Factor H to pneumococci when using species-specific anti-Factor H antibodies, albeit significantly lesser than human Factor H. However, recent findings suggested improved survival of pneumococci expressing PspC or Hic in a systemic mouse infection model, providing further evidence for the importance and versatility of PspC in different host niches (Iannelli *et al.*, 2004; Peppoloni *et al.*, 2006; Quin *et al.*, 2007). The reduced or absence of binding of mouse and rat Factor H to pneumococci as analysed by flow cytometry or immunoblots is not able to explain convincingly the increased ability of PspC expressing pneumococci to survive in mouse infection models. Therefore, further investigations are required in order to ascertain the species-specificity of pneumococcal Factor H interactions and whether there is in addition to PspC another Factor H binding protein present in *S. pneumoniae*.

Cell culture infection experiments show that Factor H bound to pneumococci significantly increased bacterial attachment to and invasion of host cells. This effect was

observed for endothelial and also for epithelial cells including nasopharyngeal cells (Detroit562), lung epithelial cells (A549), and human brain-derived endothelial cells (HBMEC). A recent study indicated that carriage isolates, which produce probably less amounts of capsular polysaccharide (CPS) than invasive isolates, recruit significantly more Factor H than systemic isolates (Quin *et al.*, 2006). Similar to this observation, our binding experiments indicated that the CPS interferes with the recruitment of Factor H. The genetic removal of the CPS increased the number of host-cell attached nonencapsulated pneumococci compared to encapsulated wild-type strains. The data suggested that the Factor H binding protein PspC is at least partially concealed below the CPS. Thus, Factor H attached to the bacterial surface acts as a molecular bridge and mediates adherence to host cells, in particular when the amount of CPS is relatively low. A similar role has been reported for Factor H and the related FHL-1 protein for adhesion and invasion of Fba expressing *S. pyogenes* (Pandiripally *et al.*, 2003)

The Factor H binding residues of the subgroup II PspC11.4 protein (Hic) were mapped to residue 29 to 269 (Janulczyk *et al.*, 2000). Interestingly, a region (amino acids 38 to 149) of Hic shows considerable sequence homology with the N-terminal sequences of subgroup I PspC proteins (Janulczyk *et al.* , 2000; Iannelli *et al.* , 2002). Respective studies on the interaction of PspC and Hic with Factor H revealed that the pneumococcal protein Hic, which is preferentially produced by serotype 3 strains, binds to SCR8-11 and SCR12-14 of Factor H whereas PspC of serotype 2 strain D39 binds to SCR6-10 and SCR13-15 of Factor H (Dave *et al.*, 2001; Duthy *et al.*, 2002). Due to inconsistency in these findings the interaction of complement regulator Factor H with the bacterial PspC protein was analysed in more detail on the molecular level. The Factor H binding site within the pneumococcal subgroup I PspC protein was mapped to a 121 amino acids long stretch in the N-terminal region of PspC comprising amino acids 38 to 158 of PspC, which is orientated towards the outside of the pathogen(Hammerschmidt *et al.*, 2007). This N-terminal Factor H binding epitope is different from the hexapeptide SC-binding site located in the R domain of PspC. The identification of Factor H- and SC-binding epitopes in different N-terminal domains of PspC is further in accordance with data from Dave et al. (2004) who demonstrated that Factor H and secretory IgA do not compete for binding to PspC. In addition, Hammerschmidt *et al.* (2007) also localized the binding sites within the host protein for PspC. It was demonstrated that the host regulator Factor H interacts with the pneumococcal PspC protein via two regions which were localized to SCR8-11 and SCR19-20. Like for PspC, SCR8-11 of Factor H has been shown to

be involved in the interaction of Factor H with Bac (β protein) of *S. agalactiae* (Jarva *et al.*, 2004). The homology of PspC, Hic, and Bac and their recognition of peptide sequences in SCR8-11 imply a general and probably conserved strategy for Factor H acquisition to bacterial and in particular streptococcal cell surfaces.

The identified contact sites in Factor H for PspC of *S. pneumoniae* differs from that of other pathogens such as *Candida albicans*, *Aspergillus fumigatus*, *S. pyogenes* and Borrelia species (Lambris *et al.*, 2008; Zipfel *et al.*, 2007; Rooijakkers and van Strijp, 2007; Kraiczy and Wurzner, 2006; Zipfel *et al.*, 2002). Theses pathogens bind to SCR6–7 and SCR19-20 or to a combination of both domains in order to recruit Factor H to their surfaces. In contrast to these pathogenic microorganisms, pneumococci do not bind FHL-1 from human plasma and none of the PspC variants interact with FHL-1.

In blocking experiments with PspC protein SH3 (amino acids 38 to 158), which includes the Factor H binding region, inhibited Factor H binding to pneumococci and Factor H mediated adherence of pneumococci to host cells. In contrast, the SC/pIgR binding domain of PspC, which includes the hexapeptide SC-binding motif, had no effect. However it is currently unclear which receptors on the host cells are involved in the Factor H-mediated adherence of *S. pneumoniae* to host cells. The RGD domain located within SCR 4 is responsible for the cell attachment activity of FHL-1/reconectin and appears to mediate binding of the protein to integrin receptors. Short synthetic peptides derived from the FH or FHL-1/reconectin RGD motif (ERGDAV) showed interference with cell spreading and binding of anchorage-dependent cells to a fibronectin matrix (Hellwage *et al.*, 1997; Zipfel and Skerka, 1999). Similarly, a matrix consisting of FHL-1/reconectin confers spreading and adhesion of anchorage-dependent cells. The identical domain is present but not active in Factor H, however, when unfolded a properly accessible RGD binding site in SCR4 may interact with specific surface receptors on host cells (Hellwage *et al*., 1997). Moreover, binding to human endothelial cells is mediated via the heparin/glycosaminoglycan-binding site within SCR20 of Factor H (Jozsi *et al.*, 2006; Jokiranta *et al.* 2005, Cheng *et al.*, 2006).

It was suggested that the interaction of Factor H with glycosaminoglycans may facilitate the tethering of this protein in tissues allowing Factor H to serve as a neutrophil adhesion ligand *in vivo* (DiScipio *et al.*, 1998). The interaction of human polymorphonuclear leukocytes (PMNs) to immobilized complement Factor H involves integrin CD11b/CD18, also termed CR3, MAC1 or $\alpha_M\beta_2$. Therefore, the role of integrin CD11b/CD18 as a potential receptor on PMNs for bacterial-bound Factor H was investigated by flow cytometry. Blocking

experiments with protein Pra1p (a *Candida albicans* protein that interacts specifically with integrin CD11b/CD18), anti CD11b or anti CD18 antibodies inhibited Factor H-bound pneumococcal association with PMNs. Although presence of Pra1p blocked association of Factor H coated pneumococci with PMNs, inhibition was more prominent in presence of blocking antibodies, suggesting the presence of additional cell surface receptor(s) of Pra1-p protein. In cell culture infection assays with A549 lung epithelial cells, the presence of Pra1p protein significantly blocked Factor H-mediated pneumococcal ingestion by host cells. No inhibitory effect on pneumococcal adherence to epithelial cells was measured. These results reiterate the existence of additional host cell surface receptor(s) of Pra1p and consequently, there is a need to investigate and identify these receptor(s) in order to understand the pathogenesis of *Candida albicans* and the role of Pra1p. In addition, absence of integrin CD11b/CD18 in CHO-K1 (Chinese hamster ovary cells) was not able to completely block Factor H mediated pneumococcal ingestion by CHO-K1 cells. The result suggested that additional host cell surface receptor(s) exist(s) that mediate(s) the interaction with bacterial bound Factor H in order to facilitate pneumococcal internalization by host epithelial cells.

Factor H is a heparin binding protein with three heparin binding sites present in SCR7, SCR13 and SCR19-20 (Pangburn *et al.* , 1991; Blackmore *et al.* , 1996, 1998). However, Factor H interacts with human endothelial cells glycosaminoglycan (Jokiranta *et al.* 2005), engagement of host cell surface glycosaminoglycans by bacteria bound Factor H to promote bacterial adherence to host cells has not been demonstrated. To assess the role of host cell glycosaminoglycans competitive inhibition experiments were performed. The present study demonstrated that the presence of heparin or dermatan sulphate significantly reduced Factor H-mediated pneumococcal adherence to and in turn ingestion by epithelial cells. This suggests the involvement of heparin binding sites of bacterial bound Factor H during pneumococcal attachment to host cells glycosaminoglycans. The blocking of pneumococcal adherence to host cells in presence of heparin or dermatan sulphate in turn resulted in decreased pneumococcal uptake by host cells. Heparin was found to interact with Factor H bound to the pneumococcal surface. Nevertheless, the presence of heparin had no influence on recruitment of Factor H by pneumococci. This is in contrast to the inhibition observed by Hammerschmidt *et al.* (2007), where the presence of heparin blocked the binding of SCR8-20 of Factor H to pneumococcal PspC protein in a dose dependent manner. This differential heparin effect observed between assays involving live bacteria and purified protein could result either from different affinities or from the presence of, in addition to PspC, a second Factor H binding

molecule on *S. pneumoniae.* Pretreatment of host cells with heparinase III, an enzyme that cleaves only heparin sulfate and not low molecular weight heparin had no effect on Factor H mediated pneumococcal adherence to or internalization into host epithelial cells.

The endothelial cells surface heparin/glycosaminoglycan-binding site has been mapped within SCR20 of Factor H (Jozsi *et al.*, 2006; Jokiranta *et al.* , 2005; Cheng *et al.*, 2006). The binding sites within bacteria-bound Factor H for its interaction with host cell surface receptor(s) were identified by employing mAbs recognizing different SCRs of Factor H as inhibitors in cell culture infection assays. These antibodies recognize the middle region of Factor H (M14), SCR14-18 (M16), C-terminal of SCR19 (CO2), or SCR 19-20 (C18). Only the antibodies mapped against the C-terminal SCR19-20, which includes CO2 and C18, blocked significantly Factor H-mediated pneumococcal adherence to host epithelial cells. In contrast, mAbs M14 and M16 showed no inhibitory effect on Factor H mediated bacterial adherence to host cells. Thus, suggesting that the SCR 19-20 of Factor H bound to *S. pneumoniae* is essential for the bacterial interaction with host epithelial cells.

The present study demonstrated for the first time the importance and the impact of host cell cytoskeleton and signalling molecules in the Factor H-mediated bacterial internalization into eukaryotic cells. Unlike PspC-hpIgR mediated pneumococcal internalization into host cells, where both the host cell actin microfilaments and the microtubules play an essential role, inhibition experiments demonstrated that the dynamics of host cell actin microfilaments but not microtubules are required for Factor H-bound pneumococcal ingestion by host epithelial cells. The presence of cytochalasin D significantly inhibited Factor H-mediated pneumococcal invasion of A549 cells. In contrast, inhibition of microtubules polymerization by nocodazole did not interfere with the Factor H-dependent pneumococcal uptake. Evidences for the role of host cell signal transductions cascade during Factor H-mediated pneumococcal infections of eukaryotic epithelial cells have been provided. Inhibition assays demonstrated that the activities of protein tyrosine kinases and PI3-kinase are essential for Factor H-mediated pneumococcal ingestion by host epithelial cells. Pretreatment of A549 lung epithelial cells with genistein, which is a general protein tyrosine kinase inhibitor or with wortmannin, a specific PI3-kinase inhibitor, blocked in a dose-dependent manner Factor H-mediated pneumococcal internalization. Further investigations are required to identify the individual kinases that are activated during Factor H-mediated pneumococcal uptake by host cells and to delineate the outside-inside and inside-outside signalling events during Factor H-mediated pneumococcal infection of eukaryotic host cells.

Since Factor H promotes pneumococcal adherence and invasion in a cell type independent manner, further investigations are required to understand whether the engagement of host cell surfaces receptors by bacteria bound Factor H are also cell type independent event.

In conclusion, Factor H binds to pneumococci via an interaction of the N-terminal part of PspC with two contact sites in Factor H. This complex formation on the pneumococcal cell surface plays dual roles in pneumococcal infections. On mucosal surfaces bacterial bound Factor H promotes adherence to and invasion of host cells. This process requires a concerted role of host epithelial cell surface glycosaminoglycans, integrins and host cell signalling pathways. Moreover, in invasive infections Factor H binding to pneumococci improves survival by inhibiting complement mediated lysis of the bacteria.

6. Material

For the successful completion and understanding of the interaction of *S. pneumoniae* with the host cells various materials and methods were employed. Some of these methods were specifically developed or modified according to the experimental requirements.

6.1. Bacterial strains and medium used

In order to investigate the interactions of *S. pneumoniae* with host cells various wild type strains and isogenic pneumococcal mutants were exploited in the infection assays. Several *E. coli* strains were utilized for the production of either recombinant proteins or as host strains for plasmids.

6.1.1. *S. pneumoniae* wild type strains

Strain	Source	Genotype	Serotype	Reference
SP36	A66	wild-type	3	(Hammerschmidt *et al.*, 2005)
SP37	NCTC10319	wild-type	35A	(Hammerschmidt *et al.*, 1997)
Sp51	ATcc11733	wild-type	2	(Hammerschmidt *et al.*, 2005)
SP173	R800 nonencapsulated	wild-type	-	(Hammerschmidt *et al.*, 2005)
SP36 variant	A66 variant	variant	-	(Hammerschmidt *et al.*, 2005)
SP257 (D39)	NCTC7466	wild-type	2	(Hammerschmidt *et al.*, 2005)
SP261 (TIGR4)	Clinical isolate	wild-type	4	(Hammerschmidt *et al.*, 2005)

Table 43 *S. pneumoniae* wild-type strains used

6.1.2. *S. pneumoniae* mutant strains used

Strain	Wild-type	Genotype	Resistance	Reference
PN8.1	NCTC10319	Δ*pspC*	erm^R	(Hammerschmidt *et al.*, 2007)
PN107	TIGR4 (P261)	Δ*cps*	$Kana^R$	(Pearce *et al.,* 2002)
PN185	D39 (P257)	Δ*cps*	$Kana^R$	(Rennemeier *et al.*, 2007)

Table 44 *S. pneumoniae* mutant strains used. *cps*: capsular polysaccharide

6.1.3. *E. coli* strains used

Strain	Genotype	Source
BL21 (DE3)	F-, *ompT*, *hsdSB* (rB-mB-), *gal*, *dcm* (DE3)	Novagen
DH5α	Δ (*lac*) *U169*, *endA1*, *gyrA46*, *hsdR17*, Φ80Δ (*lacZ*) M15, *relA1*, *supE44*, *thi-1*	Sambrook *et al.*, 1989
NovaBlue	*endA1*, *hsdR17* ($r_{K12}^{-}m_{K12}^{+}$), *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, *lac* F´ [$proA^{+}B^{+}lacI^{q}ZΔM15$ ::Tn*10*] (Tet^{R})	Novagen
XL1-Blue	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac* F´[*proAB* $lacI^{q}ZΔM15$ ::Tn*10*] (Tet^{R})	Stratagene

Table 45 *E. coli* strains used.

6.1.4. Growth medium for *S. pneumoniae*

Medium and plates	Constituents
Blood Agar-Plates (Oxoid)	23 g Peptone, 5 g NaCl, 14 g Agar, 65 ml Sheep blood, pH 7.4
THY-Medium	36.4 g THB (Todd Hewitt Broth) per Litre H_2O, 0.5 % Yeast extract autoclaved
CpH8-Medium	800 ml Pre C, 26 ml Supplement, 20 ml Glutamine (0.1 %), 20 ml Adams III, 10 ml Sodium pyruvate (2 %), 30 ml Phosphate buffer(pH 8.0), 18 ml Yeast extract (5 %), 76 ml H_2O
Individual components of CpH8 medium	
PreC	1.208 g Sodium acetate, 5 g Casein hydrolysate, 5 mg L-Tryptophan, 5 mg L-Cystein made upto 1 l with H_2O, pH 7.5 with NaOH
3 in 1-Salt	100 g $MgCl_2 \times 6\ H_2O$, 500 mg $CaCl_2$, 3.3 mg $MnSO_4 \times 4\ H_2O$
Supplement	60 ml "3 in 1" Salt, 120 ml Glucose (20 %), 6 ml Saccharose (50 %),120 ml Adenosine (2 mg/ml), 120 ml Uridine (2 mg/ml)
Phosphate buffer	947 ml K_2HPO_4 (1M), 53 ml KH_2PO_4 (1M)
Adams I	0.15 mg Biotin, 150 mg Nicotinic acid, 175 mg Pyridoxin-HCl, 600 mg Ca-Pantothenate, 160 mg Thiamine-HCl, 70 mg Riboflavin made upto1 l with H_2O
Adams II	500 mg $FeSO_4 \times 7\ H_2O$, 500 mg $CuSO_4 \times 5\ H_2O$, 500 mg $ZnSO_4 \times H_2O$, 200 mg $MnCl_2 \times 4\ H_2O$, 10 ml HCl conc. made up to 1 l with H_2O

Adams III	160 ml Adams I, 40 ml Adams II, 2 g L-Asparagine, 400 mg Choline-HCl, 17 mg $CaCl_2$

Table 46 Growth medium for *S. pneumoniae* cultures

6.1.5. Growth medium for *E. coli*

Medium and plates	Constituents
LB- Agar Plates	1 % Bacto-Tryptone, 0.5 % Yeast extract, 1 % NaCl, 1.5 % Agar pH 7.5
LB (Luria-Bertani)-Medium	1 % Bacto-Tryptone, 0.5 % Yeast extract, 1 % NaCl, pH 7.5

Table 47 Growth medium for *E.coli* cultures

6.2. Cell lines, cell culture media and antibodies

For the elucidation of the mechanism of pneumococcal interaction with eukaryotic host cells, *in-vitro* infection assays were performed. Several epithelial and endothelial cell lines were engaged in these assays. All the cell lines were propagated in specific buffered isotonic culture medium that contains the essential nutrients, minerals, salts and amino acids.

6.2.1 Epithelial cell lines used

Epithelial cell line	Source	Reference
A549	ATCC CCL-185, adherent alveolar epithelial cells (Typ II Pneumocytes) from human lung carcinoma	Giard *et al*., 1973
Detroit 562	ATCC CCL-138, human adherent epithelial cells from pharynx carcinoma	Peterson WD Jr., 1968
Calu-3	ATCC HTB-55, human adherent epithelial cells from adeno carcinoma	Fogh *et al*., 1975
MDCK	ATCC CCL-34, adherent kidney epithelial cell from a Cocker Spaniel	Madin und Darby, September 1958
MDCK- hpIgR	Stably transfected MDCK cells with human pIgR (poly immunoglobulin receptor)	Tamer *et al.*, 1995
CHO-K1	ATCC CCL-61, a hamster fibroblast cell line	Puck *el al.*, 1958

CHO-CD11b/CD18	Stably transfected CHO cell line with cDNA for full length human CD11b and CD18	Ingalls *et al.*, 1997

Table 48 Epithelial cell line used

6.2.2 Endothelial cell lines used

Endothelial cell line	Source	Reference
HBMEC	human adherent brain endothelial cells (human brain-derived microvascular endothelial cells)	Kwang Sik Kim, Johns Hopkins University School of Medicine, Baltimore, USA

Table 49 Endothelial cell line used

6.2.3 Cell culture medium used

Cell line	Medium
A549	DMEM (Dulbecco's Modified Eagle Medium) with 1 g/l Glucose (PAA), 10 % FBS (Foetal Bovine Serum "Gold" PAA), 2 mM Glutamine (PAA), 0.1 mg/ml Streptomycin (PAA), 100 Units/ml Penicillin (PAA)
Detroit 562	RPMI 1640 (PAA), 10 % FBS, 2 mM Glutamine, 1 mM Sodium pyruvat, 0.1 mg/ml Streptomycin, 100 Units/ml Penicillin
HBMEC	RPMI 1640, 10 % FBS, 10 % Nu-Serum IV (BD, Biosciences), 2 mM Glutamine, 1 % Non-essential amino acids (PAA), 1 % MEM Vitamin (PAA), 1 mM Sodium pyruvate (PAA), 0.1 mg/ml Streptomycin, 100 Units/ml Penicillin
MDCK	Eagle´s MEM (minimum essential medium) (PAA), 10 % FBS, 2 mM Glutamine, 0.1 mg/ml Streptomycin, 100 Units/ml Penicillin
MDCK-hpIgR	Eagle´s MEM (minimum essential medium), 10 % FBS, 2 mM Glutamine, 0.1 mg/ml Streptomycin, 100 Units/ml Penicillin
Calu-3	Eagle´s MEM (minimum essential medium), 10 % FBS, 2 mM Glutamine, 1 % Non-essential amino acids, 1 mM Sodium pyruvate, 0.1 mg/ml Streptomycin, 100 Units/ml Penicillin
CHO	Ham F12 medium with 2 mM Glutamine (Gibco), 10 % FBS
CHO CD11b/CD18	Ham F12 medium with 2 mM Glutamine (Gibco), 10 % FBS, supplemented with 1 mg of G418 per ml

Table 50 Cell culture growth medium for epithelial and endothelial cells

6.2.4 Additional components for cell culture

FBS	Fetal Bovine Serum "Gold"
Cell freezing medium	800 µl Cell specific medium (PAA), 100 µl FBS, 100µl DMSO (Applichem)
Penicillin/Streptomycin (PAA)	0.1 mg/ml Streptomycin, 100 Units/ml Penicillin
Trypsin/EDTA (1x) (PAA)	0.5 mg/ml Trypsin, 0.22 mg/ml EDTA
PBS/EDTA	PBS (Phosphate buffer saline) pH 7.4, 2 mM EDTA

6.3. Antibodies used

Antibody	Dilution	Source
Enzyme conjugated antibodies:		
Swine anti-rabbit, HRP (horse radish peroxidase) cojugated	1:5000	Dako
Goat anti-mouse, HRP (horse radish peroxidase) conjugated	1:2000	Jackson
Rabbit anti-goat, HRP (horse radish peroxidase) cojugated	1:2000	Dako
Fluorochrome conjugated antibodies:		
Alexa Fluor 488 Goat anti-rabbit IgG	1:300	Invitrogen
Alexa Fluor 568 Goat anti-rabbit IgG	1:350	Invitrogen
Alexa Fluor 488 Goat anti-mouse IgG	1:300	Invitrogen
Alexa Fluor 568 Goat anti-mouse IgG	1:350	Invitrogen
Cy5 Goat anti-rabbit IgG	1:100	Dianova
FITC Goat anti-rabbit IgG	1:100	Dianova
FITC Rabbit anti-goat IgG	1:300	Dianova
Unconjugated antibodies:		
Rabbit anti-*S. pneumoniae* (Strain SP139 und SP51)IgG	1:100	Eurogentec
Rabbit anti-PspC (SH2)	1:100	Eurogentec
Rabbit anti-SC (secretory component)	1:200	J. P. Vaerman Brussel, Belgium
Goat anti- human Factor H	1:200	Calbiochem
Rabbit anti-Rac1	1:1000	Cell Signalling
Rabbit anti-Cdc42	1:1000	Cell Signalling
Rabbit anti-RhoA	1:1000	Cell Signalling

Rabbit anti phospho Erk1/2	1:2000	Cell Signalling
Rabbit anti-Erk1	1:2000	Santa Cruz
Rabbit anti-phospho p38	1:1000	Santa Cruz
Rabbit anti-phospho Akt	1:2000	Cell Signalling
Rabbit anti-Akt	1:2000	Cell Signalling
Rabbit anti-Clathrin Heavy Chain	1:1000	Cell Signalling
Rabbit anti-cSrc	1:1000	Santa Cruz
Mouse anti-pTyr	1:2000	Santa Cruz
Goat anti-phospho PI3K p85α	1:1000	Santa Cruz
Mouse anti-phospho JNK	1:1000	Cell Signalling
Goat anti- JNK1/3	1:1000	Santa Cruz
Rabbit anti phospho c-Jun	1:2000	Cell Signalling
Mouse anti human CD11b	2 µg/ml	Invitrogen
Mouse anti human CD18	2 µg/ml	Invitrogen
Rabbit anti Pra1p	2 µg/ml	P.F. Zipfel, Jena, Germany
Rabbit anti FH SCR19-20 (C18)	2 µg/ml	P.F. Zipfel, Jena, Germany
Mouse anti FH SRC 14-18 (M16)	2 µg/ml	P.F. Zipfel, Jena, Germany
Mouse anti FH SRC 19 (CO2)	2 µg/ml	P.F. Zipfel, Jena, Germany
Mouse anti FH Middle region (M14)	2 µg/ml	P.F. Zipfel, Jena, Germany

Table 51 List of antibodies used

6.4. Proteins, inhibitors and other reagents used

Human Complement Factor H	Calbiochem
Heparin potassium salt	ICN
Heparin FITC	Invitrogen
Chondroitin Sulfate B (Dermatan Sulphate)	Sigma
Heparinase III	Sigma
Clostridum *difficile* Toxin B-1470	Klaus Aktories, Freiburg, Germany
Clostridum *dificile* Toxin B-10463	Klaus Aktories, Freiburg, Germany

Cytochalasin D	MP Biomedical
Nocodazole	Sigma
LatrunculinB	Calbiochem
Jasplakinolide	Calbiochem
NSC 23766 (Rac1 inhibitor)	Calbiochem
Y27632 (Rho kinase inhibitor)	Calbiochem
Secramine A (Cdc42 inhibitor)	Tomas Kirchhausen, Harvard University, USA
Wortmannin	Calbiochem
LY294002	Calbiochem
Akt inhibitor IV	Calbiochem
Genistein	Calbiochem
PP2	Calbiochem
AG957	Calbiochem
PD98059	Calbiochem
JNK inhibitor II	Calbiochem
SB2025160	Calbiochem
Chlorpromazine Hydrochloride	Calbiochem
Monodansylcadaverine	Sigma
Dynasore	Tomas Kirchhausen, Harvard University, USA
Pra1 protein	P.F. Zipfel, Jena, Germany

6.5. Plasmids and Vectors

Clone No.	Plasmid	Encoded Gene	Function/ tagging	Source	Reference
4	pRK5	Rac1-T17N	dn, myc tagged	Alan Hall, New York, USA	Nobes and Hall, 1999
5	pRK5	Cdc42-T17N	dn, myc tagged	Alan Hall, New York, USA	Nobes and Hall, 1999
12	pRK5	RhoA-T19N	dn, myc tagged	Alan Hall, New York, USA	Nobes and Hall, 1999
10	pmaxGFP	GFP	Green fluorescent protein		

59	pCDNA3.1	SrcK297M	kinase inactive c-Src	David Schlaepfer, San Diego, USA	
60	pCDNA3.1	CskK222M	kinase inactive Csk	David Schlaepfer, San Diego, USA	Sieg *et al.*, 1998
61	pCDNA3.1	Csk wt	Wild type Csk	David Schlaepfer, San Diego, USA	Sieg *et al.*, 1998
65	pEGFP-C1	h-LcaEGFP	EGFP tagged Clathrin Light chain	Tomas Kirchhausen, Harvard University, USA	

Table 52 List of Plasmids and Vectors used in transfection experiments. dn: dominant negative

6.6. Reagents and Buffers used

6.6.1. Antibiotics

Antibiotics	Concentration *E. coli* in µg ml^{-1}	Concentration *S. pneumoniae* in µg ml^{-1}	Dissolved in
Ampicillin	100	100	H_2O dest.
Kanamycin	50	200	H_2O dest.
Erythromycin	250	5	70% Ethanol

Antibiotics	Stock solution	Concentration for cell culture	Concentration for antibiotic protection assay
Penicillin	10 000 Units/ ml	100 Units/ml	100 Units/ml
Streptomycin	10 mg/ ml	0.1 mg/ml	-
Gentamicin	10 mg/ ml	-	0.1 mg/ml

Table 53 List of antibiotics and their respective concentrations used

6.6.2. Enzymes

Enzyme	Function	Company
Lysozyme	Protein-digestion	Sigma
Trypsin	Protein-digestion	Sigma

Table 54 Enzymes used

6.6.3. Oligonucleotides

siRNA used

Target gene	Target Sequence	Source
Dynamin II	CAG GAG ATT GAA GCA GAG ACC	Qiagen
Control siRNA Low GC content		Invitrogen

6.6.4. DNA ladder

Marker	DNA Ladder size (bp)	Company
1 kb DNA – Ladder	12000, 5000, 2000, 1650, 1000, 850, 650, 500, 400, 300, 200, 100	Invitrogen
Mass Ruler ™ DNA Ladder Mix	10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1031, 900, 800, 700, 600, 500, 400, 200, 100, 80 300,	Fermentas

Table 55 DNA ladder used

6.6.5. Protein Ladder

Protein	Band sizes in kDa	Company
Prestained Proteinmarker Broad Range	175, 83, 62, 47.5, 32.5, 25, 16.5, 6.5	NEB
BenchMark™ Prestained Protein Ladder	180, 115, 82, 64, 49, 37, 26, 19, 15, 6	Invitrogen

Table 56 Protein markers used

6.6.6. Buffers and solutions

6.6.6.1 Buffers and solutions for cell biology

Moviol-Solution	20 g Moviol 4-88 was dissolved in 80 ml PBS by continuous stirring. The 40 ml Glycerin was added and mixed overnight, Solution was then centrifuged at26,000 x g and the supernatant was used in assays
Paraformaldehyde (PFA)	heat 37 % PFA in PBS with continuous stirring at 70°C,

	add dropwise 0.1 M NaOH till the paraformaldehyde completely dissolves. Aliquots of the filtered solutions were stored at -20°C
PBS/EDTA	10 mM PBS, 2 mM EDTA pH 7.5
1 x PBS	37 mM NaCl, 2.7 mM KCl, 80 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4
Poly-D-Lysin	10 µg/ml in 1 x PBS
Saponin solution	1 % Saponin in cell culture medium, sterile filtered
FACS – Buffer	0.5 % FBS in 1x PBS, pH 7.4
FACS – Fixation Buffer	1 % PFA, 0.5 % FBS in 1x PBS, pH 7.4
DIF –Fixation solution	3.7 % PFA in 1 x PBS
Cacodylate Buffer	0.1 M Cacodylate (Dimethylarsenicacid sodiumsalt-Trihydrate), 0.09 M Sucrose, 0.01 M $MgCl_2$, 0.01 M $CaCl_2$)
Electron Microscopy (EM) Buffer	Cacodylate buffer, 3 % Formaldehyde, 2 % Glutaraldehyde

6.6.6.2. Buffer and solutions for Molecular biology

6x DNA loading dye	0.25 % Bromphenolblue**,** 0.25 % Xylencyannol, 40 % Sucrose in H_2O
EDTA-solution	0.5 M in dH_2O, pH 8.0
Ethidiumbromid solution	100 µl EtBr stock solution in 200 ml dH_2O
IPTG-Stock	1 M IPTG in H_2O
Lysozyme	5 mg/ml in TES
Sodium acetate solution	3 M Sodium acetate, pH 4.8 with acetic acid
Plasmid-Miniprep. Solution I	50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.1 mg/ml RNase
Plasmid-Miniprep. Solution II	0.2 N NaOH, 1 % SDS (freshly prepared)
Plasmid-Miniprep. Solution III	3 M Sodium acetate, pH 4.8
Pronase	5 mg/ml (30 min incubation at 37°C incubation)
RNase	5 mg/ml TES (15 min incubation at100°C)
50 x TAE-Buffer	2 M Tris, 0.6 M EDTA, 0.57 % Acetic acid
TE (Tris/EDTA)	10 mM Tris-HCl, 1 mM EDTA, pH 8.0

TES (Tris EDTA Saline)	10 mM Tris-HCl (pH 8,0), 1 mM EDTA, 100 mM NaCl
X-Gal	0.2 g 5-Brom-4-Chlor-3-indolyl-β-D-galactopyranosid in 10 ml Dimethylformamid (DMF) or in 10 ml DMSO

6.6.6.3. Buffers and solutions for Protein purification, SDS-PAGE und Western-Blot

Transfer Buffer (Western-Blot)	5.8 g Tris, 2.9 g Glycine, 0.37 g SDS (0.037 %), 200 ml Methanol added to 1000 ml with dH_2O
Triton X-100 Lysis Buffer	10 mM Tris-HCL, pH 7.5, 1 mM EDTA, 1 mM EGTA 100 mM NaCl, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 0.1 % SDS, 1 % Triton x 100, 10 % Glycerol, 0.5 % Deoxycholate, Complete protease inhibitor cocktail, PMSF
RIPA Lysis Buffer	50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1 % SDS, 1 % Triton x 100, 1 % Deoxycholate, Complete protease inhibitor cocktail (Roche), PMSF
Chloronaphthol	1 Tablet (30 mg) in 10 ml Methanol
Coomassie-Destainer	40 % Ethanol, 10% Acetic acid in H_2O
Coomassie-stain	50 % Ethanol, 5.8 % Acetic acid, 0.2 % Coomassie Brilliant Blue™ R250
8 x Running Buffer	0.12 M Tris, 0.96 M Glycin, 0.5 % SDS
1 x PBS	37 mM NaCl, 2.7 mM KCl, 80 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4
Protein-Probe Buffer	0.5 M Tris-HCl pH 6.8, 10 % Glycerin, 10 % SDS, 5 % 2-Mercapthoethanol, 0.05 % Bromphenolblue-Solution (10 µg/ml dH_2O)

7. Methods

7.1. Working with bacteria

7.1.1. Pneumococcal culture conditions

All strains of *S. pneumoniae* were cultivated overnight on blood agar plates at 37°C under 5 % CO_2 atmosphere. THY medium was inoculated with *S. pneumoniae* with a starting OD_{600} of 0.05 to 0.08 and was cultivated at 37°C until it reached the OD_{600} of 0.350 to 0.4.

7.1.2. *E. coli* culture conditions

E. coli were cultured overnight on LB agar plates, if required in the presence of appropriate antibiotics, at 37°C. For liquid culture, LB medium containing the appropriate antibiotics was inoculated with *E. coli* incubated at 37°C under shaking at 120 rpm.

7.1.3. Storage of bacterial strains

For long term storage, the bacterial cultures were stored in a 20 % (v/v) glycerol solution at -80°C.

7.1.4. Preparation of competent *E. coli* cells

In order to prepare competent *E. coli* cells, a 2 ml overnight culture of *E. coli* was diluted 1:250 with fresh LB medium and cultivated at 37°C, 120 rpm until an OD_{600} of 0.6 was obtained. Thereafter bacteria were briefly chilled for 15 min on ice and then centrifuged for 10 min, 5000 x g at 4°C. The sediment was gently resuspended in 1:4 culture volumes of 100 mM $CaCl_2$ and incubated on ice for 20 min. The bacterial were then centrifuged for 10 min, 5000 x g at 4°C and the sediment was gently resuspended in 1:40 culture volume of 100 mM $CaCl_2$, 15 % glycerol. Finally 50 µl aliquot were made in 1.5 ml prechilled eppendorf tubes. The tubes were briefly immersed in liquid nitrogen and stored at -80°C.

7.1.5. Transformation of *S. pneumoniae*

S. pneumoniae are naturally transformable bacteria and they acquire their competence at distinct OD and in a special type of growth medium known as CpH8 medium. In order to induce competence, CpH8 medium was inoculated with bacteria at a starting OD_{600} of 0.03-0.04 and cultivated at 37°C. Once the culture reaches the OD_{600} of 0.1 to 0.15, 1 ml bacterial suspension is taken out and is incubated with 1 µg CSP (Competence Stimulating peptide) for 20 min. at 37°C. Later the bacteria are subjected to cold shock on ice for exactly 4 minutes. After this 250 µl of the bacterial suspension was incubated with 0.5 to 5 µg of DNA, first for

30 min at 30°C followed by 90 min incubation at 37°C. Finally the bacterial suspension was platted onto blood agar plate, in presence of appropriate antibiotic resistance, and was incubated overnight at 37°C. The following day, positive colonies were isolated and further screened on the molecular level for integrity of the inserted DNA sequence.

7.1.6. Transformation of *E. coli*

The 100 µl aliquot of chemically competent cells stored at -80°C were thawed on ice and 100 ng of plasmid DNA was added. The mixture was then incubated on ice for 30 minutes. The bacterial suspension was subjected to heat shock at 42°C for 90 sec, followed by brief incubation on ice for 2 minutes. 900 µl of LB medium was added and the suspension was incubated at 37°C for 1 h. Later, 100 – 150 µl of the transformed bacteria were platted onto LB agar plates in the presence of appropriate antibiotics for selection of transformed bacteria. Recombinant clones were isolated and subcultured. Subculture comprises of 5 ml LB medium in small tubes along with appropriate antibiotic. Each clone was grown overnight in the 37°C incubator shaker followed by plasmid isolation on the following day to screen for positive clones.

7.2. Eukaryotic cell lines

7.2.1. Cell culture conditions, maintenance and cryo-conservation

All the eukaryotic cell lines were cultured in sterile tissue culture flasks containing cell line specific media and were incubated at 37°C under 5 % CO_2 atmosphere. The cell culture specific media and solutions were pre-warmed in a 37°C water bath, whereas PBS-EDTA or Trypsin-EDTA was thawed at room temperature.

Once the culture flasks have reached the confluency of 75 to 80 %, the cells were splitted into new tissue culture flasks and subcultivated. The cells were first detached from the flask by treatment with either Trypsin-EDTA or PBS-EDTA. Once in suspension, the cells were sedimented by centrifugation at 700 rpm for 3 min and then resuspended in cell culture specific media. Finally, the cells were diluted 1:6 or 1:10 and propagated further at 37°C under 5 % CO_2 atmosphere. Most of the cell lines were cultivated upto 12 passages after which new cell vials were thawed form liquid nitrogen stores.

7.2.2. Freezing of cell lines

In order to ensure a continuous supply of cell, the cell lines should be stored properly and in sufficient amount. For this, a confluent flask was taken; the cells were detached and

collected in suspension following treatment with Trypsin-EDTA or PBS-EDTA. The cells thus obtained were then resuspended in cell culture specific media containing 10 % FCS and 10% DMSO. Special types of cryo-vials were used for the storage of cell lines. From every single 75 cm² tissue culture flask, 2 aliquots of cell suspension could be made and stored. Briefly, after centrifugation of cells at 700 rpm for 3 min, the sedimented cells were resuspended in 1.6 ml of cell culture medium and 0.2 ml of FBS. Finally, 0.9 ml of this cell suspension was added to 0.1 ml of DMSO per cryo-vial. These cryo-vials were stored in a special type of storage boxes that are filled with Isopropanol at -80°C. These special boxes have the capacity to regulate the decrease in temperature, since here the temperature decreases at a rate of 1°C per min thereby preventing the formation of intracellular ice-crystals that could damage the cells. Finally the cryo-vials are taken out from the boxes and stored in tanks containing liquid nitrogen for a longer period.

7.2.3. Thawing of cell lines from liquid nitrogen storage

The cultivation of cell line stored in liquid nitrogen was accomplished by thawing the cell suspension stored in cryo-vials at 37°C water bath. Later, 1 ml of cell culture medium was added to the vial, in-order to dilute the DMSO concentration. The suspension was further diluted with 5 ml cell culture medium in a 15 ml falcon and centrifuged at 700 rpm for 3 minutes. The cell sediment thus obtained was resuspended in 1 ml of medium and splitted 1/3 and 2/3 respectively, into two 75 cm^2 tissue culture flasks containing 20 ml of cell culture medium each. The cells were cultivated by incubating the flasks at 37°C under 5 % CO_2 atmosphere.

7.2.4. Estimation of cell number using the Neubauer count chamber

For the success of the infection assay it was important to know the exact number of cells used. The estimation of the number of cells in a suspension was determined with a Neubauer counting chamber. A drop of cell suspension was added on the top of the Neubauer count chamber covered by a cover-slip and the number of cells in the central largest square was counted using a light microscope. The exact number of cell per ml of suspension was obtained by multiplying the number of cells in the large square with 10^4 (Volume of the large square is 10^{-4} ml) = Cell number/ml.

7.2.5. Determination of hpIgR expression on eukaryotic cell lines

The expression of hpIgR on eukaryotic cell lines was assessed by flow cytometry. Briefly the eukaryotic cells were cultured until confluency of 75 to 80 %. The cells were detached and collected in a suspension by treatment with accutase (PAA). The cells were washed once with FACS buffer containing 0.5 % FCS in PBS by centrifugation at 700 rpm for 3 minutes. Aliquots of approximately 1 x 10^5 cells in 100 µl FACS buffer were transferred in 1.5 ml tubes and incubated on ice. The primary antibody was added using a dilution determined earlier (Table 9) and the mixture was incubated for 45 min on ice. The cells, after being washed with FACS buffer, were resuspended in 1:300 dilution of a fluorochrome labelled secondary antibody and incubated again for 45 min on ice. Finally, the cells were washed with FACS buffer and fixed in 1 % PFA. The fluorescence was analysed by flow cytometry using a FACS Calibur or FACSCanto I or II (Becton Dickinson). Calu-3 and MDCK-hpIgR cell lines were used to assess the expression of h-pIgR.

7.3. Cell culture infection assays

7.3.1. Preparation *S. pneumoniae* for Infection Assay

Pneumococci were cultured as explained in 7.1.1. To investigate the role of Factor H on pneumococcal adherence to and invasion of eukaryotic cell, 1x 10^7 pneumococci were preincubated with, unless otherwise specified, 2 µg of purified human Factor H (Calbiochem) for 30 min at 37°C. Following the pre-incubation, infection assays were performed for 3 h with a multiplicity of infection (MOI) of 50 bacteria per cell at 37°C.

7.3.2. Preparation of eukaryotic cell lines for infection assays

Eukaryotic cells were cultured as described (7.1.2.) However, for infection assays the cells were seeded in wells of a 24-well plate (Cellstar, Greiner, Germany). Based on the kind of infection assay performed, the cells were pretreated with different proteins, antibodies or inhibitors. Unless otherwise specified, the cells were preincubated for 30 min at 37°C under 5 % CO_2 and the assays were performed in presence of these inhibitors.

7.3.3. Infection assays

Pneumococcal adherence and invasion was quantified after infecting the eukaryotic epithelial and endothelial cells. Briefly, the host cells were seeded on glass cover slips (diameter 12 mm) or directly in wells of a 24-well plate (Cellstar, Greiner, Germany) and were cultured for 2 days at 37°C under 5 % CO_2. Detroit 562 cells were seeded at a density of

7.5 x 10^4 cells per well, whereas all the other cell lines were seeded at a density of 2.5 to 3 x 10^4 cells per well and cultivated to confluency of about 70 to 80 %. The cells were washed three times with Dulbecco´s modified Eagle´s medium containing HEPES (DMEM-HEPES, PAA Laboratories, Coelbe, Germany) supplemented with 1 % fetal calf serum (FCS). Wherever specified, the cells were pre-treated with proteins or inhibitors and then infected with pneumococci. In a standardized assay, a MOI of 50 bacteria per host cell was used. The role of Factor H for adherence and or invasion was analyzed by pre-incubating pneumococci with Factor H, as described in 7.3.1 and the infection was performed for 3 h.

However, to investigate the role of hpIgR on adherence and the induced signal cascades, the infection was synchronized by centrifuging the bacteria to the host cells at 700 rpm for 3 min. The infection assays were carried out for 1 hour at 37°C under 5 % CO_2. Thereafter unbound bacteria were removed by rinsing the well three times with DMEM-HEPES supplemented with 1 % FBS. The total number of adherent and intracellular recovered bacteria was monitored by plating the bacteria on blood agar plates after detachment and lysis of cells with saponin (1 % w/v). The number of viable intracellular bacteria was quantitated by the antibiotic protection assay. The infection dose (CFU) per well was controlled by serial plating of the bacteria on blood agar plates.

7.3.4. Quantification of bacterial invasion by the antibiotic protection assay

The antibiotic protection assays were performed to quantify the total number of recovered pneumococci from the intracellular compartments of the host cells. After the infection experiments, generally 3 h or after synchronization for 1 h, the cell layers were washed thoroughly to remove unbound bacteria. To kill the extracellular adherent pneumococci, host cells were incubated for 1 hour with DMEM-HEPES containing 100 µg gentamicin and 100 U penicillin G at 37°C under 5 % CO_2. The intracellular pneumococci were released by a saponin-mediated host cell lysis (1 % w/v) for 10 min. The total number of invasive and recovered pneumococci was monitored after plating sample aliquots on blood agar plates, followed by colony formation and enumeration.

7.3.5. Association of *S. pneumoniae* with human PMNs

Isolation of human PMNs

The polymorphonuclear leukocytes (PMNs) were isolated from 10 to 20 ml blood donated by a healthy individual. The blood clotting was prevented by adding 2.5 ml of citrate buffer. In order to separate out various blood cell and components, a gradient of 12 ml

histopaque (Sigma-Aldrich) and 10 ml ficoll (GE Heathcare) was made and the blood suspension, diluted with PBS, was carefully added over the gradient at a ratio of 1:1. The blood suspension gradient mix was centrifuged at 800 x g for 20 min in a hanging basket centrifuge in order to separate out various blood components. The second layer containing the granulocytes was carefully collected in a fresh tube and diluted with 50 ml ice cold RPMI. The granulocytes suspension was centrifuged at 800 x g for 10 min at 4°C and the resulting sediment was incubated for 30 sec with 9 ml of Milli-Q water in order to lyse erythrocytes. Thereafter 1 ml of 10 x PBS was added and a final volume of 50 ml was reached by adding ice cold RPMI. This suspension was centrifuged at 800 x g for 10 min at 4°C and the sediment obtained was finally resuspended in 1 ml ice cold RPMI and incubated on ice.

Flow cytometric analysis of *S. pneumoniae* association with human PMNs

The number of PMNs isolated was determined with a counting chamber and aliquots of 5 x 10^4 PMNs were made in FACS tubes (BD). For inhibition studies, PMNs were preincubated with proteins or antibodies, unless otherwise specified, for 30 min at 37°C. For analyzing pneumococcal association, PMNs were incubated for 30 min in 100 µl PBS / 0.5 % FCS with 1 x 10^6 pneumococci at 37°C. The association of pneumococci was investigated in the absence or presence of Factor H. Following the bacterial incubation, PMNs were washed with ice cold PBS/ 0.5 % FCS by centrifugation at 1200 rpm for 5 minutes. The PMNs were then resuspended in 1:100 dilution of pneumococcal antiserum and incubated on ice for 30 min. This was performed to detect only the associated and not the phagocytosed pneumococci. The suspension was washed with ice cold PBS/ 0.5 % FBS and incubated with Alexa 488-conjugated anti-rabbit Ig antibody (Invitrogen) for 30 min on ice. Finally the PMNs were fixed with 20 0µl PBS/ 1 % FCS/ 1 % paraformaldehyde. The association of bacteria was assessed by flow cytometry using FACSCanto I and II (Becton Dickinson). The geometric mean fluorescence intensity (GMFI) x percentage of labeled bacteria was recorded as a measure for binding activity.

7.3.6. Transfection studies

For genetic interference studies, the MDCK-hpIgR cells were transiently transfected with the desired plasmid using either Fugene6 transfection reagent (Roche) or Lipofectamine LTX reagent (Invitrogen) as per the manufactures instruction. The transfection efficiency was estimated by transfecting a subset of cells with GFP encoding plasmid. 48 h post transfection, cells were infected *S. pneumoniae*.

7.3.7. siRNA studies

Similar to plasmid transfection assays, Calu-3 cells were transfected with (50 nM) Dynamin II siRNA (Qiagen) using Lipofectamine RNAiMAX transfection reagent (Invitrogen). The transfection efficiency was estimated by transfecting a subset of cells with fluorescent siRNA (Invitrogen). The infection assays were performed using S. pneumoniae wild type strain 35A, 48 h post transfection.

7.4. Microscopy

7.4.1. Preparation of cells for Immunofluorescence microscopy

To prepare the cells for immunofluorescence staining, the cells were fixed by treatment with 1 to 3 % solution of paraformaldehyde in PBS. 37 % stock solutions of paraformaldehyde are stored at -20°C and when required, an aliquot was thawed by heating the solution to 70°C.

7.4.2. Double Immunofluorescence staining for CSLM

Double immunofluorescence staining was carried out to differentiate between extracellular and intracellular bacteria. Following the infection assay, the cells were washed 3 times with PBS and fixed in presence of 3 % paraformaldehyde for 30 min at room temperature. The fixed cells were washed nicely with PBS and blocked overnight with 10 % FBS at 4°C. The extracellular, adhered pneumococci were stained with polyclonal anti-pneumococcal antiserum followed by secondary goat anti rabbit IgG coupled to Cy5 (blue) (Dianova, Germany) or Alexa 488 (green) (Invitrogen). In order to stain the intracellular bacteria, the cells were permeabilized with 0.1 % Triton X-100 solution in PBS for 10 min and then stained with polyclonal anti-pneumococcal antiserum followed by secondary goat anti rabbit IgG coupled to Alexa 568 (red) (Invitrogen). The samples were incubated for 30-45 min with antibodies and were washed nicely after the incubation. Unless otherwise specified, the host cell actin cytoskeleton was stained with phallodin Alexa 488 (Invitrogen). Finally, the coverslips were mounted on a drop of moviol and fixed with the help of nail polish onto glass slides. The slides were stored at 4°C and were viewed using a confocal laser scanning microscope (Leica TCS SP5 AOBS) and the LAS AF SP5 software was used for image acquisition.

7.4.3. Preparation of samples for Raster electron microscopy (REM)

In order to prepare the samples for raster electron microscopy, the cells were first washed nicely with PBS after the infection and later fixed in Cacodylate Buffer (0.1 M Cacodylate (Dimethylarsenicacid sodiumslat-trihydrate), 0.09 M Sucrose, 0.01 M $MgCl_2$, 0.01 M $CaCl_2$) with 3 % Formaldehyde und 2 % Glutaraldehyde (EM Buffer) over night at 4°C. Following the fixation, the samples were processed further by Dr. Manfred Rohde (HZI, Braunschweig, Germany). Briefly, the fixed samples were washed nicely with TE buffer and then stepwise dehydrated with dilutions of acetone. The samples were then dried in presence of liquid CO_2, till they reach a critical point of dryness and analyzed with gold particles in a Field emission-raster electronmicroscope (DSM 982 Gemini, Zeiss) at a voltage of 5kV. The images were digitally taken and saved.

7.4.4. Preparation of samples for Transmission electron microscopy (TEM)

To investigate the cell morphology and the cellular structures in detail, transmission electron microscopy was performed. For this the cells were washed nicely with PBS after the infection and then fixed in 0.5 % Tannin solution in cacodylate buffer with 3 % formaldehyde and 2 % gluteraldehyde for 1 h on ice. After washing the samples 3 times with PBS they were fixed with 1 % Osmium in cacodylate buffer with 3 % formaldehyde and 2 % gluteraldehyde for 1 h. Later the samples were washed nicely by PBS untill maximum osmium had been removed, by centrifuging at 13,000 rpm for 5 min and stored at 4°C. The samples were processed further by Dr. Manfred Rohde (HZI, Braunschweig, Germany).

7.5. Working with proteins

7.5.1. Over-expression of proteins in *E. coli*

For the over-expression of GST tagged proteins, GST-PBD (PAK binding domain) and GST-RBD (Rho binding domain), the plasmids containing the desired gene sequences were transformed into BL-21 strain of *E. coli*. A 5 ml overnight preculture of these bacteria was setup. The following day the pre-culture was diluted 1:100 and cultured in a 37°C incubator shaker, till it reached the OD_{600} of 0.6-0.7. Once the desired OD had been obtained, the culture was induced using 0.1 mM IPTG for 4 h at 30°C. Finally the induced bacteria were harvested by centrifuging them at 6000 rpm for 10 min and the induction of the desired protein was verified by SDS-PAGE and coomassie staining.

7.5.2. Purification of GST tagged proteins

After the induction of GST tagged proteins, the bacteria were harvested by centrifuging them at 6000 rpm for 10 min. The resulting bacterial sediments were resuspended in 10 ml of TE buffer and incubated on ice for 30 min following addition of 1 ml of lysozyme (25 mg/ml). Thereafter, 25 ml of RIPA lysis buffer (50 nM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % Triton-x100, 1 % Sodium deoxycholate, 0.1 % SDS, PMSF and protease inhibitor cocktail) was added and the samples were sonicated 5 times for 20 sec at 50 % cycle. In order to reduce nucleic acid contamination, the samples were subjected to DNase I (10 µg/ml) and RNase A (10 µg/ml) treatment. Later the samples were centrifuged at 16,000 rpm for 30 min and the supernatant obtained was collected in a fresh tube. Glutathione Sepharose^{TM} 4B Beads (Amersham Biosciences); 1ml per 500ml bacterial culture, was added to the supernatant and incubated overnight at 4°C. Next day, the samples were centrifuged at 4°C, 2000 rpm for 10 min and the beads obtained were washed once with RIPA lysis buffer and twice with TE buffer by centrifuging at 13,000 rpm for 10min at 4°C. The beads were re-suspended in PBS with 10 % glycerol and stored at -80°C.

7.5.3. Purification of IgG from the rabbit serum

Protein A-sepharose beads were used for the purification of IgG antibodies from the rabbit serum after immunization with the desired antigen. About 0.5 g of Protein A-sepharose beads were added to 20 ml of dH_2O in an elution column and were allowed to swell for about 15 min at room temperature. Once packed, the beads were equilibrated with PBS. To it 5 ml of the desired serum was added and allowed to flow through under gravity. The beads were washed once with PBS and IgG was eluted with 0.1 M Glycine/HCl (pH 3.0). The fractions of elute were then neutralized with 1 M Tris/HCl (pH 8.0) and the protein concentration was estimated spectro-photometrically at 280 nm. An OD_{280} of 1 is equivalent to 0.8 mg IgG per ml. The elution column with Protein A-sepharose beads was stored at 4°C and reused after washing with 2 M Urea solution and PBS.

7.5.4. Preparation of bacterial lysates

In order to prepare the bacterial whole cell lysate, 1 ml bacterial culture was centrifuged at 6000 rpm for 5 min. The resulting pellet was washed and then resuspended in 100 µl of PBS. To it, 100 µl of Protein probe buffer was added and the mix was boiled for 5 min at 100°C. About 15-20 µl of the lysate was subjected to SDS-PAGE.

7.5.5. Preparation of whole cell lysates of eukaryotic cells

At various time points of infection, cells were washed with with ice-cold PBS and lysed with Triton x 100 lysis buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM, Na_3VO_4, 0.1 % SDS, 1.0 % Triton-X 100, 10 % Glycerol, 0.5 % Deoxycholate) containing Complete protease inhibitor cocktail tablet (Roche). Once lysed, the cell lysates were collected with the help of a cell scraper in 1.5 ml eppendorf tubes and sonicated for 5 sec on ice at 50% cycles. The samples were centrifuged at 13,000 rpm for 10 min and the protein concentration was determined using the Bradford protein quantification reagent (Sigma).

7.5.6. Protein estimation via Bradford assay

The concentration of the isolated proteins was estimated spectrometrically by Bradford Assay (Bradford, 1976). The Bradford assay is a colorimetric protein estimation method based on an absorbance shift in the dye coomassie, whereby the red form of coomassie reagent converts into a stable coomassie blue dye upon interaction with the proteins. The complex formation results in an absorbance shift from 450 nm to 595 nm. Therefore, an absorbance reading at 595 nm is proportional to the amount of bound dye, and thus to the amount (concentration) of protein present in the sample. Briefly 5 µl of protein sample was diluted 1:100 and to it 500 µl of Bradford reagent was added. The mix was incubated for 15 min at room temperature and the absorbance at 595 nm was determined spectro-photometrically. A standard curve of 0.5 to 10 µg/ml of BSA (Bovine Serum Albumin) is used to extrapolate the concentration of protein in the samples.

7.5.7. SDS-Polyacrylamide Gel Electrophoresis (Laemmli *et al.*, 1970)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method of separating proteins according to their molecular weights. This electrophoresis is based on the principle that upon attaining uniform charge, the electrophoretic mobility of protein depends primarily on its size. A polyacrylamide gel consists of a 4 % Stacking gel followed by a resolving gel of 6 to 14 %, based upon the molecular weight of the proteins to be resolved. The main purpose of the stacking gel is to concentrate all the protein together, before they could be resolved properly in the resolving gel. In order to load the sample onto the gel, they were mixed with a protein loading dye composed of 0.5 M Tris/HCl (pH 6.8), 10 % Glycerol, 10 % SDS, 5 % ß-mercaptoethanol and 0.05 % bromophenolblue dye. SDS disrupts the secondary, tertiary and quaternary structures of protein to produce a linear

polypeptide chain coated with negatively charged SDS molecule. 1.4 mg of SDS molecule binds per 1 mg of protein. These negatively charged proteins could be easily electrophoresied under the influence of an electric field. ß-mercaptoethanol is a reducing agent that denatures the protein by reducing all the disulphide bonds.

In order to denature the sample completely the samples were boiled for 5 min at 100°C and then loaded on to the gel. The electrophoresis was performed initially at 80V, low voltage was used to concentrate the sample in stacking gel, and once in resolving gel the voltage is increased to 120V. Finally the resolving gel was carefully removed and was used either for coomassie staining or for Western blotting.

7.5.8. Coomassie Brilliant Blue staining of protein gels

The presence of proteins in the polyacrylamide gels was ascertained by staining the gels with Coomassie Brilliant Blue, which has a unique feature that it interacts only with the proteins and not with the acrylamide gel matrix. Briefly, after the completion of the poly-acrylamide gel electrophoresis, the gel was soaked in a solution of Coomassie Brilliant Blue and incubated overnight. The excess coomassie was removed from the gel by destaining the gel. The gel was stored in water till it was dried with the help of a Gel-drying apparatus.

7.5.9. Western Blot, semi dry method

Western blotting also known as immunoblotting is a procedure whereby the protein samples from an acrylamide gel is transferred over to a membrane, under the influence of electric field. Immunoblotting is performed in order to detect a specific protein of interest with the help of antibodies. Firstly the samples are resolved via SDS-PAGE and then blotted on to a nitrocellulose or methanol activated PVDF membrane using a Semi Dry Transfer Cell Trans-Blot® SD apparatus (BIO RAD) consisting of two graphite electrodes plates. The gel and the membrane are kept between the two electrodes, separated with layer of 3 Whatmann paper on both sides. The proteins are transferred under the influence of an electric field of 15V for 1 h on to the membrane.

Once the proteins have been transferred, the membrane was blocked with 5 % skimmed milk powder in PBS for 1 h at room temperature prior to overnight incubation with the desired specific primary antibody at 4°C. The membrane was washed 3 x with PBS/ 0.5 % Tween 20 for 10 min and was incubated with enzyme horseradish peroxidise conjugated secondary antibody for 1 h at room temperature. The antibody binding was detected either by using enhanced chemiluminiscence (ECL, Amersham) or by Chloronapthol (Sigma) / H_2O_2

method. Later was performed by incubating the membrane with 500 µl Chloronapthol solution and 12 µl H_2O_2 in 10 ml PBS. The peroxidase enzyme converts chloronapthol with the help of H_2O_2 into a violet colour compound at the site of reaction, thus helping the detection of protein of interest.

7.5.10. Pull-down assay

The pull-down assay is an *in-vitro* method for determination of physical interaction between two or more proteins, based on a simple principle of bait and prey. The minimal requirement for a pull-down assay is an availability of a purified, tagged protein (the bait) which is used to capture and 'pull-down' a protein-binding partner (the prey).

For pull-down assay, 100 ug of the whole cell lysates containing equivalent amounts of protein were mixed with GST-PAK or GST-RBD conjugated to sepharose beads for 1 h at 4°C. Later the beads were collected by centrifugation at 10,000 rpm for 3 min and washed twice with RIPA buffer (50 nM Tris HCl pH 7.7, 150 mM NaCl, 1 mM EDTA, 1 % Triton-x100, 1 % Sodium deoxycholate and 0.1 % SDS). The effectivity of the assay was confirmed by subjecting the protein beads complexes to SDS-PAGE and visualizing the desired protein by immunoblotting as described above.

7.6. Methods for analysing the binding of Factor H by *S. pneumoniae*

7.6.1. Flow cytometric analysis of Factor H binding to pneumococci

Binding of Factor H to viable pneumococci in competitive inhibition experiments was tested using flow cytometry. Bacteria were cultured in THY and 5 x 10^7 bacteria in 100 µl PBS were incubated in the absence or presence of PspC proteins which were used as competitors. The suspensions were incubated for 30 min at 37°C and thereafter, bacteria were washed three times. Binding of Factor H to pneumococci was detected after incubation with the Factor H antiserum for 30 min at 37°C followed by FITC-conjugated anti-goat Ig antibody (MoBiTec). Bacteria were washed and fluorescence analyzed by flow cytometry using a FACSCalibur (Becton Dickinson). The pneumococci were detected using log-forward and log-side scatter dot-plot, and a gating region was set to exclude debris and larger aggregates of bacteria. 10.000 bacteria were analyzed for fluorescence using log-scale amplification. The geometric mean fluorescence intensity (GMFI) x percentage of labeled bacteria was recorded as a measure for binding activity.

7.6.2. Analysis of Factor H binding to pneumococci by immunoblotting

The binding of Factor H to pneumococci was also analyzed by Western blotting. After incubation of pneumococci with either plasma or purified Factor H, the bacteria were centrifuged and the resulting sediment was incubated for 10 min with 2 M NaCl. After centrifugation once the supernatant was collected as an elute fraction and the bacterial sediment was resuspended in 100 µl PBS. The protein probe buffer was added to both elute and the sediment fraction and were boiled for 5 min at 100°C. The samples were then subjected to SDS-PAGE and the binding of Factor H was analyzed by immunoblotting using Factor H antiserum.

7.7. Graphical representation and Statistical analysis

The infection experiments have been performed at least 3 times, each in duplicate. The values of adherence and invasion have been expressed as mean ± standard deviation. Further the statistical significance of the results was analyzed by the 2-tailed unpaired Student´s t-test. In all analysis, p values of < 0.05 were considered statistically significant.

7.8. Working with DNA, or RNA

7.8.1. Isolation of chromosomal DNA from *Streptococcus pneumoniae*

The chromosomal DNA from *S. pneumoniae* was isolated with the help of Genomic-tip 100/G-Kit from QIAGEN, as per the manufacturer's instructions. Briefly, following the bacterial lyses, the chromosomal DNA was isolated with the help of QIAGEN Anion-Exchange Resin. The concentration of DNA thus obtained was photometrically determined and stored at -20°C.

7.8.2. Isolation of plasmid DNA from *E. coli*

A 10 ml overnight culture, cultivated at 37°C, 120 rpm incubator shaker was used for plasmid DNA isolation. Firstly the bacteria were harvested by centrifugation at 6,000 rpm for 10 min and then resuspended nicely in 200 µl (x vol) of Solution I. To the bacterial suspension 400 µl (2x vol) of freshly prepared Solution II was added and gently mixed by inverting the tube. This was followed by addition of 300 µl (1.5x vol) Solution III. After mixing gently by inverting up and down, the lysed bacterial suspension was incubated on ice for 10-15 minutes. Later the tube was centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant thus obtained was collected in a fresh eppendorf tube and to it 0.7 volume of isopropanol was added. The tube was centrifuged at 13,000 rpm for 15 min at 4°C and the

sediment thus obtained was washed with 70 % ethanol. After washing, the sediment was air dried, resuspended in sterile water and stored at -20°C.

For transient transfection studies the plasmid DNA was further purified by phenol-chloroform-isoamyl alcohol and ethanol precipitation steps. Briefly, 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the plasmid DNA solution and was centrifuged at 13,000 rpm for 10 minutes. The aqueous phase thus obtained was carefully collected in a fresh eppendorf and to it 1 volume of chloroform-isoamyl alcohol (24:1) was added. The mixture was again centrifuged at 13,000 rpm for 10 minutes. The process was repeated till the all traces of phenol have been removed. To the aqueous phase obtained, 1/10 volume of 3 M sodium acetate pH 5.4 and 2.5 volume of 100 % ice cold ethanol was added and incubated at -20°C for 1 hour. Following the incubation the tubes were centrifuged at 13,000 rpm for 15 min and the DNA sediment thus obtained was washed with 70 % ethanol, air dried and resuspended in sterile water. The plasmid DNA was the stored at -20°C.

7.8.3. Nucleic acid concentration estimation

The concentration of nucleic acid (DNA and RNA) was determined spectro-photometrically using Nano-Drop (Peq Lab System). The spectro-photometric determination of the concentration follows a simple principle that the aromatic rings of purines and pyrimidines of nucleic acids absorbs the light at 260 nm wave length. Since, the optical density at 260 nm and the concentration of nucleic acids are in linear relationship it is easy to calculate the concentration of nucleic acids in the sample.

An OD_{260} of 1 is equivalent to 50 µg/ml of double stranded DNA, or 33 µg/ml of single stranded DNA, or 40 µg/ml of RNA in the given sample

Also the ratio of OD_{260}/OD_{280} reflects the purity of the nucleic acid. Pure DNA has an OD_{260}/OD_{280} ratio of ~1.8; pure RNA has an OD_{260}/OD_{280} ratio of ~2.0. Low ratios could be caused by protein or phenol contamination.

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9. Appendix

9.1. Tables

number of invasive *S. p.* serotype 35A per well			
MDCK-hpIgR		**Calu-3**	
control	α-SC (8µg/ml)	control	α-SC (8µg/ml)
1174	111	7000	578
1276	92	2950	260
2096	90	2412	243
6960	329	4018	398

Table 57 Number of invasive pneumococci per well. The invasion and intracellular survival of pneumococci in host cells was determined in the presence of α-SC (8µg/well) or absence (Control) of antibody using the antibiotic protection assay (Figure 6).

number of invasive *S. p.* serotype 35A per well of cytoskeleton inhibitor treated MDCK-hpIgR cells							
control	Cytochalasin D (125 nM)	control	Latrunculin B (50 nM)	control	Jasplakinolide (100 nM)	control	Nocodazole (10 µM)
2634	595	1710	548	6360	170	714	292
2370	762	2066	510	3045	72	5700	1900
10150	1400	1280	245	1556	33	6000	2150

Table 58 Number invasive *S. p.* 35A per well. hpIgR mediated invasion and intracellular survival of the bacteria in MDCK-hpIgR cells was followed in the absence (control) or presence of inhibitors of actin filaments and microtubules including cytochalasin D (CytoD, 125 nM), latrunculin B (LatB, 50nM), jasplakinolide (Jasp, 100nM) and nocodazole (Noco, 10µM) by the antibiotic protection assay (Figure 7).

number of invasive *S. p.* serotype 35A per well of cytoskeleton inhibitor treated Calu-3 cells							
control	Cytochalasin D (125 nM)	control	Latrunculin B (50 nM)	control	Jasplakinolide (100 nM)	control	Nocodazole (10 µM)
3160	316	1401	250	3345	80	1272	785
5040	897	1221	335	1401	15	14350	3200
6400	1650	3810	858	5280	35	11600	4900

Table 59 Number invasive *S. p.* 35A per well. hpIgR mediated invasion and intracellular survival of the bacteria in Calu-3 cells was followed in the absence (control) or presence of inhibitors of actin filaments and microtubules including cytochalasin D (CytoD, 125 nM), latrunculin B (LatB, 50nM), jasplakinolide (Jasp, 100nM) and nocodazole (Noco, 10µM) by the antibiotic protection assay (Figure 7).

number of invasive *S. p.* serotype 35A per well of toxins treated cells							
MDCK-hpIgR				**Calu-3**			
control	TcdB1470 (100 ng/ml)	control	TcdB10463 (30 ng/ml)	control	TcdB1470 (100 ng/ml)	control	TcdB10463 (30 ng/ml)
3130	1290	2942	960	7320	5600	4800	1600
2300	788	1091	403	7200	4720	7800	1800
2320	840	4100	2440	9400	6960	8800	6000
-	-	1475	497	-	-	8000	6320
-	-	-	-	-	-	4960	4160

Table 60 Number of internalized pneumococci per well. Invasion and intracellular survival of the bacteria in MDCK-hpIgR and Calu-3 cells in the absence (control) or presence of *Clostridium difficile* toxin B, TcdB-10463 (30 ng/ml) or TcdB-1470 (100 ng/ml) was monitored by the antibiotic protection assay (Figure 8).

number of invasive *S. p.* serotype 35A per well of inhibitor treated MDCK-hpIgR cells					
control	Y27632 (50 µM)	control	NSC23766 (50 µM)	control	Secramine A (10 µM)
4416	5100	2860	3580	12900	753
4400	5800	4520	3060	32700	12900
2280	2600	1710	1230	1881	540
4410	5080	-	-	-	-

Table 61 Number of *S. p neumoniae* serotype 35A internalized by MDCK-hpIgR cells was determined in the absence (control) or presence of specific individual inhibitors of Rho family GTPases Y27632 (50 µM), NSC23766 (50 µM) or secramine A (10 µM) by the antibiotic protection assay (Figure 9).

number of invasive *S. p.* serotype 35A per well of inhibitor treated Calu-3 cells					
control	Y27632 (50 µM)	control	NSC23766 (50 µM)	control	Secramine A (10 µM)
10000	8840	7800	7400	10140	1154
10800	14400	3345	2865	15600	2322
6000	7000	3810	3600	1371	726

Table 62 Number of *S. pneumoniae* serotype 35A internalized by Calu-3 cells was determined in the absence (control) or presence of specific individual inhibitors of Rho family GTPases Y27632 (50 µM), NSC23766 (50 µM) or secramine A (10 µM) by the antibiotic protection assay (Figure 9).

number of invasive *S. p.* serotype 35A per well of transfected MDCK-hpIgR cells			
control	dn-Rac1 (Rac1-T17N)	dn-Cdc42 (Cdc42-T17N)	dn-Rho (Rho-T19N)
1300	800	500	845
1050	838	209	951
960	1090	242	845

Table 63 Number of internalized pneumococci per well. *S. pneumoniae* type 35A invasion of transiently transfected MDCK-hpIgR cells, with dominant-negative (dn) alleles of Rac1 (Rac1-T17N), Cdc42 (Cdc42-T17N) or Rho (Rho-T19N) was determined by the antibiotic protection assay (Figure 10).

number of invasive *S. p.* serotype 35A per well of PI3-kinase inhibitor treated cells							
MDCK-hpIgR				**Calu-3**			
control	Wortmannin (50 nM)	control	LY294002 (50 µM)	control	Wortmannin (50 nM)	control	LY294002 (50 µM)
2096	425	6400	220	6960	3320	21400	3500
10812	1572	3130	84	5166	1350	4695	785
2157	464	2140	233	5040	2084	6080	1080
10150	1450	2280	135	6400	2100	6540	1215

Table 64 Number of internalized *S. p.* 35 A per well. Pneumococcal invasion of MDCK-hpIgR and Calu-3 cells was determined in the absence (control) or presence of PI3-kinase inhibitors wortmannin (50 nM) or LY294002 (50 µM) by the antibiotic protection assay (Figure 14).

number of invasive *S. p.* serotype 35A per well of Akt inhibitor treated cells			
MDCK-hpIgR		**Calu-3**	
control	Akt inhibitor VIII (10 µM)	control	Akt inhibitor VIII (10 µM)
7640	460	9600	1480
9480	1040	10580	1400
-	-	1932	654

Table 65 Number of invasive pneumococci per well. Pneumococcal invasion of MDCK-hpIgR and Calu-3 cells was monitored in the absence (none) or presence of Akt Inhibitor VIII (Akt i, 10 µM) by the antibiotic protection assay (Figure 17).

number of invasive *S. p.* serotype 35A per well of PTK inhibitor treated cells			
MDCK-hpIgR		**Calu-3**	
control	Genistein (50 µM)	control	Genistein (50 µM)
1890	790	3780	2250
1898	1026	3184	1941
2468	1422	3398	1800
1276	952	2950	1323
2157	1265	5166	2937

Table 66 Number of *S. pneumoniae* serotype 35A internalized by MDCK-hpIgR and Calu-3 cells was determined in the absence (none) or presence of protein tyrosine kinase inhibitor genistein (50 µM) by the antibiotic protection assay (Figure 18).

number of invasive *S. p.* serotype 35A per well of PTK inhibitor treated cells							
MDCK-hpIgR				**Calu-3**			
control	PP2 (5 µM)	control	AG957 (10 µM)	control	PP2 (5 µM)	control	AG957 (10µM)
6960	2073	6360	1348	6400	2050	3345	1133
2157	470	3045	668	7500	1300	5280	2440
10150	1600	4700	397	6000	1330	3810	1202

Table 67 Number of pneumococci ingested by MDCK-hpIgR and Calu-3 cells in the presence or absence of *Src* family of protein-tyrosine kinase inhibitor PP2 or bcr/abl kinase inhibitor AG95 as determined by antibiotic protection assay (Figure 19).

number of invasive *S. p.* serotype 35A per well of transfected MDCK-hpIgR cells		
control	Csk wt	Csk(K222M)
5010	3030	2700
9480	6960	9000
9720	6240	12640
6600	6420	6420

Table 68 Number of invasive pneumococci per well of wild-type Csk or kinase-inactive Csk (Csk K222M) transfected MDCK-hpIgR cells, respectively (Figure 20).

number of invasive *S. p.* serotype 35A per well	
control	Src(K297M)
5010	3035
9480	8100
9720	6680
6600	1305

Table 69 Number of ingested pneumococci per well of kinase-inactive c-Src (Src K297M) transfected MDCK-hpIgR cells (Figure 21).

number of invasive *S. p.* serotype 35A per well					
control	PD98059 (100 µM)	control	JNK inhibitor II (5 µM)	control	SB202190 (10 µM)
1475	1060	2096	561	3855	422
5760	3120	2157	1269	6960	603
3360	2280	2370	872	10812	1842
-	-	10150	4200	2157	222

Table 70 Number of ingested pneumococci per well. Invasion and intracellular survival in MDCK-hpIgR cells, of *S. pneumoniae* serotype 35A, was monitored in the absence (control) or presence of MAP kinase kinase (MEK) inhibitor (PD98059, 100 µM), c-Jun N-terminal kinase inhibitor (JNK inhibitor II, 5 µM) or p38 MAP kinase inhibitor (SB202190, 10 µM) by the antibiotic protection assay (Figure 24).

number of invasive *S. p.* serotype 35A per well					
control	PD98059 (100 µM)	control	JNK inhibitor II (5 µM)	control	SB202190 (10 µM)
6300	3330	4880	1164	3984	897
9400	5600	5166	1710	3396	875
7800	4440	5040	2428	6000	1200
-	-	6400	1700	-	-

Table 71 Number of ingested pneumococci per well. Invasion and intracellular survival in Calu-3 cells, of *S. pneumoniae* serotype 35A, was monitored in the absence (control) or presence of MAP kinase kinase (MEK) inhibitor (PD98059, 100 µM), c-Jun N-terminal kinase inhibitor (JNK inhibitor II, 5 µM) or p38 MAP kinase inhibitor (SB202190, 10 µM) by the antibiotic protection assay (Figure 24).

number of invasive *S. p.* serotype 35A per well			
control	PP2 (5 nM)	JNK inhibitor II (5 µM)	PP2 (5 nM) + JNK inhibitor II (5 µM)
2709	282	576	38
3500	330	1110	55
3130	498	508	100

Table 72 Number of invasive pneumococci per well. Pneumococcal invasion of MDCK-hpIgR cells was monitored in the absence (control) or presence of Src kinase inhibitor (PP2, 5 µM), c-Jun N-terminal kinase inhibitor (JNK inhibitor II, 5 µM) or combination of both inhibitors by the antibiotic protection assay (Figure 27).

number of invasive *S. p.* serotype 35A per well of inhibitor treated MDCK-hpIgR cells			
control	Wortmannin (50 nM)	PP2 (5 µM)	Wortmannin (50 nM) + PP2 (5 µM)
2709	576	1187	252
3500	110	1400	619
3130	508	1880	509

Table 73 Number of invasive pneumococci per well. Invasion and intracellular survival of *S. p neumoniae* serotype 35A in MDCK-hpIgR cells was determined in the absence (control), presence of PI3-kinase inhibitor wortmannin (WM, 50 nM), or Src kinase inhibitor (PP2, 5 µM) and in assay with a combination of both inhibitors by using the antibiotic protection assay (Figure 28).

number of invasive *S. p.* serotype 35A per well							
MDCK-hpIgR				Calu-3			
control	BAPTA/AM (10 µM)	control	Thapsigargin (1 µM)	control	BAPTA/AM (10 µM)	control	Thapsigargin (1 µM)
2860	7200	6360	1124	7800	11600	3345	765
4520	6800	3045	705	6360	12720	1401	295
6360	15660	2066	234	2433	6150	5280	1540
3103	4485						

Table 74 Number of invasive pneumococci per well of hpIgR expressing host epithelial cells. Antibiotic protection assay was performed in the presence or absence of BAPTA/AM (10 µM) or Thapsigargin (1 µM), respectively to determine the number of internalized pneumococci (Figure 29).

number of invasive *S. p.* serotype 35A per well							
MDCK-hpIgR				Calu-3			
control	MDC (50µM)	control	Chlorpromazine (10 µM)	control	MDC (50µM)	control	Chlorpromazine (10 µM)
5440	1710	7640	2010	6180	2120	4020	2090
2870	346	4180	285	1585	329	9600	3430
9400	1520	10140	4530	2400	412	12900	2025
				4020	1625	1932	367

Table 75 Number of invasive pneumococci per well of hpIgR-expressing host epithelial cells, pretreated with inhibitor of clathrin machinery (Figure 30).

number of invasive *S. p.* serotype 35A per well			
MDCK-hpIgR		Calu-3	
control	Dynasore (80 µM)	control	Dynasore (80 µM)
32700	11220	15600	2452
1881	231	1371	24

Table 76 Number of internalized *S. p.* 35A per well of Dynasoe (80 µM) treated hpIgR expressing host epithelial cells (Figure 31).

number of invasive *S. p.* serotype 35A per well	
control	siRNA Dynamin (25 nM)
14820	3855
12480	2880

Table 77 Number of internalized *S. p.* 35A in dynamin knocked-down Calu-3 cells as monitored by antibiotic protection assay (Figure 32).

Cell lines	Number of internalized *S. p.* serotype 35A per well	
	none	Factor H [3 µg]
Detroit 562	827 ± 161.22	1176.25 ± 170.06
A549	61.33 ± 12.73	117.33 ± 19.29
HBMEC	134 ± 12.73	512 ± 63.64

Table 78 Number of internalized *S. p.* 35A in host cells as determined by antibiotic protection assay. Pneumococci were pretreated with or without Factor H before 3 h infections (Figure 44).

Preincubation with Factor H [µg]	adherent bacteria *S. p.* serotype 35A per cell	
	Detroit 562	A549
0	4.67	26.5
2	60.46	204.83
2 + PspC SH3 (2.5 µg)	6.46	62.28

Table 79 Number of attached *S. p.* 35A per cell. Blocking of Factor H-mediated pneumococcal attachment to host epithelial cells by PspC-SH3 derivative as determined by immunofluorescence microscopy (Figure 47).

9.2. Abbreviations

α	Anti
Amp	Ampicillin
bp	Base pair
°C	Degree Celsius
Ca	Calcium
CFU	Colony Forming Units
Chlorp.	Chlorpromazine
CLSM	Confocal Laser Scanning Microscope
CO_2	Carbon dioxide
Conz.	Concentration
CPS	Competence stimulating peptide
CR3	Complement receptor 3
CSP	Competence stimulating peptide
Ctrl	Control
CytoD	Cytochalasin D
dH_2O	Distilled Water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DS	Dermatan Sulphate
EDTA	Ethylenediaminetetraacetic acid
e.g.	Example
EGTA	Ethylene glycol tetraacetic acid
EM	Electron Microscopy
ERK	Extracellular signal-regulated kinases
Erm	Erythromycin
et al.	And others
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
g	Gram
GMFI	Geometric Mean Fluorescence Intensity
h	Hour
hpIgR	Human-polymeric immunoglobulin receptor
IB	Immunoblot
i.e.	That is
Ig	Immunoglobulin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Jasp	Jasplakinolide
JNK	c-Jun N-terminal kinases
Kb	Kilobase
kDa	Kilo Dalton
L	Litre
LB	Luria bertani
m	milli (10^{-3})
M	Molar (mol/l)
mAb	Monoclonal antibodies

MDCK	Madin Darby canine kidney
mg	Milligram
ml	Millilitre
min	Minute
MOI	Multiplicity Of Infection
MW	Molecular weight
n	nano
NaCl	Sodium chloride
nm	Nanometer
n.s.	Not significant
OD	Optical density
Ω	Ohm (SI unit for electrical resistance)
%	percent
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer salaine
PFA	Paraformaldehyde
pIgR	Polymeric immunoglobulin receptor
PMN	Polymorphonuclear leukocytes
PMSF	Phenylmethylsulphonylfluoride
PspC	Pneumococcal surface protein C
PVDF	Polyvinylidene Fluoride
rpm	Revolution per minute
RT	Room temperature
SCR	Short Consensus Repeats
SDS	Sodium Dodecyl Sulfate
sec	Seconds
siRNA	Small interfering RNA
S. p.	*Streptococcus pneumoniae*
SSC	Side scatter
TEMED	Tetramethylethylenediamine
THY	Todd Hewitt media with 0.5 % yeast extract
TRIS	Tris-(hydroxymethyl)-aminomethane
TRIS-HCl	Tris-(hydroxymethyl)-aminomethane-hydrochloride
µ	Micro
U	Units
µg	Microgram
µl	Microlitre
V	Volts
Vol	volume
v/v	Volume percent
WT	Wild-type
w/v	Weight per volume
w/w	Weight percent

9.3. Instruments Used

Agarose Gel-Electrophoresis apparatus	Peqlab and Gibco
Autoclave	Varioklav
CO_2 incubator (37°C)	Heraeus
CO_2-Incubator (37°C, 5 % CO_2)	Heraeus
CO_2-Incubator (37°C, 5 % CO_2)	BINDER
Ice machine	Sierra
Ice machine	Scotsman
FACS-Calibur	Becton Dickinson
FACS-Canto I and II	Becton Dickinson
Fine balance	Chyo
Film cassette	BLB
French Press	SLMAminco
Gel documentation	Bio-Rad
Gel Drying System	Bio-Rad
Glass wares	Schott, VWR Brand
Heat block (Thermomixer 5436)	Eppendorf
Heat block	Techne
Incubator shaker	B. Braun Biotech
Fridge, 4°C	Liebherr
Magnetic stirrer heat able, RCT basic	KIKA
Magnetic stirrer heat able	Labortechnik
Magnetic stirrer M20/1	Franco®
Microscope	
Phase contrast microscope ID 02	Zeiss
Fluorescence microscope Axioskop	Zeiss
Confocal Microscope	Zeiss
Confocal Microscope	Leica
Microwave	AEG
Neubauer chambers	Brand
PCR-Thermocycler	Eppendorf
PCR-Thermocycler	PerkinElmer
pH-Meter	WTW
Bio Photometer	Eppendorf
Pipettes	Gilson
Pipettes	Eppendorf
Power Supply 200/2.0 und Power Pac 300	Bio-Rad
Pump with UV-Detector for Affinity chromatography	Bio-Rad
Quartz cuvette	Hellma
Shaker	Biometra
SDS-Gel electrophoresis apparatus	Peqlab
Sterile Working bench	Heraeus
Sterile Working bench	BDK
Sterile Working bench Lamin Air®HLB 2427	Heraeus
Fridge	
-20°C	Privileg
-20°C	BOSCH
-20°C	Liebherr
-80°C	Heraeus

Transblot SD Semidry Transfer Cell	Bio-Rad
Sonificator (Sonifier 250)	Branson
Vortex Genie 2	Scientific Industries
Weighing balance	Kern & Sohn
Water bath	GFL®
Time piece	Junghans
Centrifuge	
Sorvall T6000B	DuPont
Sorvall RC 5B	DuPont
Biofuge fresco	Heraeus
CP Centrifuge	Beckmann
Centrifuge 5417R	Eppendorf
Centrifuge 5810	Eppendorf

9.4. Consumables

Name	**Company**
Pipettes 5 ml, 10 ml und 25 ml (sterile, Plastik)	Greiner
Falcon-tubes, 15 ml und 50 ml	Greiner
Gloves	Flexam
Microtiter plates, Maxisorp F96	Nunc
Paper napkins	ZVG
Parafilm „M“®	ANC
Pasteur-Pipettes	Brand
Petri-dishes for Bacterial culture	Greiner
Petri-dishes for cell culture	Greiner
Pipette Tipps 10 µl, 200 µl und 1000 µl	Sarstedt
Plastic cuvettes 1 ml	Greiner
PVDF-Membrane	Millipore
Reaction tubes 0.5 ml	ABgene
Reaction tubes 1.5 ml und 2 ml	Eppendorf
X-ray films	Amersham
Scalpell	Braun
Sterile-Filter (0.2 µm)	Schleicher & Schuell
Vinyl-gloves	Sempermed
Whatman-Filter paper	Schleicher & Schuell
Cell culture flasks, 25 cm^2 und 75 cm^2	Greiner
Cell culture plates (6 and 24 well)	Greiner
Transwell system	Corstar
Centrifugation tubes 14 ml	Greiner

9.5. Chemicals

Name	**Company**
Accutase	PAA
Acetic acid	Roth
Acrylamid-N, N-Methylene-Bisacrylamid (30 % / 0.8 %)	Roth

Active coal	Roth
Agar Agar	Difco
Agarose	Peqlab
Agarose	Seakem
Ammoniumsulphate	Applichem
Ampicillin	Sigma
APS	Applichem
Bacto-Agar	Difco
Bacto-Trypton	Difco
Biotin	Sigma
Blood agar-Plates	Oxoid
BSA	Sigma
$CaCl_2$-Dihydrate (~99.5 %)	Merck
Chloramphenicol	Serva
Chloronaphthol	Sigma
Coomassie Brilliant Blue™ R250	Bio-Rad
L-Cystein	Sigma
Deoxycholacid	Sigma
D-+-Glucose	Sigma
DMEM	PAA
DMSO	Applichem
DTT	Sigma
EDTA	Riedel-de-Haën
EGTA	Sigma
Erythromycin	Sigma
Ethanol (~99.8 %)	Roth
FBS (Fetal Bovine Serum)	PAA
Ficoll	GE Healthcare
Formamide	Merck
FUGENE-6	Roche
Gentamicin solution	Sigma
Glutamine	PAA
Glycerine	Applichem
Glycine	Roth
H_2O_2	Merck
HAM F12 medium	Gibco
HCl	Roth
Histopaque	Sigma-Aldrich
Imidazole	Sigma
Immersion oil	Zeiss
IPTG	Applichem
Iron sulphate	Sigma
Iso-propanol	Roth
Kanamycin	Serva
KCl	Merck
KH_2PO_4	Sigma
K_2HPO_4	Sigma
Lipofectamine LTX reagent	Invitrogen
Lipofectamine RNAiMAX	Invitrogen
Liquid Nitrogen	Linde

$MgCl_2$-Hexahydrate	Merck
MEM Vitamin	PAA
2-Mercaptoethanol	Applichem
Methanol	Roth
Methanol	Merck
Milk powder (Blotting grade)	Roth
Moviol	Hoechst
NaCl	Roth
Na_2HPO_4 (water free)	Applichem
NaH_2PO_4	Applichem
NaOH	Sigma
Na-Pyruvate	PAA
NH_4-Acetate	Riedel-de-Haën
Non essential aminoacids	PAA
NU-Serum	Becton Dickinson
Paraformaldehyde	Fluka
Peptone	Merck
Penicillin-Streptomycin-Solution	PAA
pH-Meter Caliberation-solution (pH 4.0, 7.0, 10.0)	Applichem
PMSF (Phenylmethylsulfonylfluoride)	Applichem
Polyethylenglycol (8000)	Merck
Riboflavin	Sigma
RPMI 1640	PAA
Saponin	Applichem
SDS	Roth
Sodium acetate	Roth
Sodium citrate	Roth
Sucrose	Applichem
TEMED	Bio-Rad
Todd Hewitt Broth	Roth
Triton X-100	Applichem
Trypsin-EDTA	PAA
Trypton	Merck
Tween® 20	Applichem
X-Gal	Applichem
Xylencyanol	Applichem
Yeast-Extract	Difco
Zinc sulphate	Sigma

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Publication (peer review):

1. Sven Hammerschmidt*, **Vaibhav Agarwal**, Anja Kunert, Steffi Haelbich, Christine Skerka and Peter Zipfel 2007. The host immune regulator Factor H binds via two contact sites with the PspC Protein of *Streptococcus pneumoniae* and mediates complement control and adhesion to host epithelial cells. **J. Immunology** 178: 5848-5858. **(I.F. 6.068)**
2. Simone Bergmann, Anke Lang, Manfred Rohde, **Vaibhav Agarwal**, Claudia Rennemeier, Carsten Grashoff, Klaus T. Preissner and Sven. Hammerschmidt*. Integrin-linked kinase is required for vitronectin-mediated internalization of *Streptococcus pneumoniae* by host cells. **J. Cell Sciences (in press**) **(I.F. 6.383)**
3. **Vaibhav Agarwal**, Sven Hammerschmidt. Pneumococcal internalization into host cells via the PspC-polymeric Ig receptor requires activation of Cdc42 and a signalling pathway via the PI3-kinase/Akt. (Manuscript under preparation)
4. **Vaibhav Agarwal**, Peter Zipfel and Sven Hammerschmidt. Complement regulator Factor H mediates *S. pneumoniae* invasion of host epithelial cells by interaction of its C-terminal SCRs with polyanionic cell surfaces. (Manuscript under preparation)

Presentations at Conferences:

Vaibhav Agarwal*, Sven Hammerschmidt. Pneumococcal invasion into host cells via the PspC-polymeric Ig receptor requires activation of Cdc42 and a signalling pathway via the phosphoinositide-3-kinase and protein kinase B. ISPPD-6, June 8-12, 2008 Reykjavik, Iceland **(Poster)** (**Scholarship award to participate in ISPPD-6**)

Vaibhav Agarwal*, Sven Hammerschmidt. Pneumococcal invasion into host cells via the PspC-polymeric Ig receptor requires activation of Cdc42 and a signalling pathway via the phosphoinositide-3-kinase and Akt. Annual Conference VAAM/GBM, March 9-11, 2008 Frankfurt/Main, Germany **(Oral presentation)**

Sven Hammerschmidt*, **Vaibhav Agarwal**, Anja Kunert, Steffi Haelbich, Christine Skerka and Peter F. Zipfel. Factor H interacts via two contact sites with the PspC and mediates adhesion of pneumococci to host cells. 8th conference on The Molecular Biology of the Pneumococcus (EUROPNEUMO 2007), April 14-17, 2007 Lissabon, Oeiras, Portugal. **(Oral presentation)**

Sven Hammerschmidt*, Anja Kunert, **Vaibhav Agarwal**, Larry McDaniel, Christine Skerka and Peter Zipfel. Complement regulator Factor H interacts via two contact sites with the PspC Protein of Streptococcus pneumonia and mediates adhesion to host epithelial cells. Young Investigator Meeting ZINF Würzburg - ZIBI Berlin March 2-3, 2007 Wuerzburg, Germany **(Poster)**

Sven Hammerschmidt*, Anja Kunert, **Vaibhav Agarwal**, Larry McDaniel, C. Skerka and Peter Zipfel. Factor H interacts via two contact sites with the PspC protein of Streptococcus pneumoniae and mediates adherence to host cells. ASM Conference on Streptococcal Genetics June 18-21, 2006 Saint-Malo, France **(Poster)**

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