CONTRIBUTIONS OF PNEUMOCOCCAL VIRULENCE FACTORS TO SECONDARY *STREPTOCOCCUS PNEUMONIAE* INFECTION FOLLOWING INFLUENZA INFECTION

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Quinton Oliver King

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APPROVAL

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Quinton Oliver King

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Dr. Allen G. Harmsen

Approved for the Department Veterinary Molecular Biology

Dr. Mark T. Quinn

Approved for the Division of Graduate Education

Dr. Carl A. Fox

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ABSTRACT

Influenza infection increases susceptibility to secondary infection with *Streptococcus pneumoniae* resulting in significantly increased morbidity and mortality. Whereas viral contributions to this synergism have been explored, little is known concerning contributions of the bacterium, specifically those provided through bacterial virulence factors. To assess the contributions of the known pneumococcal virulence factors hyaluronidase (Hyl), neuraminidase (NanA) and pneumococcal surface protein A (PspA) to secondary *S. pneumoniae* infection following influenza infection, mutants lacking these proteins were administered with wildtype pneumococci in a competitive growth model. Whereas mutants lacking the Hyl and NanA proteins did not exhibit attenuation, mutants lacking PspA were severely attenuated in mice without influenza infection and significantly more so in mice with a prior influenza infection. Additionally, mice received intranasal immunization with recombinant PspA protein and subsequently received primary and secondary challenges with serotypes 2, 3 and 4 pneumococci. Immunization with PspA significantly reduced bacterial burdens of all three challenge serotypes in primary and secondary pneumococcal infection and significantly reduced lung damage markers in mice receiving secondary pneumococcal challenges. In addition to known virulence factors, two surface-exposed proteins, Spr0075 and Spr1345, were assessed for virulence contributions to primary and secondary pneumococcal infections. Mutants lacking Spr0075 or Spr1345 were found to be severely attenuated in both primary and secondary pneumococcal challenges. Whereas immunization with either recombinant Spr0075 or Spr1345 significantly reduced primary pneumococcal burdens, only immunization with Spr0075 significantly reduced secondary pneumococcal burdens. Together these results indicate virulence contributions to both primary and secondary pneumococcal challenges for the PspA, Spr0075 and Spr1345 proteins. However, whereas immunization with PspA and Spr0075 significantly reduced both primary and secondary pneumococcal burdens, immunization with Spr1345 did not significantly impact secondary pneumococcal burdens. This result illustrates that a virulence contribution and/or an ability to protect against primary infection does not necessarily translate into a protein's capacity to protect against secondary infection. The results presented here are the first experimental evidence demonstrating virulence roles for the Spr0075 and Spr1345 proteins and are the first reports of immunization with pneumococcal proteins, specifically PspA and Spr0075, providing protection against secondary pneumococcal infection following influenza.

INTRODUCTION

Influenza

History and Virus Discovery

Our use of the word "influenza" to describe both the respiratory disease and its etiological virus is rooted in Italian, (inferring an "influence" of astrological origin) with its first recorded usage during a mid-18th century epidemic [1, 2]. The historical and ubiquitous nature of influenza epidemics and pandemics has resulted in the symptoms attributable to this particular pathogen comprising a gold standard for comparison (i.e. "influenza-like"). However, identification of this specific virus, like so many scientific discoveries, was a slow process precipitated by the necessary progression of incremental advances. Influenza pandemics of 1781-82, 1789-1799, 1830-33, 1847-51 and 1889-94 all contributed to a concerted recognition of a specific etiological agent for this respiratory disease and, with the latter two occurring in an era of public health statistics, an understanding of this pathogen's epidemiology and associated mortality [3]. Between 1892 and 1893, working under Koch, researchers Pfeiffer and Kitasato published the discovery of a culturable bacterium, *Bacillus influenzae* (now *Haemophilus influenzae*), in which the authors had apparently fulfilled Koch's postulates and prompting their claim of having identified the causative agent of pandemic influenza [3].

The great Spanish Influenza of 1918-1919 constitutes perhaps the single most devastating infectious disease event in recorded human history. Global mortality

estimates have been consistently upgraded from initial estimates of ~21 million offered in the 1920's to current estimates of at least 50 million [4]. While the latter and larger figure represents the best of available data, it too is suspected of significantly under representing the death toll attributable to Spanish flu which may have well exceeded 100 million, the majority having occurred within 18 months from the initial outbreaks. The 1918-1919 influenza pandemic provided ample opportunity to replicate Pfieffer and Kitasato's findings, yet attempts to culture "Pfeiffer's bacillus" were routinely unsuccessful despite significant improvements in required technique and growth media and were particularly unsuccessful when sampling influenza victims who had succumb within three days of symptom onset [5]. While it was clear *H. influenzae* was a pathogenic bacterium, the accumulating evidence founded a growing consensus that *H. influenzae* was likely recovered as a secondary bacterial pathogen (as were pneumococci, streptococci and staphylococci routinely recovered) and that the true etiological agent of pandemic influenza was likely a virus [5]. This notion of a filterable virus capable of passing through a Berkefeld filter (comprised of diatomaceous earth and porcelain), and perhaps the most significant blow to *H. influenzae* as the etiological agent of pandemic influenza, was directly supported by experiments reported by Olitsky and Gates in 1921 [6]. In this study, filtered nasal secretions recovered from Spanish Flu patients shortly after the onset of symptoms and without secondary complications were serially passed through rabbits where lung lesions indicative of influenza infection were observed in the absence of *H. influenzae*.

From this point, two factors impeded the discovery of the influenza virus. First, available virological techniques were primarily limited to disease transmission with inocula having been filtered and a concurrent absence of culturable bacteria. In the early twentieth century, the concept of viral pathogens, first experimentally described by the Tobacco Mosaic Virus work of Ivanovski and Beijerinck in 1892 and 1898, respectively, was still competing with concepts of filterable atypical bacteria (e.g. Mycoplasma spp.) [7]. Advances were forthcoming in the understanding of viruses as obligate intracellular pathogens, but confusion remained as to the nature of these pathogens and whether they were comprised of a set of macromolecules, a replicating toxin or a true, albeit wholly invisible, organism with these questions remaining topical through the 1950s [7]. A second factor slowing discovery of the influenza was simply that, as the Spanish Flu pandemic faded remarkably fast into memory considering its devastating effects, the ever present focus on translational research pulled attention away from the etiological agent of influenza and shifted efforts toward pathogens of more immediate concern [5].

The influenza pandemic of 1918-1919 was not limited to humans and a concurrent respiratory disease bearing a marked similarity to that of humans had been observed in swine and was colloquially dubbed “hog flu”. This swine influenza was again observed in 1928 and 1929 occurring in animals of eastern Iowa and provided source material for further attempts at elucidating the etiological agent. From these samples, Lewis and Shope successfully cultivated a bacterium resembling *B. influenzae* which they dubbed *B. influenzae suis* [8]. However, in support of the previous work of Olitsky and Gates, when this bacterium alone was used to inoculate healthy animals,

disease was not observed. Shope then employed filtered inocula which produced an infectious, although milder, form of the natural disease which he termed "filtrate disease", but then continued further to demonstrate inoculation with both filtrate and cultured *B. influenzae suis* resulted in a far more severe pathology typical of natural infection [9]. While clearly correlated in the epidemiological and autopsy data obtained from the 1918 pandemic [10-12], Shope's work may represent the first experimental induction of direct pathological synergism between influenza infection and a concurrent bacterial infection. Using similar techniques, researchers from the National Institute for Medical Research in the United Kingdom in 1933 reported success using filtered samples recovered from influenza patients in producing a catarrhal illness indicative of influenza infection in ferrets [13]. These researchers were also successful in neutralizing the infection by incubating influenza filtrates with convalescent sera from humans with prior influenza infection. Leaving little doubt as to the viral nature of influenza, these earlier findings inspired a large body of subsequent research aimed at further defining the nature of the influenza virus resulting in vastly improved methodologies in serology, passive immunotherapy and vaccine development and significantly shaped the modern incarnations of virology and immunology.

The Virus

Influenza viruses belong to the *Orthomyxoviridae* family (Greek, *orthos*, meaning "standard, correct" and *myxa*, meaning "mucus") which is comprised of 4 genera, influenza viruses A, B and C and thogotovirus (or influenza D) [14]. Whereas both influenza B and C are predominantly restricted to humans with exceptions observed for

seals (influenza B) and pigs (influenza C), influenza A viruses are known to naturally infect a broad range of hosts including humans, pigs, horses, minks, seals, whales and a variety of domestic and wild birds with waterfowl and shorebirds providing a natural reservoir [15, 16]. Influenza viruses are characterized by a segmented single-stranded RNA genome (8 segments for A and B, 7 segments for C), an envelope derived from host plasma membrane and, as the family name implies, are distinguished from the *Paramyxoviridae* family by their ability to bind mucus [14]. As mRNA is conventionally referred to as a positive strand, the viral RNA (vRNA) is characterized as negative-stranded given it provides the template for both viral mRNA and for antigenome positive strands (cRNA) where cRNA in turn provides template for the subsequent negative vRNA copies incorporated during viral assembly [14].

The influenza A vRNA segments encode 11 proteins (Table 1.1): the two envelope glycoproteins, hemagglutinin (H) and neuraminidase (N), matrix protein 1 (M1) and matrix/ion-channel protein 2 (M2), nucleocapsid protein (NP), non-structural protein 1 (NS1), non-structural protein 2/nuclear export protein (NS2/NEP), three polymerase proteins (PA, PB1 and PB2) and a pro-inflammatory/pro-apoptotic protein (PB1-F2) [14, 17, 18]. Despite retention of its nomenclature, NS2/NEP has been observed in mature virions and is no longer considered to be non-structural [19].

Influenza A, B and C viruses are antigenically distinguishable by their NP and M proteins with influenza A viruses further subtyped on the basis of H and N combinations (e.g. H5N1) where together, these two proteins represent the major surface antigens observable as rod-shaped (H) and mushroom-shaped (N) projections emanating from the

enveloped virus [14]. Currently, 16 distinct H subtypes and 9 distinct N subtypes have been described [16].

Table 1.1. Influenza A RNA Segments and Encoded Proteins

Segment	Length (nucleotides)	Encocded Polypeptide(s)	Mol. Weight (kD)	Function
1	2,341	PB2	85.7	M^7GpppX^m host mRNA recognition; component of RNA transcriptase complex
2	2,341	PB1	86.5	Endonuclease; nucleotide addition; component of RNA transcriptase and replicase complex
		PB1-F2	10.5	Induction of apoptosis in alveolar macrophages
3	2,233	PA	84.2	Component of RNA transcriptase and replicase complex
4	1,778	H	61.5	Major surface glycoprotein; major antigenic determinant; host cell binding receptor; low pH-induced conformational change fuses viral envelope with host endosomal membrane
5	1,565	NP	56.1	Binds vRNA forming coiled nucleoprotein complexes; regulates switch from viral mRNA transcription to cRNA and subsequent vRNA replication
6	1,413	N	50.1	Surface glycoprotein; antigenic determinant; sialidase activity releases bound newly formed viral progeny
7	1,027	M1	27.8	Major structural protein; interacts with RNPs and NS2/NEP
		M2	11.0	Integral membrane protein; ion channel activity involved in uncoating
8	890	NS1	26.8	Highly abundant nonstructural protein involved in suppression of host anti-viral defenses
		NS2/NEP	14.2	Minor structural protein; interacts with M1; facilitates nuclear export of newly formed RNP complexes

Infection and Viral Replication

As its name implies, hemagglutinin (H) present on the viral surface is capable of agglutinating erythrocytes via interaction with cell surface sialic-acid containing glycoconjugates [14]. This interaction with host cell sialic-acid bearing glycoconjugates begins the infection cycle by allowing binding to host cells, however, host species

restriction and tissue tropism has been linked to this protein's specificity in binding sialic acid moieties distinguished by the α-linkages originating from the sialic acid 2-carbon to the underlying galactose [20]. Human influenza viruses preferentially bind the α2-6 linkage and equine and avian viruses preferentially bind α2-3, whereas swine viruses bind both [20]. These observations correlate to the known tissue tropisms of the respective viruses where human trachea predominantly expresses the α2-6 linkage, equine trachea and avian intestinal mucosa express the α2-3 linkage and swine trachea expresses both [20]. While in low abundance, human respiratory epithelium also expresses the α2-3 linkage, thus making it amenable to avian influenza infection [21]. Susceptibility of hosts to infection with viruses of different subtypes and the segmented nature of the influenza genome provide a means for reassortment of RNA segments between coinfecting strains which can potentially produce an "antigenic shift" resulting in a virus with novel antigenicity (via a newly acquired H or N segment) and potentially enhanced pathogenicity and/or transmissibility [14, 22].

Hemagglutinin is initially expressed as an inactive precursor, H_0, requiring proteolytic cleavage to yield the active H_1 and H_2 proteins which are linked via a disulfide bond [23]. Differences in amino acid sequences of the cleavage site among the 16 H_0 subtypes and the availability of suitable host tissue proteases have been correlated to both tissue tropism and viral pathogenicity [14, 23, 24]. Internalization of the virus within host cell endosomes is followed by acidification via the action of host proton pumps where a flow of ions from the endosome to the virion interior through the M2 ion channel induces dissociation of the ribonucleoprotein complexes (RNP, comprised of

viral RNA, NP, PA, PB1 and PB2) from the M1 matrix protein [14, 21]. This reduced endosomal pH also induces an irreversible conformational change in the H_2 protein which in turn fuses host and viral membranes releasing the virion contents to the host cytoplasm [14, 15]. The importance of the M2 protein in viral uncoating is made evident by the effectiveness of the amantidine-class of anti-influenza ion-channel blocking drugs [21]

Following their release, viral RNPs are transported to the host nucleus via the presence of nuclear localization signals present on the four proteins within the RNPs where primary viral mRNA transcripts are produced, principally those of NP and NS1 [14, 15, 21]. Unique among RNA viruses, transcription and replication of influenza RNA occurs within the host cell nucleus granting access to two requisite host factors: first, viral mRNA transcription requires M^7GpppX^m-capped RNA primers scavenged from the host which are provided by a virus-encoded cap-dependent endonuclease and second, two of the eleven proteins encoded by the viral genome (M2 and NS2/NEP) require host nuclear RNA splicing machinery [14]. This primary transcription occurs via the action of the RNA-dependent RNA polymerase complex comprised of PA, PB1 and PB2 where the requisite "cap snatching" is accomplished by concerted efforts between the M^7GpppX^m recognition domain of PB2 and an endonuclease domain most recently reported to be present on PA (previous studies had independently implicated PB1 and PB2 to house this function) [25]. Following this primary transcription, cRNA of the vRNA segments is transcribed in relatively equal amounts; however, subsequent transcription of new vRNA is preferentially dominated by the NP and NS1 encoding segments where this differential regulation of new vRNA transcripts in turn correlates to

the rate of further viral mRNA transcription [14]. Given the absence of proof reading within the RNA replicase, replication of the viral genome is error prone where point mutations in the major H and N surface proteins results in “antigenic drift”, providing an escape from acquired immunity and represents the source of annual influenza epidemics [14, 15, 26].

The role of NS1 in viral replication is multifaceted where it has been shown to preferentially block host mRNA maturation and translation, to inhibit signaling pathways required for the induction of the host interferon response, to inhibit 2’-5’-oligoadenylate synthetase (OAS) activated RNase L and to inhibit dsRNA-dependent serine/threonine protein kinase R (PKR) directed protein synthesis inhibition [14]. NS1 has also been shown to inhibit early host cell apoptosis and to regulate splicing of segment 8 vRNA mRNAs (within which NS1 is encoded), thereby regulating expression of the NS2/NEP protein [27]. After its early synthesis, NP is transported back to the nucleus where it regulates the switch from primary transcription of viral mRNAs to viral cRNAs which in turn is followed by further vRNA replication [14]. Later stages of infection are dominated by the translation of M1, H and N proteins where the H and N proteins are subsequently transported to the host cell plasma membrane [14]. Given its sensitivity to low pH, the M2 ion-channel protein’s presence in membranes of the trans-Golgi network and associated vesicles assists in maintaining proton concentrations below the threshold for induction of the H protein’s irreversible conformational change [14]. Nuclear accumulation of M1 initiates RNP complex formation via interactions with vRNA and

NP and where further interaction of M1 with NS2/NEP directs nuclear export of the RNP complexes [15, 21].

Previously, it was suspected that viral RNA segment packaging was a random event, however, virion production has been observed to be most efficient when progeny included eight RNA segments where it was further observed that a signal sequence within the coding region of the N encoding vRNA segment drove its selective incorporation and suggests similar mechanisms for the other seven segments [28]. Following the encasement of a complete viral genome by the M1 protein on the cytoplasmic side of the host plasma membrane, viral budding is initiated where it has been suggested interactions of M1 with cytoplasmic domains of H, N or M2 proteins provides some requisite signal although the specific interactions among viral proteins and with those of the host in this process remain unclear [14, 15]. As in the initial binding step of infection, H proteins present on the newly formed enveloped virus bind to sialic acid structures on the host cell surface requiring the sialidase activity of the N protein for the release of viral progeny and represents the final step in virion formation [21].

Pandemics, Disease and Host Response

Combinations of the H1, H2 and H3 subtypes and the N1 and N2 subtypes have been responsible for the human pandemics of the 20th century: the “Hong Kong Flu” H3N2 virus in 1968, the “Asian Flu” H2N2 virus in 1957 and the “Spanish Flu” H1N1 virus in 1918 where these three pandemic strains resulted in excessive mortalities of 56,000, 86,000 and 675,000, respectively, within the United States [11]. Despite speculation of an avian source of the 1918 pandemic virus, phylogenetic analysis has

indicated this was not the case and the true source remains undefined [29]. Nonetheless, this virus, or at least parts of it, has continued to circulate as epidemic and pandemic influenza where reassortment and acquisition of avian H2, N2 and PB1 genes resulted in the 1957 pandemic and a further reassortment acquisition of avian H3 and PB1 genes produced the 1968 pandemic virus [15, 30]. The emergence of the H2N2 strain was marked by a disappearance of circulating H1N1 descendents of the 1918 virus, however, this virus "reemerged" from a laboratory freezer in 1977 and along with the H3N2 virus, their descendents represent the current endemic viral subtypes [30]. In addition to the dramatic effects of reassortment between different viral subtypes, intra-subtype reassortment is also a common event providing a rapid means for antigenic escape with this process having been implicated in the production of epidemic H1N1 viruses in 1947 and 1951 [31].

In 1997, 18 infections and 6 deaths were associated with avian influenza subtype H9N2 and whereas the hemagglutinin and neuraminidase proteins were different, the authors noted strong similarity between the six gene segments encoding the internal viral components to that of the H5N1 subtype [32]. In recent years, human infections with highly pathogenic avian influenza of H5N1 subtype have resulted in 340 confirmed cases across Eurasia and Africa resulting in a 61% fatality rate with at least some epidemiological data indicating limited human-to-human transmission [33]. In light of the ability of influenza to acquire virulence and pathogenicity factors through reassortment and the ability of human and other hosts to be coinfected with different viral

subtypes, the H5N1 avian subtype has received considerable attention as a potential pandemic strain [34-36].

While the threat of pandemic influenza garners significant interest, annual influenza A epidemics are estimated to cause upper respiratory infections in 5-15% of the global population and ultimately result in 250,000 - 500,000 deaths [37]. An average annual influenza season in the United States will result in illness severe enough to require hospitalization in >200,000 individuals and will claim the lives of 36,000 [38] . Direct annual medical costs have been estimated at 10.4 billion dollars with total annual economic impact due to lost productivity estimated at 87.1 billion dollars [39].

After an initial incubation period lasting 1-4 days, influenza presents as an acute respiratory disease characterized by the rapid onset of high fever, coryza, headache, cough, prostration, malaise and an inflammation of the upper respiratory tract with symptoms continuing for 7-10 days [26, 40]. Viral shedding generally peaks 48 h following symptom onset but can occur 24 h prior to noticeable symptoms [41]. Disease severity is greatest among the very young, those 65 or older and those with preexisting conditions, including, chronic cardiac or pulmonary disease or those with diabetes [40]. Additionally, whereas most cases are self-limiting and do not involve pneumonia, severe complications including dyspnea, hemoptysis, pulmonary edema, cyanosis, hemorrhagic bronchitis, pneumonia and death can occur within 48 h following onset of symptoms [40].

Recent human isolates of subtypes H3N2 and H1N1 have been shown to preferentially attach to ciliated epithelial cells, followed by goblet cells and then non-ciliated cuboidal cells with cells of the trachea and bronchi favored over those of the

bronchioles [42]. Additionally, these human isolates showed a preference for type I pneumocytes over type II within alveoli [42]. Avian isolates were shown to exhibit an inverse predilection for non-ciliated cuboidal cells in the bronchioles and a preference for type II pneumocytes [42]. Influenza infection is cytotoxic within 20-40 h following infection with replication within these aforementioned cell types resulting in multifocal destruction and desquamation often leaving only basal epithelial cells, exposed basement membrane and hyaline membrane formation [40, 43, 44]. Early stages of epithelial infection are marked by cellular shrinkage and vacuolization and whereas this stage is without neutrophil infiltrates, neutrophil influx follows soon after in response to epithelial necrosis with later stages characterized by inflammatory mononuclear cells present in the walls of transitional airways [40]. Regeneration of airway epithelium can be observed as early as the fifth day following illness onset and is marked by basal cell migration and differentiation into ciliated epithelium [40, 43]. Early infection of the alveolar epithelium is characterized by capillary thrombosis, focal alveolar wall necrosis and hyaline membrane formation and is associated with a hemorrhagic edematous pneumonia [40]. As infection continues, cytoplasmic vacuolization, nuclear pyknosis and desquamated cells within the luminal space are observed along with macrophages and neutrophils where these phagocytes may display degeneration due to either phagocytic exhaustion or direct influenza infection [40]. Later stages of infection are characterized by luminal and interstitial lymphocyte infiltrates and alveolar regeneration is observable with both hyperplasia and metaplasia of type II pneumocytes [40].

Influenza infection of epithelial cells results in the activation of numerous transcription factors including nuclear factor kappa B (NF-κB), activating protein 1 (AP-1), interferon regulatory factors (IRF) 1, 7 and 9, signal transducers and activators of transcription (STATs), and nuclear factor-IL-6 (NF-IL-6) [44, 45]. Induction of these transcription factors is mediated via Toll-like receptor 3 (TLR3) and RNA helicases retinoic acid-inducible gene-I (RIG-I) through dsRNA and 5' triphosphorylated ssRNA, respectively [46]. This activation results in the production of type I interferons, RANTES (for regulated upon activation, normal T cell expressed and secreted), monocyte chemotactic protein-1 (MCP-1) and interleukin 8 (IL-8) [44, 45, 47]. Infection of monocytes/macrophages results in the production of type I interferons, RANTES, MCP-1 and 3, macrophage inflammatory protein-1 alpha and beta (MIP-1α, MIP-1β), macrophage inflammatory protein-3 alpha (MIP-3α), interferon gamma inducible protein-10 (IP-10), tumor necrosis factor alpha (TNF-α) and interleukins 1β, 6 and 18 (IL-1β, IL-6, IL-18) [44, 45].

The production of type I interferons is observed early in influenza infection and directly correlate to observable symptoms of infection [47, 48]. In addition to an autocrine positive feedback inducing increased MHC expression and an antiviral state, inflammatory and chemotactic cytokines function to recruit and activate neutrophils, NK cells and T cells where NK/T cell activation results in interferon gamma (IFN-γ) production [44, 45]. NK cells, which can be directly activated by influenza hemagglutinin, represent a critical early innate cytolytic defense against influenza infection with this role augmented by IL-18 [49-51]. In similar fashion, a critical role for

cytolytic CD8+ T cells in clearance of influenza-infected cells, via FasL and perforin, has been demonstrated [52, 53]. Ig-deficient mice depleted of CD8+ T cells displayed significantly greater viral replication [54] and adoptive transfer of CD8+ T cells from sublethally challenged mice has been shown to rescue B-lymphocyte deficient mice from lethal challenge [55]. An anti-influenza contribution of CD4+ T cells has also been observed where their depletion from Ig-deficient mice exacerbated viral replication and disease [54]. Whereas CD4+ T cells are not required for primary CD8+ T cell development and expansion in response to influenza infection, CD8+ T cell secondary and memory responses are defective in the absence of CD4+ T cells [53]. CD4+ populations generated by influenza infection are heterogeneous in phenotype, tissue homing and function where this diversity may constitute distinct sources for memory subsets, nonetheless, those migrating to the infection site appear most differentiated and produced greater levels of IFN-γ than populations recovered from secondary lymphoid tissues [56]. While the role of IFN-γ production by these infection-homing CD4+ T cells remains unclear, the role of IFN-γ produced by Th1-type CD8+ T cells has been shown to play a pivotal role in mitigating pulmonary immunopathology resulting from influenza infection [57]. Additionally, a substantial role for gamma delta (γδ) T cells was observed when comparing survival in lethally challenge mice in wild type and γδ T cell KO mice depleted of CD4+ and CD8+ [54].

B cells and neutralizing antibody are clearly important in response to influenza infection demonstrated by a dramatically increased susceptibility in B-lymphocyte deficient mice [55, 58]. The B cell response to influenza can be divided into an innate

"natural antibody" producing B-1 population and an adaptive B-2 population where the B-1 population is characterized by relatively high and steady-state serum IgM levels before and after influenza challenge whereas the B-2 population serum IgM levels are significantly increased in response to the virus [59]. Further examination of this dichotomy revealed that whereas B-1a IgM levels remained stable in serum, there was a robust induction of IgM levels recovered from bronchioalveolar lavage fluid (BALF) with BALF B-1b IgM levels remaining virtually unchanged and indicating a tissue specific role of the B-1a subset [60]. Furthermore, it was observed that the absence of either of these two B-1 cell subsets significantly increased susceptibility to influenza and that both are required for induction of an antiviral IgG response [61].

Anti-Influenza Vaccines and Therapeutics

In light of an ever present pandemic threat, considerable effort has focused on development of vaccine candidates with broad heterosubtypic protection [62-64]. Vaccination remains the single most effective measure against influenza infection and the most recent release of the CDC's Advisory Committee on Immunization Practices has recommended annual vaccination for all individuals six months of age or older [65]. Currently licensed vaccines are available in a trivalent inactivated virus vaccine preparation delivered intramuscularly and a live, attenuated influenza vaccine delivered intranasally. Both vaccines contain the two currently recommended strain antigens of Influenza A (A/Brisbane/59/2007 (H1N1)-like and A/Brisbane/10/2007 (H3N2)-like) and one from the recommended strain of Influenza B (B/Florida/4/2006-like) [65]. Efficacy of these vaccines varies depending upon age, preexisting conditions, schedule compliance

and the viral antigens matching those of the prevailing influenza strains within any given flu season. However, efficacy determination of these vaccines is complicated by the various means employed in its assessment where serum H-specific antibody levels, neutralizing antibody levels and clinical presentations are routinely used as indicators [65]. Despite reduced efficacies in the very young, the elderly and those with underlying medical conditions, retrospective studies of vaccine efficacies with both optimal and suboptimal vaccine antigen matches to circulating influenza strains have consistently reported improved protection against hospitalization for pneumonia and influenza and against medically attended acute respiratory illness [65].

In addition to vaccination, a number of antiviral medications are licensed for the treatment of influenza and fall into two basic categories, M2 protein ion-channel blockers and viral neuraminidase inhibitors. Amantidine and rimantidine comprise the ion-channel blocking medications, however, as of January 2006, they are no longer recommended in treatment of influenza given widespread viral resistance [65, 66]. Following their discovery in the early 1960s and FDA approval in the 1970s, these ion-channel blockers enjoyed application with viral resistance in the 1990s below 1% [66, 67]. However, since 2002, rates of viral resistance have dramatically increased, reaching >90% by the 2005-2006 influenza season due to a S31N substitution in the viral M2 protein [66, 68]. Oseltamivir and zanamivir comprise the neuraminidase inhibitors which function by their competitive inhibition of the sialic-acid binding pocket of the viral N protein [65, 68]. Initial viral resistance rates to these drugs were low and remained so as recently as the 2006-2007 influenza season, but analysis of circulating H1N1 influenza

from the most recent 2008-2009 season revealed greater than 98% viral resistance to oseltamivir though remaining susceptible to zanamivir [68, 69]. This resistance is linked to a H274Y mutation in the neuraminidase which precludes oseltamivir binding in group 1 neuraminidases (N1, N4, N5 and N8) but not in group 2 (N2, N3, N6, N7 and N9) which remain susceptible [68]. These observations have particular relevance to influenza pandemic planning where antiviral drug stockpiling strategies predominantly reliant on oseltamivir would exhibit decreased efficacy against a pandemic strain having acquired a resistant group 1 neuraminidase [68].

Streptococcus pneumoniae

Discovery and Description

As with the history of influenza, the research focused on understanding the etiological agent of bacterial pneumonia would be a driving force in shaping microbiology and immunology and would ultimately provide the foundational discoveries of molecular biology. In 1880, Sternberg and Pasteur independently described a bacterium recovered from rabbits having been inoculated with saliva (Pasteur from a child succumbing to hydrophobia and Sternberg from his own) though it is likely the organism had been observed in pulmonary secretions by Klebs in 1875 [70, 71]. These initial reports described characteristic features of the bacterium in that the organism was most frequently observed in pairs and displayed a translucent capsule [70, 71]. Early work referred to the organism by a variety of names including, “Micrococcus”, “Capsulecoccus” and “Diplococcus” though Sternberg objected to these names having

observed the organism form chains in culture indicating it was, in fact, a Streptococcus [71]. The bacterium's association with pneumonia came quickly thereafter in 1882 with Friedlander observing the organism in lung sections of patients dying of the disease, however, Friedlander placed the etiological role for the pneumonia with *Klebsiella pneumoniae* which was also observed [71]. In 1884, Frankel isolated a bacterium from a fatal case of pneumonia which did not exhibit similar growth characteristics to Friedlander's purported etiological agent, *K. pneumoniae* [71]. Weichselbaum would two years later resolve the conflicting reports, however earlier histological staining work of Gram (working under Friedlander) had already found that in 19 of 20 fatal pneumonia cases, the infecting bacterium was not decolorized (i.e. it was Gram positive) and that Friedlander's assertion for *K. pneumoniae* was based primarily on the single case in which it was (i.e. Gram negative) [71]. The accumulating knowledge of bacterial morphology and pulmonary tropism ultimately resulted in providing the bacterium's accepted name, *Streptococcus pneumoniae,* with additional work revealing multiple additional sites of extrapulmonary infection producing sinusitis, otitis media, endocarditis and meningitis [71].

Frankel, in 1885, observed that rabbits who had recovered from pneumococcal ear infection were refractory to subsequent challenge whereas in 1891, Metchinkoff (the father of cellular immunity) had observed the agglutination of pneumococci by immune serum [71]. The protective nature of pneumococcal antiserum had also been demonstrated in rabbits and lead to the first use of serotherapy in humans by the Klemperers [70, 71]. In 1888, Gamaléia had suggested a protective role for phagocytes

in the lung during pneumococcal infection while in 1893, Issaef reached a concordant conclusion in that while protective, immune sera was neither antitoxic or antibacterial, but rather promoted this phagocytosis [70]. However, the success of serotherapy applied to pneumococcal pneumonia was inconsistent [72]. By 1913, pneumococci were known to be serotypically distinct and were divided into types I, II, III and IV where type IV represented a "catch-all" for strains not falling within the first three [70, 71, 73]. In 1925, Avery and Morgan, Avery and Neil and Avery and Heidelberger definitively linked these serotypes to the polysaccharide components of their respective capsules [74-76]. Two years later, Schiemann and Casper reported the immunogenicity of capsular polysaccharide in the mouse with similar findings in humans reported by Francis and Tillett [70]. However, the first definitively successful application of pneumococcal polysaccharide as a vaccine to prevent type-specific infection would not come until 1945 when MacLeod, Hodges, Heidelberger and Bernhard reported their use of a tetra-valent polysaccharide vaccine (serotypes I, II, V and VII) in service members attending an Army Air Force technical school [77]. This work represents the strategy still applied today in the currently licensed 7-valent protein-conjugate and 23-valent pneumococcal polysaccharide vaccines.

The role of capsule in pneumococcal virulence had been reported by Stryker in 1916, in which Type I and II pneumococci cocultured with type-specific antiserum selected for bacteria which lacked capsular polysaccharide and, as opposed to the encapsulated parental strains, displayed a dramatically reduced virulence and were readily phagocytosed [78]. However, these attenuated strains regained capsule,

resistance to phagocytosis and an increased virulence upon serial animal passage [78]. This observation of the specific contribution of capsule to virulence led Avery and others to explore enzymatic degradation of pneumococcal capsule as an alternative means to treat pneumococcal pneumonia [73, 79-81]. In a classic experiment reported in 1928, Griffith demonstrated that stable unecapsulated (rough colonies, R) pneumococcal strains could be transformed by co-inoculating this avirulent form with heat-killed virulent encapsulated (smooth colonies, S) strains to produce a lethal virulent encapsulated form in mice [82]. Furthermore, this work demonstrated that the initial type I or II R strain could be transformed to yield S strains producing capsules of type I, II or III and lead Griffith to suggest this capacity for transformation represented an adaptive trait [83, 84]. This hypothesis would eventually be validated in our current understanding of the competence mechanisms underlying pneumococcal transformation and its impact on vaccine efficacy and antibiotic resistance described in a later section. The nature of pneumococcal transformation received further scrutiny through which it was observed that transformation *in vitro* was also possible given appropriate preparations of heat-killed cultures [85, 86]. This prior work inspired Avery to investigate the phenomenon culminating in his group's landmark 1944 paper revealing the transforming agent to be DNA and founding the modern discipline of molecular biology [87].

Pneumococcal Virulence Factors

S. pneumoniae employs a variety of virulence factors known to contribute to this bacterium's growth in the host (Table 1.2). Chief among pneumococcal virulence factors is its polysaccharide capsule. Even before the structural and chemical diversity of

pneumococcal capsules was fully appreciated, specific antibody responses observed in convalescent serum allowed for discrimination between distinct strains based on their capsular serotype [70, 71]. Early on, it was recognized to play a major role in virulence by the aforementioned work of Stryker in which selection by growth in broth containing capsular antiserum produced spontaneous mutants lacking capsule and which were severely attenuated and readily phagocytosed [78]. Furthermore, targeting of the pneumococcal capsule would eventually lead to the first validated vaccine against pneumococcal pneumonia [77]. Currently, over 90 different serotypes have been identified, each with its own unique repetitive carbohydrate structure and all providing the same primary function of reducing complement-mediated phagocytosis [88, 89]. Pneumococcal capsular genes responsible for serotypic diversity are encoded as unique operons which in turn regulate unique capsular synthesis pathways and include proteins involved in monosaccharide cellular uptake/synthesis, coordinated sequential transfer to the oligosaccharide repeats, polymerization and the subsequent export and attachment to the cell surface [89-91]. Capsule expression is niche specific in that initial colonization of mucosal surfaces is accompanied by a relatively thin capsule (increasing bacterial surface protein exposure) while later disseminated stages of disease are characterized by relatively thick capsule production (presumably in an anti-phagocytic response to inflammation) [92, 93]. These phenotypes correlate to a natural phase variation between transparent and opaque colony forms [93, 94].

S. pneumoniae is endowed with a single, though highly effective, toxin, pneumolysin. Pneumolysin is a 53-kD multifunctional protein present in all clinical

Table 1.2. *S. pneumoniae* Virulence Factors.

Pneumococcal Virulence Factor	Function/Mechanism
Polysaccharide Capsule	Primary virulence factor; antiphagocytic properties; over 90 serotypes identified based on carbohydrate composition
Pneumolysin	Cholesterol binding cytotoxin; oligomerization forms pores in host cells, activates phospholipase A; potent inflammatory mediator
Autolysin	Degrades peptidoglycan; functions in cell wall remodeling; results in cell lysis and release of inflammatory mediators
Choline Binding Protein A	Binds secretory IgA, complement factor H and complement C3; binds polymeric Ig receptor facilitating bacterial translocation across respiratory epithelium
Pneumococcal Surface Protein A	Inhibits complement activation, deposition and complement-mediated clearance; binds to and prevents killing by (apo)lactoferrin
Hyaluronidase	Digests hyaluronic acid component of host extracellular matrix; facilitates pneumococcal invasion and dissemination
Neuraminidase	Cleaves terminal sialic-acid residues decorating respiratory epithelium; exposes preferred ligands increasing adhesion and colonization
Pneumococcal Surface Antigen A	Substrate binding lipoprotein of an ABC transporter system with specificity for manganese
PiaA and PiuA ABC Transporter	Lipoprotein components of an ABC transporter system with specificity for iron
IgA1 Protease	Zinc metalloprotease; cleaves host immunoglobulin A
Pneumococcal Adhesion and Virulence Factor A	Bacterial adhesin binding host fibronectin

isolates with a primary role in host cell lysis [88, 95]. Lacking a typical secretion signal sequence, it was originally inferred that pneumolysin was released upon pneumococcal autolysis; however, this toxin is present extracellularly in the absence of the major pneumococcal autolysin [96]. Pneumolysin binds to cholesterol present on host cell membranes where oligomerization of up to 50 monomers and a conformational change induced by cholesterol binding results in pore formation [88]. Pneumolysin is also known to bind and sequester complement though this pneumolysin contribution to virulence appears limited to later stages of pulmonary infection and the transition to a

secondary bacteremia [88]. In addition to these primary roles, pneumolysin is a potent inflammatory mediator which: activates phospholipase A present on respiratory epithelium (which in turn targets host membrane phospholipids leading to tissue damage) and induces IL-8 production; induces increased superoxide, elastase, prostaglandin E_2 and leukotriene B_4 production in neutrophils; induces TNF-α and IL-6 production in macrophages via TLR 2 and 4; and induces apoptosis in neuronal cells [88, 95, 97-100]. The adaptive benefit for such a proinflammatory toxin would seem paradoxical, yet its contribution to virulence via pneumolysin-deficient mutants has been demonstrated in animal models of pneumonia [101-103], bacteremia [102, 104] meningitis [102, 105, 106] and sepsis [107] suggesting that the induced inflammation is balanced by this pathogen's ability to mitigate the host response and provides an overall increased fitness [88].

S. pneumoniae requires choline for growth as phosphocholine is an integral component of bacterial cell where it is covalently linked to teichoic and lipoteichoic acids (LTA) [108, 109]. While cell wall LTA is inflammatory and contributes to virulence, the phosphocholine constituent too is involved in virulence via adherence to platelet-activating factor receptor (PAFr) decorating respiratory epithelium which is known to facilitate pneumococcal intracellular transport via endocytosis [92, 110]. In addition, cell wall phosphocholine also serves as an anchor for a number of pneumococcal choline-binding proteins (identified by their C-term choline binding motifs) which are known to contribute to virulence [108]. The three best characterized of these choline-binding proteins are autolysin A (LytA), Choline-binding protein A (CbpA) and Pneumococcal Surface Protein A (PspA) [108]. LytA is the major autolysin of pneumococci

contributing to cell wall remodeling during division but ultimately induces bacterial cell lysis [92, 108]. This autolysis releases proinflammatory cell wall components and mutants lacking this protein have reduced virulence in mouse models of pneumonia [102], bacteremia [102], meningitis [102, 106] and sepsis [107]. CbpA is known to bind secretory IgA [111], complement regulatory factor H [112] and complement C3 [113]. Additionally, CbpA is known to bind the human polymeric Ig receptor of human epithelium and facilitates bacterial translocation [114]. Mutants lacking this protein were attenuated in mouse models of pneumonia and meningitis [102], bacteremia [104] and in a mouse model of sepsis [115].

Among these choline-binding proteins, PspA has received the greatest attention and has been shown to inhibit complement activation and deposition [116-120] as well as binds to and prevents killing by lactoferrin [121-123]. Mutants lacking this protein are attenuated in mouse models of pneumonia [101] and bacteremia [104, 117, 124] and PspA contributes additively with Ply to sepsis [107, 125]. PspA is present on all clinical isolates; however, it displays a wide serological diversity [126] and ranges in size between 66-99 kD [127]. The mature protein is comprised of a charged N-term alpha-helical coil-coil structure, a proline-rich region and 10 choline-binding repeats [109, 128]. At least 31 distinct PspA types exist and have been categorized into six clades further divided into three families based on mAb reactivity and phylogenetic and amino acid homologies [126, 128]. Despite this diversity, broad serum cross-reactivity exists in immunized animals and immunization with PspA is protective against challenge with pneumococci bearing heterologous PspA [129-132].

Depending on the strain examined, between 13 and 20 pneumococcal surface proteins bearing the LPXTG sortase motif of gram-positive cell wall-linked proteins have been identified through genomic analysis including the known virulence factors hyaluronidase (Hyl) and neuraminidase (NanA), a serine protease (PrtA), as well as a number of other proteins of putative and unknown function [133-135]. Hyl is present in virtually all clinical isolates where it digests the hyaluronic acid component of the host extra cellular matrix and is believed to facilitate tissue invasion as has been shown for other bacterial pathogens [88, 136]. Furthermore, a strong correlation exists between pneumococcal hyaluronidase activity and meningitis such that a murine pneumococcal meningitis model depends on its exogenous addition [137]. Mutants lacking this virulence factor are attenuated in mouse models of pneumonia [138] and sepsis [139]. Similar in function to the neuraminidase of influenza, NanA cleaves terminal sialic acid residues decorating respiratory epithelium where this action is known to enhance colonization [140]. Mutants lacking this virulence factor are attenuated in mouse models of nasopharyngeal colonization/otitis media [102, 141], pneumonia [102, 142] and bacteremia [142]. PrtA is highly conserved among clinical isolates and though it bears the LPXTG sortase motif, a truncated form was recovered from bacterial supernatants suggesting both cell-surface and extracellular roles for this protein [143]. A mutant lacking this protein was attenuated in a mouse model of sepsis [143].

S. pneumoniae is additionally endowed with a variety of lipoproteins demonstrated to contribute to virulence. Pneumococcal surface antigen A (PsaA) was originally thought to be an adhesin based on both amino acid sequence homology to the

fimbrial adhesins of *Streptococcus sanguis* and *Streptococcus parasanguis* [144]. This virulence function was initially supported in the observation of reduced adherence *in vitro* [145]. However, further examination has revealed PsaA to be the substrate-binding protein of an ATP-binding cassette (ABC) transport system with specificity for manganese [146]. Mutants lacking this protein are attenuated in murine models of pneumonia and sepsis [145]. Three pneumococcal ABC transporter operons have been identified to be involved in iron uptake, *pia*, *piu* and *pit* of which, pia and piu appear to be more critical [92]. PiaA and PiuA are the lipoprotein substrate binding proteins within the transporter and mutants lacking these proteins are attenuated in mouse models of pneumonia and sepsis [147, 148].

S. pneumoniae is also endowed with a number of zinc metalloproteases, the best characterized of which is IgA1 protease [149]. Mutants lacking this protein are attenuated in mouse models of sepsis [138] and *in vitro* adherence [150]. Additionally, investigations of pneumococcal adhesion and virulence factor A (PavA), which binds immobilized fibronectin, have demonstrated its role in virulence where mutants lacking this protein are attenuated in mouse models of sepsis [151] and meningitis and *in vitro* adherence [152]. Furthermore, contributions to pneumococcal virulence have been demonstrated for pneumococcal superoxide dismutase [153], pneumococcal NADH oxidase [154] and a host of two-component signaling systems [83, 155-157].

Pneumococcal Disease

S. pneumoniae is a ubiquitous microbe whose only known natural reservoir is the human upper respiratory tract and with nasopharyngeal carriage rates ranging from 5-

30% in healthy adults and 20-50% in healthy children in the United States [158-160]. This pathogen remains the leading cause of community-acquired pneumonia and is responsible for greater than a million deaths annually in children under five around the world [161, 162]. Despite its lack of motility, this organism presents in a wide variety of clinical disease ranging from the relatively mild to that which is severe and life threatening [163]. The spectrum of pneumococcal disease has been categorized into three basic categories: that arising from primary colonization, that arising from primary and secondary bacteremia and that arising from this hematogenous dissemination [163].

The two most prominent disease conditions arising from the first of these three categories are otitis media and sinusitis in which *S. pneumoniae* is the most frequently encountered etiological agent for both adults and children [158, 163]. That >90% of children will have experienced otitis media by the age of five years (with most occurring before the age of two years) speaks to this pathogen's ubiquitous distribution [159]. In the absence of underlying risk factors (e.g. smoking, viral infection), pneumococci reaching the eustachian tubes or sinuses are generally cleared through ciliary action, however, inflammation and occlusion of these areas provides a medium for proliferation and infection [163]. While the economic impact of *S. pneumoniae* induced sinusitis has not been assessed, an annual cost of two billion dollars has been attributed to treatment of acute otitis media where this treatment is clearly indicated in the fact that spontaneous clearance of infection occurs in only 20% of cases in the absence of therapy [159]. Similar to sinusitis and otitis media, underlying risk factors affecting normal tracheobronchial clearance, particularly those which undermine mucociliary function (e.g.

smoking), have also been associated with dissemination from a primary pneumococcal nasopharyngeal colonization and can result in an acute purulent tracheobronchitis [163]. However, the most severe disease associated with non-hematogenous disseminated nasopharyngeal colonization is pneumococcal pneumonia where community-acquired pneumonia in the U.S. is the fifth leading cause of death and the most frequent cause of infection-related mortality in those older than 65 years [159]. While significant in the developed world, pneumococcal pneumonia in the developing world results in five million deaths annually [159]. Symptoms of pneumococcal pneumonia include cough, fatigue, fever and shortness of breath which are all more prominent in younger patients [163]. Again, incidence of pneumococcal pneumonia is strongly correlated with reduced pulmonary clearance (e.g. chronic obstructive pulmonary disease, HIV/influenza infection), diabetes, smoking and alcohol abuse [163]. Given that pneumococci are not readily adherent to ciliated epithelium; it is presumed that colonization of the lower respiratory tract results from aspiration from the nasopharynx to the alveolus where *S. pneumoniae* readily adheres to Type II pneumocytes [164, 165]. From here, disease progression is marked by three distinct histopathologic features beginning with an edematous engorgement of alveoli, followed by localized hemorrhagic edema (Red Hepatization) and ending with a strong leukocyte infiltration and consolidation (Grey Hepatization) [164]. These features are spatially defined within distinct lobes of the lung (lobar pneumonia) and while not exclusively diagnostic, are hallmarks of pneumococcal pneumonia when present in pulmonary X-ray and computed tomography (CT) scans [164]. In addition to disease resulting from dissemination from the nasopharynx to other

respiratory mucosal sites, *S. pneumoniae* is known to colonize the conjunctiva resulting in conjunctivitis and is known to colonize the female reproductive tract resulting in endometritis, salpingitis and if permitted access through perforative injury, can cause a secondary peritonitis [164].

Pneumococcal bacteremia represents the second broad categorization of pneumococcal disease and has been defined as either primary, in which invasion occurs from a site of colonization (i.e. nasopharynx), or as secondary, in which invasion occurs from a site of established infection [163]. Pneumococcal bacteremia is the most frequently encountered invasive pneumococcal disease in children less than two years old accounting for approximately 70% of cases [166]. And while blood infection can produce a life-threatening sepsis, the hematogenous dissemination of *S. pneumoniae* (representing the third broad categorization of pneumococcal diseases) is notably linked to unfavorable outcome particularly when resulting in pneumococcal meningitis [159, 163, 167]. With the introduction of effective vaccination against *H. influenzae*, *S. pneumoniae* has become the leading cause of bacterial meningitis in the developed world, and despite effective antibiotic therapy, mortality rates still reach 34% [159, 166, 168]. Following an established pneumococcal bacteremia, bacterial invasion occurs at either the choroid plexus where choroidal epithelium separates fenestrated vascular endothelium from the cerebral-spinal fluid (CSF) or at the blood-brain barrier where pericytes and astrocytes combine with tightly joined vascular endothelium in the barrier function [168]. Following inflammatory signaling due to bacterial presence, induced host cell-surface receptors serving as bacterial adhesion foci are thought to provide a cellular

translocational mechanism granting bacteria access to the underlying tissues [168]. When death does not result from pneumococcal meningitis, 50% of those surviving will face long-term sequelae including hearing loss/deafness, cerebral palsy and cognitive impairment [167-169]. Hematogenous pneumococcal dissemination has also been implicated in the development of endocarditis, pericarditis, primary peritonitis, septic arthritis, osteomyelitis, empyema and soft tissue infections where this last presentation generally warrants an assessment of HIV status [105].

Host Response

The host response to *S. pneumoniae* infection is primarily one of an acute inflammation mediated through the binding and activation of innate soluble factors, pathogen-associated molecular pattern (PAMP) recognition receptors (PRR) and subsequent cytokine and chemokine signaling [170-172]. The soluble factors contributing to pneumococcal defense include antibody, complement, acute phase proteins and surfactant proteins [172]. As with influenza, innate antibody responses to pneumococcal polysaccharide are dependent on the two subsets of B-1 B cells, B-1a and B-1b [173]. However, whereas the secondary signaling which drives the systemic T cell-independent responses of B-1b and tissue specific responses of B-1a (as observed in influenza infection [59]) remain unclear [174], CD19 expression has been demonstrated to play a role in proper development of the B-1a and B-1b subsets and their respective responses to pneumococcal polysaccharide [173]. While also key to initiating the broader inflammatory response, a critical role for innate soluble factors is their opsonin function in that absent this contribution, the pneumococcal polysaccharide capsule

effectively resists phagocytosis [175, 176]. The role of natural antibody and C-reactive protein (CRP) in pneumococcal defense has been shown to be in their binding to bacterial polysaccharide and phosphocholine, respectively, and the subsequent activation of complement [177, 178]. CRP was first described in 1930 in the analysis of sera from patients with acute inflammation following pneumococcal infection where it was found to precipitate with a somatic component fraction (fraction C) of pneumococci [179]. CRP-activated complement is critical for protection against lethal pneumococcal challenge and whereas FcγR was not required for this protection [180], CRP-opsonized *S. pneumoniae* interaction with FcγR is known to result in increased TNF-α and IL-1β production in peripheral blood mononuclear cells (PBMC) and induces increased antibody responses via CRP targeting of opsonized pneumococci to dendritic cells [181, 182]. While all three complement pathways are known to contribute to complement deposition on *S. pneumoniae*, the classical pathway appears most relevant for pneumococcal clearance where mice lacking C1q were more susceptible than those lacking factor B and where additive susceptibility indicated the contribution from the alternative pathway was primarily one of amplification [183]. The role of the lectin pathway is evident in the increased susceptibility to pneumococcal disease experienced by those with inherited defects in mannose-binding lectin (MBL) [184] and while MBL has been demonstrated to bind to *S. pneumoniae* [185], the observation of similar susceptibilities in C4-/- and C1q-/- mice leave this pathway's specific contribution unresolved given an additive effect would be expected within the C4-/- group [183]. The resulting complement activation and deposition provides a target for CD11b/CD18/CR3

mediated phagocytosis and a chemotactic signal in C5a [186, 187]. That complement represents a critical component of the innate response is further underscored by the targeting of this host defense mechanism by a number of the pneumococcal virulence factors described above. The acute-phase lipopolysaccharide binding protein (LBP) has also been shown to bind pneumococcal cell wall where it facilitates signaling through TLR1, 2 and 6 and CD14 pathogen recognition receptors [188]. Surfactant proteins A and D (SP-A and SP-D) produced by alveolar type II pneumocytes also contribute to defense against pneumococcal infection where they are known to agglutinate pneumococci of multiple capsular serotypes [189], augment phagocytosis by alveolar macrophages[190], prevent dissemination and ultimately contribute to pneumococcal clearance in the respiratory tract [191].

A variety of PRRs are involved in the host recognition of *S. pneumoniae* [170, 192]. TLR1 and TLR2 expression and signaling was rapidly induced within respiratory epithelium exposed to pneumococci (via lipoteichoic acid components of the cell wall) and synergistically resulted in IL-8 production whereas signaling through TLR4 was restricted to an interaction with Ply [193]. *S. pneumoniae* infection has also been shown to upregulate nucleotide-binding oligomerization domain 1 (Nod1) and Nod2 expression in bronchial epithelial cells suggesting a role for these intracellular pattern recognition proteins in host defense [194]. Similar TLR-mediated responses have been described for macrophages where engagement of TLR2 and TLR4 results in production of TNF-α, IL-6, IL-8 [100, 171, 195] and the engagement of TLR9 enhances macrophage pneumococcal

uptake and killing [196]. It has also been shown that TLR4 mediates macrophage apoptosis and that this response was beneficial in pneumococcal challenge [197, 198]. A role for TLR2 signaling has also been described for B cells and CD4+ T cells where if either group is derived from a TLR2-/- source, T cell-dependent type 1 IgG targeting pneumococcal phosphocholine is significantly reduced [199]. CD14 expressed on cultured monocytes has also been shown to respond to purified pneumococcal cell wall with production of TNF-α, IL-1β and IL-6 [200]. Macrophage expressed specific intracellular adhesion molecule-grabbing nonintegrin (SIGN)-R1 has been shown to contribute to pneumococcal clearance via capsular polysaccharide binding [201] and through this work, has led to the discovery that, in partnership with C1q, this PRR forms a C3 convertase [202]. Additionally, the macrophage scavenger receptor for collagenous structure (MARCO) expressed by alveolar macrophages has been shown to contribute to bacterial clearance and survival [203].

When assessing various *in vitro* and *in vivo* models, pneumococcal pulmonary infection and mechanisms of pathogen recognition by the host are known to induce a rapid and potent inflammatory cytokine response [172]. Respiratory epithelium has been shown to upregulate a number of proinflammatory cytokines and chemokines in response to pneumococcal adhesion including IL-1β, IL-6 and CXCL1-3 and has been shown to produce prostaglandin E_2 (PGE_2) via cyclooxygenase 2 (COX-2) induction [204, 205]. Within four hours following pulmonary infection, significant increases in TNF-α, IL-1α and IL-6 are observed with an associated increase in recruited neutrophils [206]. An early role for CD4+ T cells in the host response was made evident in that their absence

has been shown to lead to a rapid increase and persistence of pulmonary pneumococcal infection when compared to controls [207]. Furthermore, it was shown that two distinct subpopulations of activated CD4+ cells were induced to migrate in response to pneumococci, one of which secreted IL-4 and the other secreting IFN-γ [207]. TNF-α, IL-1α and IL-6 levels were observed to continue increasing through 12 hours following pneumococcal infection and were associated with still further neutrophil recruitment, increased nitric oxide (NO) release and increased BALF malondialdehyde (MDA), myeloperoxidase (MPO) and leukotriene B_4 (LTB_4) concentrations [206]. This increasing inflammatory response is correlated with an upregulation of CD18 and alveolar PAFr and, with an accompanying influx of erythrocytes and fibrin, is characterized by red hepatization [97, 164]. Depletion of NK cells revealed their involvement in the early pro-inflammatory response to pneumococcal pneumonia in which the NK population contributed to IFN-γ and IL-6 production through an, as yet, undefined pneumolysin-dependent mechanism [208]. Between 12 and 72 hours following pneumococcal infection, neutrophil populations remain steady and macrophage and lymphocyte populations increase and whereas TNF-α and IL-1α levels in the lung steadily decrease, IL-6 levels are maintained [206]. Employing *in vitro* macrophage assays, pneumococcal stimulation induced granulocyte colony-stimulating factor (G-CSF), macrophage chemoattractant protein (MCP)-1, IFN-γ and IL-10 responses which were all observed to increase between 24 and 48 hours [209]. Analysis of NKT cell responses to pneumococcal infection found that mice lacking this cell population (which displayed an increased susceptibility to infection after 48 hours) and treated with

exogenous IFN-γ, a wildtype phenotype emerged and indicated a role of IFN-γ production for this population [210]. These latter stages of the host response (2-5 days post-infection) are characterized by grey hepatization in which erythrocytes have been lysed and the consolidation of dense leukocyte infiltrates and fibrin fill the alveoli [97]. Resolution typically occurs between five and ten days post-infection and is marked by efficient opsonization via B-1b capsule-specific antibody [173], fibrin dissolution, reduction of pro-inflammatory mediators and a return to normal lung architecture [97]. A contribution of lung γδ T cells was demonstrated in this resolution phase post-pneumococcal infection in which a >30-fold increase in γδ T cell numbers correlated with a cytotoxic reduction of alveolar macrophages and pulmonary dendritic cells and an enhanced return to normal lung architecture when compared to that observed in TCRδ-/- mice [211]. Additionally, a role for Th17 CD4+ T cells has been demonstrated in the mitigation of IL-10-mediated suppression of pneumococcal whole cell antigen (WCA)-induced inflammation required for nasopharyngeal clearance of colonizing pneumococci [209]. Furthermore, this Th17 CD4+ T cell role has been demonstrated to function primarily through enhanced neutrophil killing where adoptive transfer of WCA-immunized Th17 CD4+ T cells into RAG1-/- mice was shown to be capable of clearing pneumococcal nasopharyngeal colonization in an antibody- and serotype-independent fashion [212].

Vaccines and Antibiotic Resistance

The success of the tetravalent pneumococcal polysaccharide vaccine developed by MacLeod, Hodges, Heidelberger and Bernhard at the close of World War II [77]

prompted the development of a hexavalent vaccine in the late 1940s, but unfortunate timing resulted in its release at the dawning of the antibiotic era and their vaccine garnered little interest [213]. However, in the ensuing decades, the incidence and severity of invasive pneumococcal disease, particularly in those of highest risk (i.e. children < 2 years old, the elderly and those who are immunocompromised) led to a renewed interest in a prophylactic intervention [213]. In 1977, a 14-valent polysaccharide vaccine was introduced with an expanded 23-valent vaccine following in 1983 [214]. The currently licensed pneumococcal polysaccharide vaccine (PPV) is comprised of capsular serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9N/V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A/F, 20, 22F, 23F and 33F) which account for 90% of invasive disease in Europe and the United States [214]. However, while this formulation is effective in healthy adults, those at highest risk for invasive pneumococcal infection are also those who are most likely to respond poorly to these T cell-independent antigens [214]. The five serotypes most commonly associated with invasive disease in children (6A/B, 14, 18C, 19F and 23F) are particularly poor immunogens in children <2 years old where responses do not reach comparable adult levels until eight to ten years of age [214]. Additionally, there exists a geographic discrepancy in serotype distributions associated with invasive disease and while the current formulation protects against 90% of the serotypes associated with disease in the west, it is estimated to cover only 60% of serotypes causing disease in Asia [214]. Furthermore, in the absence of T cell help, polysaccharide antigens fail to invoke long-lived or anamnestic responses [214]. Primarily for these reasons, a protein-conjugate vaccine (PCV) was developed in which 7

serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) are individually conjugated to the cross-reactive material (CRM)-197 nontoxic variant of diphtheria toxin [166]. With predicted serotype cross-reactivity, this formulation would cover serotypes responsible for 86% of bacteremia, 83% of meningitis and 65% of acute otitis media (AOM) disease however, these estimates are again based on serotype prevalence and distribution in Europe and the United States [166]. For instance, this current formulation is predicted to cover only 41% of serotypes in Israeli children and 22% in Bedouin children, whereas simply including serotypes 1 and 5 would improve this coverage to 67% and 63%, respectively [215]. Nonetheless, efficacy of this formulation was found to be 90-100% protective against invasive pneumococcal disease caused by both targeted and non-targeted serotypes in healthy children in the United States [166].

Despite the success of the PCV, there remain two causes for concern regarding its extended utility. A Finnish study assessing PCV efficacy against AOM reported a 57% reduction in disease caused by targeted serotypes, however, this study also revealed a 33% increase in nasopharyngeal colonization by non-targeted serotypes, many of which are linked to invasive disease [216]. Additionally, as first demonstrated by Griffith in 1928, potential for recombinational exchange of capsule biosynthesis loci *in vivo* exists and is known to have resulted in immunologic escape of highly transmissible and antibiotic resistant pneumococcal strains [82, 217, 218].

Optochin was a chemical commonly used in the early part of the 20th century to distinguish pneumococci from other α-haemolytic streptococci in that *S. pneumoniae* was optochin-sensitive and would form a zone of inhibition around paper discs seeded with

the chemical [219]. However, in 1912, an insidious trait common to *S. pneumoniae* and countless other pathogens became apparent in that optochin-resistant pneumococcal strains had evolved [71]. Whereas penicillin-resistant pneumococcal strains had been recovered from mice as early as 1933, the first documented penicillin-resistant strain isolated from a human would not be reported until 1971 [70, 220]. Currently pneumococcal strains with resistance to aminoglycosides, cephalosporins, chloramphenicol, macrolides, quinolones, tetracyclines and vancomycin have been reported [70, 221]. Additionally, multi-drug resistant strains have been identified some of which are resistant to all FDA approved therapeutics for the treatment of AOM [222, 223]. And as with antigenic escape from serotype-specific immunity, recombinational genetic exchange between pneumococcal strains provides a readily available mechanism for further acquisition of antibiotic resistance [224].

Following the introduction of the pneumococcal PCV in 2000, invasive pneumococcal disease caused by single and multiple antibiotic-resistant *S. pneumoniae* declined dramatically [225]. This observation provided still further motivation to develop a pneumococcal protein vaccine capable of providing both the benefits of a T cell-assisted antibody response and at the same time, a broader coverage of clinically relevant pneumococcal strains than that currently offered by the PPV and PCV formulations [214]. This effort has primarily focused on pneumococcal proteins with established roles in virulence and include the aforementioned Ply, CbpA, PspA, PsaA, and NanA proteins [226]. Subcutaneous (SC) immunization with recombinant detoxified-Ply has been shown to protect mice against lethal intraperitoneal (IP) and

intranasal (IN) pneumococcal challenges with nine different serotypes [227]. Ply delivered IP has also been shown to protect against a rat model of lobar pneumonia [228]. Additionally, intravenous (IV) administration of three Ply mAbs provided passive protection against a lethal IN challenge [229]. Both passive administration of anti-CbpA antibodies and IP CbpA immunization protected mice from lethal IP pneumococcal challenge [230, 231]. PspA delivered IN was protective against lethal pneumococcal challenge administered IV, IN, IP and intratracheally (IT) and was cross-protective against nasopharyngeal colonization by two heterologous PspA bearing strains [232]. Anti-PspA monoclonal and polyclonal antibodies were shown to protect against lethal IV pneumococcal challenge and were also capable of rescuing mice from established lethal bacteremia [233]. PspA-GM-CSF and PspA-IL2 fusion peptides differentially induced IgG1 and IgG2 anti-PspA antibodies, respectively, which passively protected against a lethal IV pneumococcal challenge [234]. Similarly, PspA delivered IN with IL-12 produced anti-PspA antibodies which passively protected against a lethal IP pneumococcal challenge [235]. Live vaccines comprised of PspA-expressing *Lactobacillus* and *Salmonella* delivered via nasal and oral routes, respectively, induced protective anti-PspA antibody responses which protected against lethal IP pneumococcal challenges [236, 237]. Additionally, DNA vaccines expressing PspA have been protective against lethal IV pneumococcal challenges [238, 239]. Furthermore, PspA incorporated into a poly(ethylene oxide) matrix induced anti-PspA antibodies which protected against a lethal IV pneumococcal challenge [132]. PspA administered IN to murine dams was shown to induce passive immunity in neonatal mice which protected

against a lethal IP pneumococcal challenge [240]. PsaA administered SC protected against lethal IV pneumococcal challenge [241] and PsaA-cholera toxin B fusion-protein delivered IN inhibited pneumococcal nasopharyngeal colonization [242]. NanA administered IN protected against pneumococcal nasopharyngeal colonization and reduced the incidence of otitis media in a chinchilla model [243, 244]. Lastly, additive protection has been witnessed using various combinations of these protective antigens [231, 245-247].

Influenza-Induced Susceptibility to Secondary *S. pneumoniae* Infection

The first formalized recognition that influenza predisposed individuals to secondary disease is attributed to William Farr who in 1847 observed increased influenza mortality not attributable to influenza infection itself [10]. Farr developed the foundational methodology to assess "excess mortality" during influenza epidemics which, with some additional refinement in the early part of the 20th century, remains in use today [10]. The 1918-1919 Spanish influenza pandemic occurred in an era primed to further investigate the etiological agents of both the pandemic and its secondary complications ultimately resulting in the identification of the influenza virus and an understanding of the secondary nature of the frequently encountered bacterial infections [5, 6, 9-13]. Among secondary pathogens most frequently encountered following influenza infection both then and now are *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Haemophilus influenzae* ("Pfeiffer's bacillus", *Bacillus influenzae*) with *S. pneumoniae* the being most common [5, 11, 12, 248-250].

The possible mechanisms by which influenza infection induces susceptibility to secondary bacterial pathogens are numerous but can be divided into two broad categories of virally-induced modification of the host respiratory tract and viral suppression of host innate phagocytic effector cell populations [10].

As described previously, viral neuraminidase cleaves terminal sialic-acid moieties decorating respiratory epithelium and through this action has been shown to increase pneumococcal adhesion and colonization through exposure of preferred bacterial ligands and increased access to the underlying host cell surface [251-254]. Additionally, influenza infection induces an upregulation of numerous host cell surface molecules and molecules secreted into the extracellular matrix, such as PAFr and C3, which can lead to increased pneumococcal adherence and intracellular invasion [113, 164, 200, 255, 256]. Mucociliary function has been shown to be impaired by influenza infection with a concomitant increase in bacterial colonization [10, 257, 258]. As mentioned above, the cytotoxic nature of influenza infection results in multifocal destruction of respiratory epithelium where *S. pneumoniae* was found to be associated with these damaged areas [259] and where denuded epithelium, exposing a ligand-rich basement membrane, was observed to be foci of pneumococcal adherence [43].

Influenza infection is also known to suppress macrophage chemotaxis, phagocytosis and killing of bacteria [260-264]. Additionally, the influenza PB1-F2 protein has been shown to induce apoptosis in human monocytes and macrophages [17, 265]. Similarly, influenza infection is known to suppress neutrophil chemotaxis, phagocytosis, phagolysosomal fusion, oxidative burst and killing of bacteria [263, 264,

266-270]. Furthermore, influenza infection has also been shown to synergistically induce neutrophil apoptosis upon exposure to *S. pneumoniae* [271]. A role for increased levels and persistence of the inflammatory suppressing cytokine IL-10 has been linked to susceptibility to secondary pneumococcal infection [272]. While the role of neutrophils is critical in clearing secondary pneumococcal infection originating within three days of the initial influenza infection, this role is significantly diminished when secondary pneumococcal challenge occurred six days following influenza infection and indicated that influenza suppression of both cellular and non-cellular defenses significantly contribute to increased secondary pneumococcal colonization and infection [270].

Hypothesis

Both influenza and *S. pneumoniae* have evolved numerous mechanisms facilitating their infection of the human respiratory tract and it is clear that influenza infection significantly predisposes to secondary pneumococcal infection. Whereas the mechanisms of viral contributions to this synergism have been explored, little is known concerning contributions originating with specific *S. pneumoniae* virulence mechanisms. We hypothesized that secondary *S. pneumoniae* infection following influenza infection involves enhanced or novel application of known pneumococcal virulence factors. We further hypothesized that *S. pneumoniae* surface-exposed proteins currently uncharacterized regarding their potential virulence contributions may also contribute to secondary pneumococcal infection. To address these hypotheses, virulence of mutant *S. pneumoniae* strains with insertion-duplication mutations in genes encoding surface

proteins of known and unknown virulence contributions were tested in competitive growth experiments in which individual mutant strains were coadministered with the parental wildtype strain into a mouse model of primary pneumococcal infection and secondary pneumococcal infection following established influenza infection.

Development of a viable pneumococcal protein vaccine has focused on pneumococcal surface-exposed virulence factors; however, while successful in providing candidates capable of protecting against pneumococcal challenge alone, these efforts have not assessed their ability to provide protection against the far more dangerous scenario of a secondary pneumococcal infection in an influenza-infected respiratory tract. We additionally hypothesized that pneumococcal proteins demonstrating virulence contributions to secondary pneumococcal infection following established influenza infection could potentially serve as vaccine targets capable of protecting against secondary pneumococcal infections. To address this hypothesis, surface proteins demonstrating virulence contributions to secondary pneumococcal infection in competitive growth experiments were employed in a model of intranasal immunization to test their ability to protect against secondary pneumococcal infection following established influenza infection.

PNEUMOCOCCAL SURFACE PROTEIN A CONTRIBUTES TO SECONDARY *STREPTOCOCCUS PNEUMONIAE* INFECTION FOLLOWING INFLUENZA INFECTION

Introduction

Influenza A infection dramatically increases susceptibility to secondary *Streptococcus pneumoniae* infection resulting in significantly greater morbidity and mortality [10]. Viral effects implicated include disruption of innate-effector responses [262, 270] and modification of respiratory mucosa [43, 253]. Whereas viral contributions to secondary pneumococcal infection have received considerable attention, contributions of *S. pneumoniae* virulence factors remain unclear. Much effort has focused on pneumococcal virulence factors as targets for vaccine development in primary infection [226, 273, 274]. Among those explored are hyaluronidase (Hyl), neuraminidase A (NanA) and pneumococcal surface protein A (PspA). Hyl targets hyaluronic acid in host connective tissues and extracellular matrix enhancing access for tissue invasion and colonization [226, 275]. Like influenza neuraminidase, pneumococcal NanA cleaves terminal sialic acid residues on respiratory-surface glycoconjugates promoting adhesion and colonization of bacteria [141, 276]. PspA is a choline-binding surface protein, which inhibits complement-mediated phagocytosis and binds to and prevents killing by lactoferrin [117, 123]. Numerous studies have documented attenuation in mutants lacking PspA [101, 107, 117, 277], and PspA protein is protective in a variety of delivery and challenge models [132, 230, 245-247, 278].

Current pneumococcal vaccines have been effective in decreasing incidence of invasive pneumococcal disease [279, 280]; however, protecting the elderly and selection driven shifts in colonizing and disease causing serotypes remain prominent concerns [279, 281, 282]. As *S. pneumoniae* virulence factors are attractive candidates for vaccine development, further examination of their roles in the influenza-infected lung will help identify targets critical for secondary pneumococcal infection. This study examined the relative contributions of Hyl, NanA and PspA to *S. pneumoniae* virulence in healthy and influenza-infected mice. Additionally, the ability of immunization with PspA to reduce serotype 2, 3 and 4 primary and secondary pneumococcal growth and lung damage was assessed.

Materials and Methods

Mice

Healthy 6-8 week old female C57BL/6 mice were obtained from the NCI-Frederick Animal Production Area, Frederick, MD. All procedures were approved by the Montana State University Institutional Animal Care and Use Committee.

Bacterial Strains

S. pneumoniae strains D39 (serotype 2), WU2 (serotype 3) and TIGR4 (serotype 4) were cultured at 37°C/5% CO_2 in Todd Hewitt broth with 0.5% yeast extract (THY). Insertion-duplication mutants of D39 lacking PspA (PspA-), Hyl (Hyl-) and NanA (NanA-) were generously provided by Dr. J. C. Paton. These mutants have been previously described [107] and while these and other insertion-duplication mutants have

been routinely employed [101, 102, 142], there exists the possibility of polar effects. Bacteria were cultured in THY supplemented with 0.2 μg/ml erythromycin when appropriate and harvested in mid- to late-log phase, and aliquots containing 20% glycerol were snap frozen and stored at -80ºC. To determine stock CFU concentrations, 10-fold serial dilutions of aliquots were plated on 5% sheep blood agar plates containing 25 μg/ml neomycin, supplemented when appropriate with 0.2 μg/ml erythromycin, and incubated at 37°C/5% CO_2 for 24 h. Aliquots were thawed, washed with sterile PBS and diluted to desired concentrations for inoculation.

Infection Model

Groups of 5-7 mice were infected via oropharyngeal aspiration [283] with 400 PFU of the H1N1 mouse-adapted influenza A/Puerto Rico/8/34 (PR8) influenza virus in 50 μl PBS or received a sham infection of PBS alone. On day 6 post-influenza or sham inoculation, mice were infected via oropharyngeal aspiration with a mixture of 5×10^5 CFU of D39 and 5×10^5 CFU of PspA-, Hyl- or NanA- mutant in 50 μl PBS. Numbers of mutant and total bacteria in each inoculum were determined by selective and non-selective plating, respectively, as described above. At 24 h after the pneumococcal infection, mice were euthanized and lungs were lavaged with 5 ml PBS. Lungs were removed, placed in recovered bronchioalveolar lavage fluid (BALF), homogenized, snap frozen and stored at -80ºC. Numbers of mutant and total bacteria present in lung homogenates were again determined by selective and non-selective plating as described above. Competitive index (CI) values were calculated by dividing recovered mutant/total bacteria ratios in lung homogenates by mutant/total bacteria ratios present in inocula as

described previously [284]. To quantify PFU, lung homogenates were serially diluted, and plaque assay performed as previously described [57].

As insertion-duplication mutants are subject to reversion, *in vivo* stability was assessed by infecting mice with the individual Hyl-, NanA- and PspA- mutants. Lung homogenates recovered after 24 h were serially diluted and plated on both selective and non-selective blood agar plates. Bacteria numbers obtained from selective plating were divided by those recovered from non-selective plating to assess increases in non-selected populations due to revertants. Mean values calculated for the Hyl- (.9904), NanA- (1.018) and PspA- (1.025) mutants were not significantly different than the expected value of 1 when analyzed by one-sample t-test ($\alpha = .05$) indicating reversion rates were not confounding during *in vivo* growth through 24 h.

Intranasal Immunization with PspA

To assess inhibition of primary and secondary pneumococcal growth by PspA immunization, mice under light isoflurane anesthesia received intranasal immunizations twice weekly for three weeks. Groups of 10-14 mice received 1 µg recombinant N-terminal His-tagged PspA protein (rPspA/Rx1), generously provided by Dr. J.C. Paton, along with 4 µg cholera toxin B subunit (CTB, List Biological Laboratories) in 20 µl sterile saline. Control groups received PBS or adjuvant alone. Mice receiving PspA were immunized with adjuvant for two weeks receiving only protein in the third week with PBS and adjuvant controls receiving only saline in the third week. Twenty days following immunization, mice in the three groups were divided into subgroups (5-7 mice) receiving either 400 PFU PR8 influenza or a sham PBS infection. Six days after

influenza or sham infection, all mice received 1×10^6 CFU D39, WU2 or TIGR4 pneumococci via intratracheal instillation. Twenty-four hours following bacteria inoculation, BALF and lungs were harvested. Viral and bacterial loads in lung were determined as described above. PspA-specific IgG and IgA titers in sera were determined by ELISA.

For ELISA, high binding 96-well plates were coated with 1 μg/ml PspA in 0.05 M carbonate buffer, pH 9.6, for 3 h at 37ºC and then at 4ºC overnight. Plates were washed 3 times with wash buffer (0.005% Tween 20 in PBS) and non-specific binding sites were blocked with 5% nonfat dried milk in PBS at 37ºC for 1 h. After 3 washes, 2-fold serial dilutions of serum samples in wash buffer were added and incubated at 37ºC for 1 h. Following 3 washes, alkaline phosphatase-conjugated, anti-mouse, isotype-specific secondary Ab (IgG, Sigma-Aldrich; IgA, Serotec) was added and incubated at 37ºC for 2 h. After 5 washes, *p*-nitrophenyl phosphate in diethanolamine buffer was added and incubated at 37ºC for 30 min. Endpoint titers were defined as the highest reciprocal dilution exhibiting absorbance (405 nm) above 0.100 OD units above negative controls [285].

To evaluate PspA immunization effects on lung pathology, levels of albumin and lactate dehydrogenase (LDH) in recovered BALF were determined using commercially available kits (631-2; Sigma Diagnostics, St. Louis, MO, and CytoTox 96; Promega, Madison, WI).

Statistical Analyses

Analyses of one-sample t-test, two-tailed Mann-Whitney, one-way ANOVA with Bonferroni post-test and two-way ANOVA with Bonferroni post-test were performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, California).

Results

Competitive Primary and Secondary Growth of PspA-, NanA- and Hyl- Mutants

Prior to administration of mutant/D39 mixtures on day 6 after flu and control inoculations, mice having previously received influenza exhibited symptoms and behavior consistent with influenza infection including clustering, ruffled fur and a wasted appearance, whereas controls appeared symptom-free. Influenza-infected mice contained $1 \times 10^6 – 1 \times 10^7$ PFU in their lungs day 7 after viral inoculation, whereas sham treated mice revealed no detectable virus (Fig. 2.1). Median total bacteria numbers recovered from mice with prior influenza infection were significantly greater (Fig. 2.2 A, B and C) compared to mice without prior influenza infection for all three mixed mutant and wildtype inocula with increases ranging from 15,000-fold (Hyl- & D39) to 29,000-fold (PspA- & D39).

Competitive growth is a common method for identifying fitness defects between co-administered bacterial strains [284, 286, 287]. To better resolve interactions of pneumococcal virulence factors with prior influenza infection, we chose this method to normalize lung deposition of mutant and wild type strains in influenza-infected mice given the synergistic nature of this superinfection can amplify small initial variation in

bacteria numbers. We calculated CI values as a measure of mutant fitness where a CI value of 1 indicates both strains grew equally well; a CI value of 0.1 indicates a 10-fold reduction in mutant growth and/or survival relative to wild type.

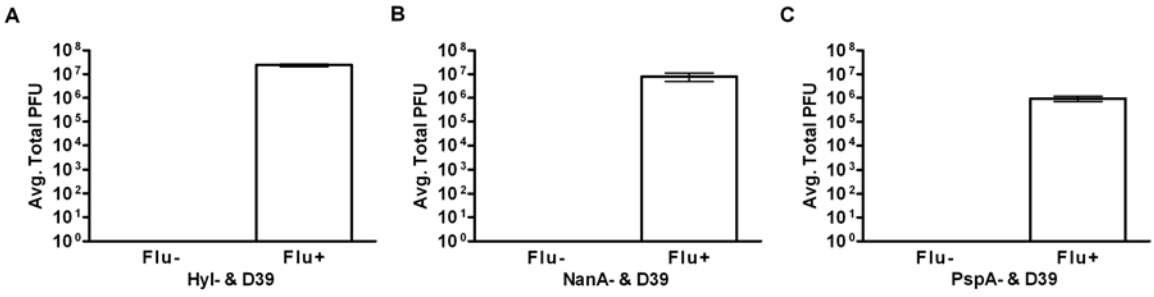

Figure 2.1. Average Total PFU Recovered from Mouse Lung 24 h following Hyl- & D39 (A), NanA- & D39 (B) or PspA- & D39 (C) Infection. Following inoculation with either a sham PBS infection (Flu-) or 400 PFU PR8 influenza (Flu+), mice received approximately 5×10^5 CFU of the D39 parental strain concurrently with approximately 5×10^5 CFU of Hyl-, NanA- or PspA- mutant strain (1×10^6 CFU total) day 6 post-influenza or sham treatment. Columns represent group means with error bars indicating SEM. Data presented were pooled from at least two independent replicates.

Median CI values of Hyl- in mice with and without prior flu infection at 24 h were 1.39 and 1.6, respectively, indicating mutant fitness was not attenuated relative to the wild type (Fig. 2.2 D). The difference between these CI values was not statistically significant (P = 0.0566), suggesting that prior influenza infection did not impact the role of this virulence factor. Similarly, NanA- exhibited median CI values of 1.37 and 1.43 in mice with and without influenza infection, respectively, and thereby failed to exhibit attenuation (Fig. 2.2 E). Again, difference in the CI values of NanA- in mice with and without flu infection was not significant (P = 0.9817). Contrary to Hyl- and NanA-, the

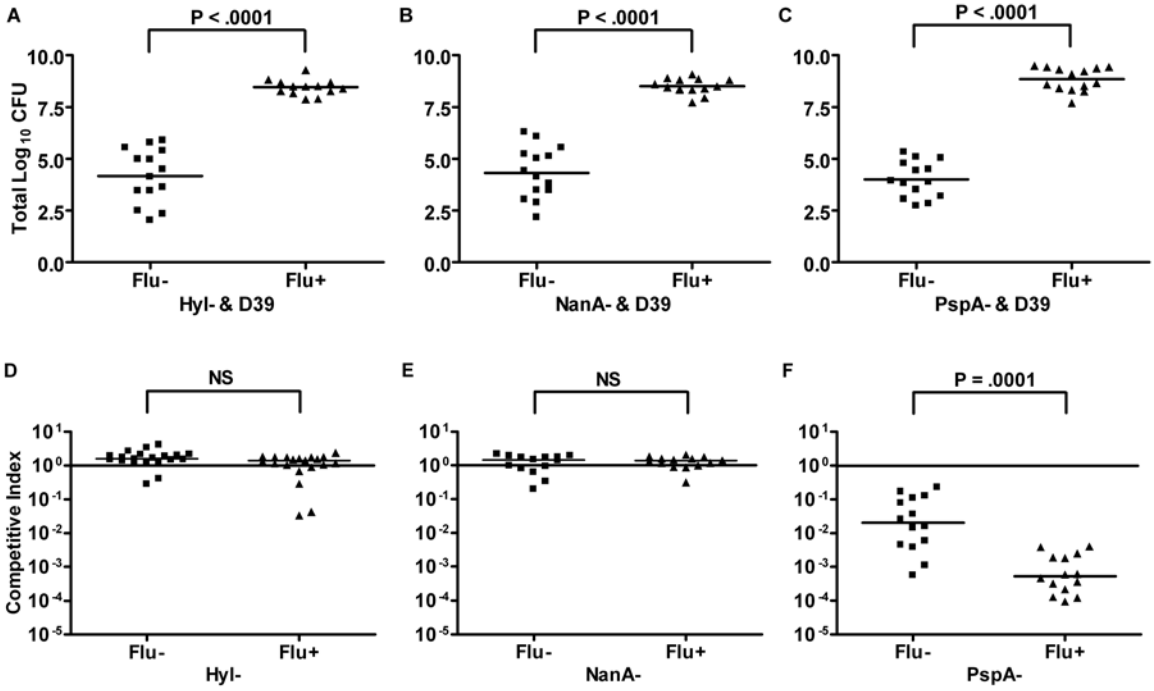

Figure 2.2. Total CFU and Competitive Indices of Hyl-, NanA- and PspA- Recovered from Mouse Lung 24 h following Pneumococcal Infection. Total CFU recovered from mouse lung 24 h following Hyl- & D39 (A), NanA- & D39 (B) or PspA- & D39 (C) infection. Mice received approximately 5×10^5 CFU of the D39 parental strain concurrently with approximately 5×10^5 CFU of Hyl-, NanA- or PspA- mutant strain (1×10^6 CFU total) day 6 post-influenza (▲) or sham treatment (■). Competitive indices of Hyl- (D), NanA- (E) or PspA- (F) growth from panels A, B and C, respectively, in mice with (▲) or without (■) prior influenza infection. Horizontal bars represent group medians. Data presented were pooled from at least two independent replicates and were analyzed with a two-tailed Mann-Whitney.

PspA- mutant displayed growth attenuation in both mice with and without antecedent influenza infection (Fig. 2.2 F). The median CI value of PspA- in mice without prior flu infection was 0.021, representing a 47-fold reduction in fitness relative to the D39 strain. The median CI value for PspA- in influenza-infected mice was 0.00053, representing a greater than 1800-fold reduction in relative growth of the PspA- mutant which was highly significant compared to PspA- growth in mice without prior flu infection.

Reduction of Primary and Secondary
Pneumococcal Infections with D39,
WU2 and TIGR4 by PspA Immunization

Mice receiving influenza again displayed visible symptoms of infection on day 6 post-influenza infection and had $1 \times 10^5 – 1 \times 10^6$ PFU in their lung, whereas sham treated mice appeared healthy and did not have detected virus (Fig. 2.3). PspA-specific IgA and IgG were detected in the sera of PspA-immunized mice but not in the sera of PBS or adjuvant-only treated controls (Fig. 2.4).

At 24 h following primary D39 challenge, PspA-immunized mice exhibited significant 15-fold and 14-fold reductions in mean bacterial titers when compared to

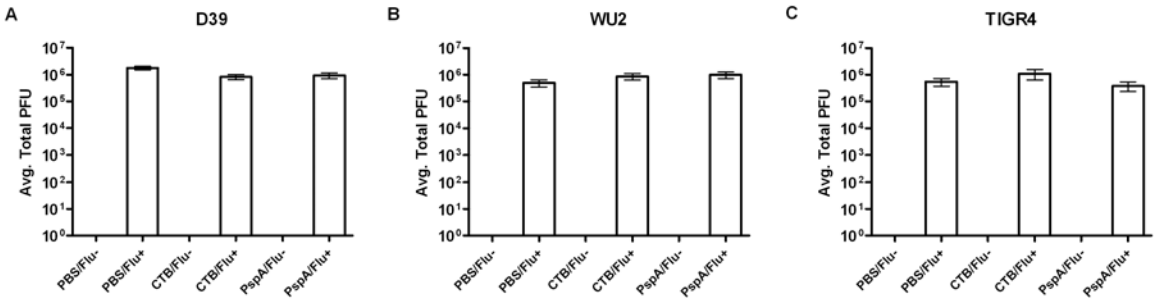

Figure 2.3. Average Total PFU Recovered from Lungs of Mice Immunized Intranasally with PspA 24 h Post-Pneumococcal Challenge. Following PBS-only (PBS), CTB-only (CTB) or CTB and PspA (PspA) immunization, mice received either 400 PFU PR8 influenza (Flu+) or sham infection (Flu-). Six days post-influenza or sham infection, all mice received 1×10^6 CFU D39, WU2 or TIGR4. Columns represent group means with error bars indicating SEM. Data presented were pooled from at least two independent replicates.

PBS-treated and adjuvant-treated controls, respectively (Fig. 2.5 A). In influenza-

infected groups receiving secondary D39 challenges, PspA-immunized mice exhibited significant 35-fold and a 53-fold reductions compared to PBS-treated and adjuvant-

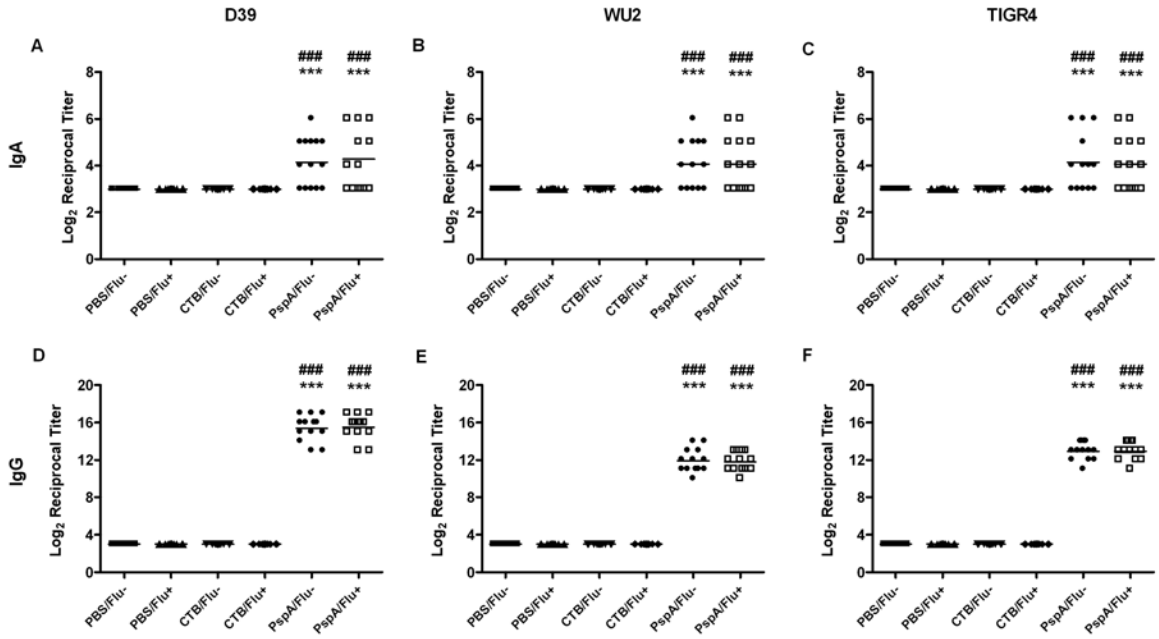

Figure 2.4. IgA and IgG Serum Responses from Mice Receiving Intranasal PspA Immunization as Determined by ELISA. Mice received intranasal immunizations with PBS-only (PBS), adjuvant-only (CTB) or in combination with PspA protein (PspA). These treatment groups were then divided into subgroups receiving 400 PFU influenza (Flu+) or sham PBS (Flu-) infections. Six days post-influenza or sham infection, all mice received 1×10^6 CFU D39, WU2 or TIGR4. Significance is as follows: For all panels, ###, $P < 0.001$ comparing PspA-treated groups to CTB-treated groups; ***, $P < 0.001$ comparing PspA-treated groups to PBS-treated groups. Horizontal bars represent group means. Data presented were pooled from at least two independent replicates and were analyzed by two-way ANOVA with Bonferroni post test.

treated controls, respectively (Fig. 2.5 A). No significant differences were detected between PBS-treated and CTB-treated groups in either primary or secondary D39 challenges. Despite significant reduction in secondary D39 infection in the PspA-immunized group, mean bacteria titers for this group remained significantly higher than

PBS-treated, CTB-treated and PspA-immunized mice receiving primary D39 infection when analyzed by one-way ANOVA with Bonferroni post-test (Fig. 2.5 A; all $P < 0.001$).

As albumin and LDH levels in BALF have been shown to be specific markers of lung pathology [288], they were measured to assess mitigation of lung damage by PspA immunization after 24 h primary and secondary pneumococcal infections. Albumin concentrations and LDH activity in BALF recovered from mice receiving primary D39 infection were very low, suggesting that D39 infection alone does not cause extensive

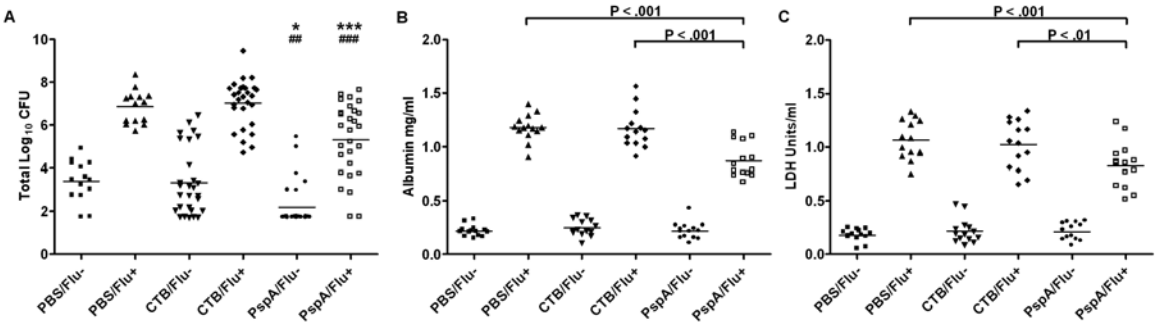

Figure 2.5. D39 Bacteria Titers (A), Albumin (B) and LDH (C) Concentrations Recovered from Lungs of Mice Immunized Intranasally with PspA 24 h Post-Pneumococcal Challenge. Following PBS-only (PBS), CTB-only (CTB) or CTB and PspA (PspA) immunization, mice received either 400 PFU PR8 influenza (Flu+) or sham infection (Flu-). Six days post-influenza or sham infection, all mice received 1×10^6 CFU D39. Significance values for panel A: *, $P < 0.05$ PspA/Flu- vs. PBS/Flu-; ***, $P < 0.001$ PspA/Flu+ vs. PBS/Flu+; ##, $P < 0.01$ PspA/Flu- vs. CTB/Flu-; ###, $P < 0.001$ PspA/Flu+ vs. CTB/Flu+. CTB groups were not significantly different than PBS groups when compared between respective influenza treatments in panels A, B or C. All Flu+ groups yielded significantly higher ($P < 0.001$) bacteria titers and lung damage marker concentrations when compared to Flu- groups within their respective immunization treatments in panels A, B and C with a single exception in panel C where PspA/Flu+ vs PspA/Flu- yielded a significance of $P < 0.01$. Horizontal bars represent group means. Data presented were pooled from at least two independent replicates and were analyzed by two-way ANOVA with Bonferroni post test.

lung damage after 24 h. Thus, no significant differences were detected in albumin or LDH levels between groups receiving primary D39 infections (Fig. 2.5 B and C.). Mice receiving D39 infection following influenza all exhibited significantly increased levels of albumin compared to their respective immunization treatment group lacking prior influenza infection (Fig. 2.5 B; all $P < 0.001$). Similar results were observed for LDH levels (Fig. 2.5 C; PBS and CTB, $P < 0.001$, PspA, $P < 0.01$). Whereas both lung damage markers were pronounced in secondary D39 infections, PspA immunization significantly reduced levels of albumin (Fig. 2.5 B) and LDH (Fig. 2.5 C).

To determine whether PspA immunization reduces infections caused by strains of other serotypes, the immunization and challenge experiments were repeated for strains WU2 (serotype 3) and TIGR4 (serotype 4). PspA immunization significantly reduced recovered bacteria titers 3100-fold and 2800-fold from primary WU2 infection when compared to PBS-treated and CTB-treated controls, respectively (Fig. 2.6 A). PspA-immunized mice also exhibited significant 62-fold and 91-fold reductions in secondary WU2 titers relative to PBS-treated and CTB-treated controls, respectively (Fig. 2.6 A). No significant differences were detected between PBS-treated and CTB-treated control groups in either primary or secondary WU2 challenges. PspA-immunized mice receiving a secondary WU2 challenge following influenza again exhibited significantly greater WU2 titers than PBS-treated, CTB-treated and PspA-immunized groups receiving WU2 infection alone when analyzed by one-way ANOVA with Bonferroni post-test (Fig. 2.6 A; all $P < 0.001$). Primary WU2 infections did not yield substantial albumin or LDH levels

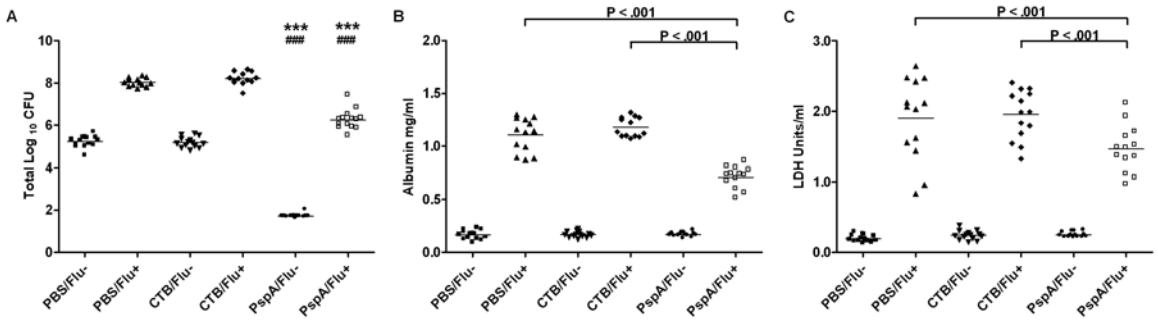

Figure 2.6. WU2 Bacteria Titers (A), Albumin (B) and LDH (C) Concentrations Recovered from Lungs of Mice Immunized Intranasally with PspA 24 h Post-Pneumococcal Challenge. Following PBS-only (PBS), CTB-only (CTB) or CTB and PspA (PspA) immunization, mice received either 400 PFU PR8 influenza (Flu+) or sham infection (Flu-). Six days post-influenza or sham infection, all mice received 1×10^6 CFU WU2. Significance values for panel A: ***, $P < 0.001$ PspA/Flu- vs. PBS/Flu- and PspA/Flu+ vs. PBS/Flu+; ###, $P < 0.001$ PspA/Flu- vs. CTB/Flu- and PspA/Flu+ vs. CTB/Flu+. CTB groups were not significantly different than PBS groups when compared between respective influenza treatments in panels A, B or C. All Flu+ groups yielded significantly higher ($P < 0.001$) bacteria titers and lung damage marker concentrations when compared to Flu- groups within their respective immunization treatments in panels A, B and C. Horizontal bars represent group means. Data presented were pooled from two independent replicates and were analyzed by two-way ANOVA with Bonferroni post test.

in recovered BALF while secondary WU2 infections yielded significantly greater levels of both lung damage markers (Fig. 2.6 B; all $P < 0.001$, and C; all $P < 0.001$). PspA immunization resulted in a significant reduction in albumin concentrations in mice receiving secondary WU2 infection relative to both PBS-treated and CTB-treated controls (Fig. 2.6 B) with parallel results observed for LDH concentrations (Fig. 2.6 C). Primary infection with the TIGR4 pneumococcal strain was also found to be significantly reduced in PspA immunized mice 135-fold and 424-fold compared to PBS-treated and CTB-treated controls, respectively (Fig. 2.7 A). PspA immunization significantly reduced lung burdens 12- and 6-fold in mice receiving secondary TIGR4 infections

following influenza when compared to PBS-treated and CTB-treated controls (Fig. 2.7 A). No significant differences were detected between PBS-treated and CTB-treated control groups in either primary or secondary TIGR4 challenges. Again, one-way ANOVA with

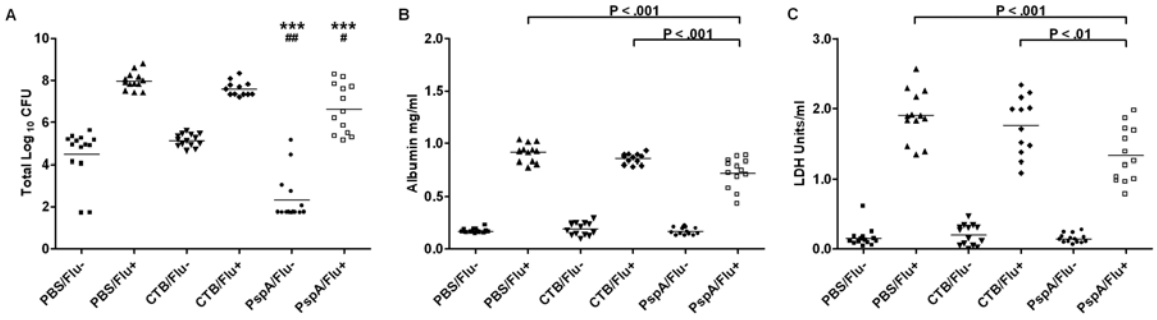

Figure 2.7. TIGR4 Bacteria Titers (A), Albumin (B) and LDH (C) Concentrations Recovered from Lungs of Mice Immunized Intranasally with PspA 24 h Post-Pneumococcal Challenge. Following PBS-only (PBS), CTB-only (CTB) or CTB and PspA (PspA) immunization, mice received either 400 PFU PR8 influenza (Flu+) or sham infection (Flu-). Six days post-influenza or sham infection, all mice received 1×10^6 CFU TIGR4. Significance values for panel A: ***, $P < 0.001$ PspA/Flu- vs. PBS/Flu- and PspA/Flu+ vs. PBS/Flu+; ##, $P < 0.01$ PspA/Flu- vs. CTB/Flu-; #, $P < 0.05$ PspA/Flu+ vs. CTB/Flu+. CTB groups were not significantly different than PBS groups when compared between respective influenza treatments in panels A, B or C. All Flu+ groups yielded significantly higher ($P < 0.001$) bacteria titers and lung damage marker concentrations when compared to Flu- groups within their respective immunization treatments in panels A, B and C with a single exception in panel C where PspA/Flu+ vs PspA/Flu- yielded a significance of $P < 0.01$. Horizontal bars represent group means. Data presented were pooled from two independent replicates and were analyzed by two-way ANOVA with Bonferroni post test.

Bonferroni post-test revealed that PspA-immunized mice with prior influenza receiving secondary TIGR4 infection exhibited significantly greater bacterial lung burdens than PBS-treated ($P < 0.001$), CTB-treated ($P < 0.01$) and PspA-immunized ($P < 0.001$) mice receiving primary TIGR4 challenges (Fig. 2.7 A). As with D39 and WU2, primary

infection with TIGR4 did not induce substantial albumin or LDH levels in recovered BALF, however, significant increases were observed when comparing influenza-infected mice receiving TIGR4 to mice receiving primary TIGR4 infection (Fig. 2.7 B; all $P < 0.001$, and C; PBS and CTB, $P < 0.001$, PspA, $P < 0.01$). Albumin levels of PspA-immunized mice were significantly reduced when compared to PBS-treated and CTB-treated controls receiving secondary TIGR4 infection (Fig. 2.7 B) with similar results observed for LDH levels (Fig. 2.7 C).

Discussion

Influenza infection is known to denude respiratory epithelium exposing pneumococcal ligands on the basement membrane [43], while bacterial hyaluronidases are believed to facilitate invasion through targeting of hyaluronic acid, a constituent of the extra cellular matrix [275, 289]. We hypothesized that herein may lay an opportunity for enhanced application of pneumococcal Hyl. A strong correlation exists between clinical isolates and hyaluronidase production, specifically in pneumococcal meningitis [290]. A serotype 19F Hyl- mutant is attenuated in a pneumonia model [138] and whereas a serotype 6 Hyl- mutant is attenuated in intraperitoneal infection [139], a D39 mutant was not [107]. Our results show that growth of the Hyl- mutant of D39 in the lung is not attenuated in mice with or without prior influenza infection and while these results do not indicate a secondary infection contribution for Hyl, they may support suggestions that the hyaluronidase virulence role is serotype specific [138, 139].

NanA- relative fitness was not compromised in either primary or secondary pneumococcal infection. Lack of attenuation in the primary challenge was a surprise to us given NanA cleaves terminal sialic acid from host glycoconjugates decorating respiratory epithelium where this action enhances colonization [142, 291]. We suspected contribution of pneumococcal NanA to colonization would be superseded by the influenza neuraminidase in secondary bacterial challenge but that this virulence factor might then reveal some novel application, however, secondary competitive growth of the NanA- mutant failed to support this hypothesis. NanA- mutants of D39 reported little to no attenuation in intraperitoneal challenges [107, 273] in which colonization is not critical for infection. In intranasal challenges, NanA of serotype 2 strains is important for virulence in two reports [102, 142] but not in another [273]. It has been suggested that among methodological differences, susceptibility of mouse strains employed might explain dissimilar results [273]. This may be the case in our use of C57Bl/6 mice rather than the BALB/c employed by others. Furthermore, our results might also reflect our competitive growth model where the NanA- mutant and D39 strains were co-administered in a mixed inoculum. NanA of the wild-type D39 strain might modify the surface of the respiratory tract for both D39 and NanA- bacteria. If this is the case, the competitive growth method may serve to better identify those cis virulence factors and better potential vaccine targets, such as PspA, required of each individual bacterium.

PspA is well established as both a crucial virulence factor [101, 107, 117, 277] and a broadly protective antigen in a variety of immunization and challenge models [132, 230, 245-247, 278]. Given the lower respiratory tract inflammatory response

accompanying influenza infection, we hypothesized this virulence factor's function in inhibiting complement mediated clearance would become critical in a secondary pneumococcal infection. Fitness of the PspA- mutant was reduced 47-fold relative to D39 in the absence of influenza infection in agreement with observations that PspA is required for pulmonary *S. pneumoniae* infection [101]. Additionally, in support of a significant contribution of PspA to secondary pneumococcal infection, we observed an 1800-fold reduction in relative PspA- growth in mice with prior influenza infection.

PspA immunization experiments further supported a significant contribution of PspA to secondary infection. Intranasal PspA immunization significantly reduced numbers of D39, WU2 and TIGR4 in the lungs of mice both with and without prior influenza infection when compared to controls. Consistent with these results and a unique role for PspA in secondary infections, BALF levels of LDH and albumin were significantly reduced in PspA-immunized groups but not in PBS-treated or CTB-treated controls receiving D39, WU2 and TIGR4 infections following prior influenza infection. Despite substantial sequence variability, both cross-reactivity and protection have been observed between immunized sera and heterologous PspA proteins [131, 132]. The rPspA/Rx1 (family 1, clade 2 and type 25) PspA antigen used in this present study has been shown to induce immune mouse serum cross-reactive with PspA proteins present on D39, WU2 and EF3296 pneumococcal strains [17] where the N-terminal region of the EF3296 PspA protein is identical to that of TIGR4 [292]. Additionally, it has been shown that immunization with the D39 type 25 PspA can provide protection against lethal challenges with the D39 and WU2 strains and perhaps at least some, albeit not

statistically significant, protection against EF3296 [129]. While this earlier work may support our observation of rPspA/Rx1 immunization reducing 24 h lung titers in primary and secondary infections with the D39, WU2 and TIGR4 strains, another study has specifically assessed this antigen with respect to protection against WU2 and TIGR4 [292]. In this prior study, the JAS218 PspA/Rx1 fragment elicited protection against lethal challenge with the family 1 bearing WU2 strain but not the family 2 bearing TIGR4. The observations we present here may again reflect the different methodologies and endpoints employed in that, whereas our model is examining the ability of a PspA antigen to reduce pneumococcal infection in the respiratory compartment alone and at a defined endpoint early in primary and secondary challenges, the aforementioned study is assessing this PspA antigen in its capacity to protect against a complex systemic disease outcome. With this in mind, we would additionally suggest, that while informative within the context of our model's narrow temporal and tissue focus, observations we present here may not serve the extrapolation required for comparison to studies examining protection against disease resulting from established infections, including those assessing the protective efficacy of current pneumococcal vaccines. Our focus on a 24 h endpoint in this study reflects our belief that a vaccine developed to effectively prevent pneumococcal infection would likewise serve to limit pneumococcal disease and that this distinction between early and later events may prove particularly relevant to secondary pneumococcal infection following influenza infection. However, while effective in limiting early primary and secondary infection in the lung compared to controls, PspA immunization was not sufficient to overcome influenza-induced

susceptibility when comparing primary and secondary D39, WU2 and TIGR4 lung titers in PspA-treated animals. Despite these findings, that pneumococcal growth in the influenza-infected lung can be significantly reduced by a targeted humoral response despite presumed virally-induced phagocyte deficiencies is encouraging. Furthermore, it is probable, given previous observations [120, 230, 246], that combination of different PspA clades with other pneumococcal virulence factors into a single protein vaccine would significantly increase overall efficacy against both primary pneumococcal infection and a synergistic secondary pneumococcal infection following prior influenza infection.

Recent analyses of the 1918 Spanish influenza pandemic indicated the unusually high mortality associated with this pandemic was likely the result of increased susceptibility to secondary bacterial pathogens, including *S. pneumoniae* [11, 12]. We strongly believe that identification of pneumococcal vaccine candidates which offer protection against both pneumococcal infection alone and the significantly greater threat of pneumococcal infection following influenza infection may serve to reduce expected increases in morbidity and mortality attributable to secondary *S. pneumoniae* infections during what many believe to be inevitable future influenza pandemic events.

CONTRIBUTIONS OF TWO CELL WALL-ANCHORED PROTEINS TO PRIMARY PNEUMOCOCCAL INFECTION AND SECONDARY PNEUMOCOCCAL INFECTION FOLLOWING INFLUENZA INFECTION

Introduction

Streptococcus pneumoniae is a ubiquitous human pathogen with over 90 serotypes identified. Infection with this bacterium may produce relatively moderate disease, such as sinusitis and otitis media, but can also result in life-threatening invasive disease including pneumonia and meningitis. In addition to the evolution of antibiotic resistance, a reduced efficacy of pneumococcal vaccines targeting capsular polysaccharides has been observed in the very young and the elderly populations with the highest risk of pneumococcal infections [293-295]. Protein conjugate vaccines have been shown to be more effective in preventing disease and carriage; however, they have also been associated with increasing prevalence of carriage and disease attributable to non-targeted serotypes [296]. For this reason, conserved pneumococcal surface exposed proteins have received considerable attention as putative virulence factors and potential targets for development of vaccines with broad protection [98, 226, 273, 274].

Genomic analysis of the nonencapsulated pneumococcal R6 strain, a derivative of the virulent type 2 D39 strain, revealed that in addition to known cell wall-linked surface-exposed virulence factors (i.e. NanA, Hyl and PrtA), a number of hypothetical proteins bearing the LPXTG sortase motif are also present [134]. Among these putative cell wall-linked proteins are Spr0075, Spr0328, Spr0400, Spr1345, Spr1403, Spr1652 and Spr1806.

Spr0075 has been detected in a pneumococcal phage-display library using immune mouse sera and convalescent human sera and has been shown to be surface-exposed and antigenic [297]. Spr1345 is a homolog of the TIGR4 strain Sp1492 mucin-binding protein and, like Spr0075, is surface-exposed and antigenic in mice [298]. However, the contributions of these two proteins to *S. pneumoniae* virulence have not been assessed.

Another consideration in development of an efficacious pneumococcal vaccine is the increased susceptibility to pneumococcal infection following influenza infection [299] which is known to significantly increase morbidity and mortality [10]. Influenza infection has been shown to compromise both the respiratory mucosal barrier and innate cellular immunity [43, 258, 262, 267, 270]. Recently, we have reported a contribution of pneumococcal surface protein A (PspA) to *S. pneumoniae* virulence in influenza-infected mice where we further showed immunization with this protein resulted in significantly reduced secondary bacterial burdens after 24 h [300]. To our knowledge, this is the only pneumococcal protein vaccine candidate to have been examined specifically for its ability to protect against secondary pneumococcal infection following influenza. As virulence contributions of the Spr0075 and Spr1345 proteins remain unclear, it is yet further unknown what, if any, contribution they might make to the far more severe scenario of secondary pneumococcal infection following influenza.

We hypothesized that pneumococcal proteins at the *S. pneumoniae*-host interface are potential candidates for virulence contributions and as potential vaccine targets. We further hypothesized that either or both of these traits may change between the contexts of a healthy respiratory tract and that of one previously infected with influenza. To

address these questions, insertion-duplication mutants lacking the ability to express the Spr0075 (Spr0075-) and Spr1345 (Spr1345-) proteins were constructed and competitive growth of these mutants relative to the parental D39 strain was assessed in mice with or without antecedent influenza infection. Additionally, the ability of intranasal immunization with N-terminal fragments of the Spr0075 and Spr1345 proteins to reduce primary and secondary pneumococcal infections was also examined. Both Spr0075- and Spr1345- mutants displayed severe attenuation in primary pneumococcal infection and secondary pneumococcal infection following prior influenza infection. We additionally found that immunization with recombinant Spr0075 and Spr1345 proteins both significantly reduced D39 numbers recovered from lungs of mice receiving a primary pneumococcal challenge; however, only immunization with Spr0075 significantly reduced D39 numbers in mice secondarily challenged with *S. pneumoniae* following established influenza infection.

Materials and Methods

Mice

Pathogen free 6-8 week old female C57BL/6 mice obtained from the NCI-Frederick Animal Production Area, Frederick, MD were maintained in ventilator cages in the Montana State University Animal Resource Center. All animal procedures were approved by the MSU Institutional Animal Use and Care Committee.

Bacteria Strains and
Insertion-Duplication Mutagenesis

Virulent type 2 *S. pneumoniae* strain D39 was cultured at 37°C/5% CO_2 in Todd Hewitt broth with 0.5% yeast extract (THY). Cultures were harvested in mid to late log-phase and aliquots containing 20% glycerol were snap frozen in liquid nitrogen and stored at -80ºC. To determine stock CFU concentrations, 10-fold serial dilutions of sample aliquots were plated on 5% sheep blood tryptic soy agar (TSA) plates containing 25μg/ml neomycin (to inhibit gram-negative organisms).

Insertion-duplication mutagenesis (IDM) was used to construct mutants of D39 that are defective in the *spr0075* (Spr0075-) and *spr1345* (Spr1345-) genes. An internal 300-bp fragment of each gene was amplified from the D39 parental strain using primers designed to include SphI or BamHI restriction sites (see Table 1). The PCR products were inserted into the pEVP3 suicide vector (generously provided by Dr. Donald Morrison) [301] at the SphI and BamHI sites. The inserts of the resulting recombinant plasmids were sequenced to confirm the insertion. The plasmid DNA was introduced into *S. pneumoniae* D39 as previously described [302]. Briefly, early log phase D39 cultures were diluted 1:10 in brain-heart infusion broth (BHIB) containing competence stimulating peptide-1 (again, generously provided by Dr. Donald Morrison) at a final concentration of 0.1 μg/ml, 10 mM glucose and 10% horse serum. Cultures were incubated for 15 min at 37° C/5% CO_2 after which ~4 μg plasmid DNA was added. Cultures were then incubated for 1 h before selective plating on 5% sheep blood TSA plates supplemented with 5μg/ml chloramphenicol. Resulting transformants were then screened via PCR using IDM confirmation primers designed to flank the internal IDM

targeting fragment of the *spr0075* or *spr1345* gene to identify insertional mutation of each gene (Table 3.1). An additional primer was designed, in combination with the reverse IDM confirmation primers, to produce a fragment bridging the pEVP3 vector to

Table 3.3. Primers Employed in the Construction and Screening of Insertion-Duplication Mutants Targeting the *spr0075* and s*pr1345* Genes. Also listed are primers employed in the construction of the pSpr0075 and pSpr1345 expression vectors used to express the recombinant Spr0075 and Spr1345 proteins for immunizations.

Primer Set	Primer	Primer 5'→3'[a, b]	ORF Position (Accession #)
Spr0075 IDM Construction	F	*NGCATGC*TCGGATAATGCTCCTTGGTC	616 – 635
	R	*NGGATCC*CAACGGCTTTTTCGACAGTT	897 – 916 (AE008391)
Spr0075 IDM Confirmation	F	CTGCCGGTGTAAACCAAGTA	539 – 558
	R	CACCTGCGAAGAGTTCGTAT	1014 - 1033 (AE008391)
Spr1345 IDM Construction	F	*NGGATCC*ACAACAGGAAACTGGGACGA	172 – 191
	R	*NGCATGC*AGTCGGAACTTCTGGGTCTG	455 – 474 (AE008504)
Spr1345 IDM Confirmation	F	GGTCACGGGAGTTGTAACCT	141 – 160
	R	CCAGCACTAGCCAAGGTAGC	526 – 545 (AE008504)
pEVP3 IDM Confirmation	F	AAAGTTCATTTGATATGCCTCCT	Ref. [301]
Spr0075 Protein Expression	F	*NNNCATATG*GATGTGGTCAATCCGACCCCAG	127 – 148
	R	*NNNGAATTC*GGTGCGAAGAGTTCGTATGGAATCACATG	1003 – 1029 (AE008391)
Spr1345 Protein Expression	F	*NNNCATATG*GTGGTTCCCAAAACAGCAACCT	1 – 22
	R	*NNNGAATTC*GGTGTGTTTGGCAATTCCTCTCTCTTA	486 – 510 (AE008504)

a - IDM construction primers *italics*: 5' SphI restriction site on forward primer; 5'BamHI restriction site on reverse primer

b - Protein expression primers *italics*: 5' NdeI restriction site on forward primer; 5'EcoRI restriction site on reverse primer. Underlined bases added to maintain reading frame.

the flanking genomic sequence and thereby confirming targeted IDM vector insertion. Primers targeting the pneumolysin (Ply) gene were also employed as positive control for DNA extraction. Mutants were then cultured at 37°C/5% CO_2 in THY broth with the addition of 5μg/ml chloramphenicol. Cultures were then stored and CFU concentrations determined as with the D39 strain. Growth curves for Spr0075- and Spr1345- IDM mutants cultured in THY media supplemented with 5μg/ml chloramphenicol were measured to assess whether these mutants have in vitro growth defects.

As insertion-duplication mutants are subject to reversion, *in vivo* stability was assessed by infecting mice (N = 4-5) with ~1×10^8 CFU of the individual Spr0075- and Spr1345- mutants. Lung homogenates recovered after 24 h were serially diluted and plated on both selective and non-selective blood agar plates. Bacteria numbers obtained from selective plating were divided by those recovered from non-selective plating to assess increases in non-selected populations due to revertants. Mean values calculated for the Spr0075- (.9882) and Spr1345- (.9780) mutants were not significantly different than the expected value of 1 when analyzed by one-sample t-test ($\alpha = 0.05$) indicating reversion rates were not confounding during *in vivo* growth through 24 h. Furthermore, while insertion-duplication mutants have been routinely employed [101, 102, 142], there exists the possibility of polar effects.

Infection Model

Groups of 5-7 mice were infected via oropharyngeal aspiration [283] with 400 PFU of the H1N1 mouse adapted influenza A/Puerto Rico/8/34 (PR8) influenza virus in 50 μl PBS or received a sham infection of PBS alone. Briefly, mice under isoflurane gas

anesthesia were suspended from a rubber band by their upper incisors on a 60° incline board. Using forceps, the tongue was pulled to the side of the open mouth after which the viral inocula was delivered to the distal oropharynx. With the tongue extended, mice were incapable of swallowing and the inoculum was aspirated into the lower respiratory tract. On day 6 post-influenza or sham infection, mutant and wildtype bacteria stocks were thawed, washed in PBS and diluted to the desired inoculum concentrations. Prior to mouse infections, inocula were plated on both selective and non-selective blood agar plates to determine mutant to wildtype ratios. Mice were then infected via oropharyngeal aspiration with a combined inocula of 5×10^6 CFU of the D39 wild type *S. pneumoniae* strain and 5×10^6 CFU of the Spr0075- or Spr1345- IDM mutants (total of 1×10^7 CFU) in a total volume of 50µl PBS. Twenty-four h post-pneumococcal infection, mice were euthanized, and lungs were lavaged with 5 ml sterile PBS. Lungs were then harvested, homogenized in recovered bronchioalveolar lavage fluid (BALF) and aliquots containing 20% glycerol were then snap frozen in liquid nitrogen and stored at -80º C.

To assess relative virulence factor mutant growth, 10-fold serial dilutions of lung homogenates were plated in triplicate on both selective (5 µg/ml chloramphenicol) and non-selective 5% sheep blood TSA agar plates containing 25µg/ml neomycin followed by culture at 37º C for 24 h. CFU numbers were then multiplied by total homogenate volumes to yield total CFU recovered. Competitive indices (CI) were obtained by dividing the number of recovered mutants from the selective plating by the total number of recovered bacteria from the non-selective plating and then dividing this figure by the ratio of mutants/total bacteria present in the inocula as described previously [284]. To

confirm influenza infection and quantify PFU, lung homogenates were 10-fold serially diluted and plaque assay performed on Madin-Darby canine kidney (MDCK) cells as previously described [57]. Data presented is pooled from two independent experiments.

Cloning, Expression and
Purification of Spr0075 and Spr1345

PCR products encoding the Spr0075 fragment of amino acids 43-296 (~36 kD) and the Spr1345 fragment of amino acids 1-170 (~23 kD) were digested with NdeI and EcoRI and then ligated to vector pET-21b (Novagen) at the NdeI and EcoRI sites to yield pSpr0075 and pSpr1345 (Table 3.1). Ligation of the Spr0075 and Spr1345 PCR fragments into this expression vector results in the addition of a 6×His tag at the C-terminus of the recombinant proteins. The gene clones were sequenced to confirm reading frame and rule out spurious mutations.

The recombinant Spr0075 and Spr1345 fragments were expressed in *E. coli* strain BL21 (DE3) containing pSpr0075 and pSpr1345, respectively. The BL21 strains were grown at 37°C in 3 L of Luria-Bertani broth supplemented with 100 mg ampicillin/liter to OD_{600} of 0.5, and 0.5 mM IPTG was added to induce protein production. Twelve hours later, bacteria were harvested by centrifugation. The bacterial pellet was resuspended in 40 ml of 20 mM Tris-HCl, pH 8.0 and sonicated on ice for 15 min. The sample was centrifuged at 10,000 rpm for 10 min. For purification of the Spr0075 fragment, the supernatant obtained was brought to 0.5 M NaCl and loaded onto a Ni-NTA column (2.5 × 3.0 cm). The column was washed with 50 ml of 0.5 M NaCl in 20 mM Tris-HCl, pH 8.0, and eluted with 100 mM imidazole in 0.5 M NaCl in 20mM Tris-HCl. All fractions

containing the protein were combined and dialyzed against 3.0 L of 20 mM Tris-HCl pH 8.0. The dialyzed fractions were then loaded onto a DEAE column (2.5 × 4.5 cm), washed with 80 ml of 20 mM Tris-HCl, pH 8.0, and eluted with a 150-ml gradient of 0-200 mM NaCl in 20 mM Tris-HCl. Fractions containing the protein with >70% purity were pooled and brought to 1.2 M $(NH_4)_2SO_4$. The sample was then loaded onto a phenyl column (1.5 × 4 cm), washed with 50 ml of 1.2 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl, and eluted with a 120-ml gradient of 1.2-0 M $(NH_4)_2SO_4$. Fractions with > 90% purity were pooled, dialyzed against 20 mM Tris-HCl, and concentrated using a Millipore PL-10 filter device.

For purification of the Spr1345 fragment, the supernatant obtained was loaded onto a DEAE column (2.5 ×4 cm) and washed with 100 ml of 20 mM Tris-HCl, pH 8.0. The protein was eluted with a 100-ml increasing gradient of NaCl. Spr1345 was the first protein to come off the column at 40 mM NaCl. Fractions containing the protein with >70% purity were pooled, brought to 1.0 M $(NH_4)_2SO_4$ and loaded onto a phenyl column (2.5 × 4.0 cm). Protein was eluted with a 100-mL decreasing gradient of $(NH_4)_2SO_4$ starting with 1.0 M. Spr1345 came off the column immediately. Fractions with >85% pure protein were pooled, dialyzed, brought to 0.5 M NaCl and loaded onto a nickel column (3.0 × 2.5 cm). The column was washed with 50 ml of 0.5M NaCl in 20 mM Tris-HCl, pH 8.0. Protein was eluted with 60mL of 100 mM imidazole in 0.5 M NaCl in 20 mM Tris-HCl. Fractions containing the protein with >90% purity were pooled, dialyzed against 3 L of 20 mM Tris-HCl, pH 8.0, and concentrated using a Millipore PL-10 filter device.

Intranasal Immunization
with Spr0075 and Spr1345

To assess inhibition of primary and secondary pneumococcal growth by Spr0075 and Spr1345 immunization, mice under light isoflurane anesthesia received intranasal immunizations twice weekly for three weeks. Groups of 10-14 mice received 1 μg recombinant N-terminal His-tagged Spr0075 or Spr1345 proteins along with 4 μg cholera toxin B subunit (CTB, List Biological Laboratories) in 20 μl sterile PBS. Control groups received adjuvant alone. Mice receiving Spr0075 and Spr1345 proteins were immunized with adjuvant for two weeks then received only protein in the third week with adjuvant controls received only saline in the third week. Twenty days following immunization, mice were divided into subgroups (5-7 mice) receiving either 400 PFU PR8 influenza or sham PBS infection. Six days after influenza or sham infection, all mice received 1×10^6 CFU D39 pneumococci via intratracheal instillation. Twenty-four hours following bacteria inoculation, BALF and lungs were harvested. Viral and bacterial loads in lung were determined as described above. Spr0075-specific and Spr1345-specific IgG and IgA titers in sera were determined by ELISA. Data presented is pooled from two independent experiments.

For ELISA, high binding 96-well plates were coated with 1 μg/ml of either Spr0075 or Spr1345 in 0.05 M carbonate buffer, pH 9.6, for 3 h at 37ºC and then at 4ºC overnight. Plates were washed 3 times with wash buffer (0.005% Tween 20 in PBS) and non-specific binding sites were blocked with 5% nonfat dried milk in PBS at 37ºC for 1 h. After 3 washes, 2-fold serial dilutions of serum samples in wash buffer were added and

incubated at 37ºC for 1 h. Following 3 washes, alkaline phosphatase-conjugated, anti-mouse, isotype-specific secondary Ab (IgG, Sigma-Aldrich; IgA, Serotec) was added and incubated at 37ºC for 2 h. After 5 washes, *p*-nitrophenyl phosphate in diethanolamine buffer was added and incubated at 37ºC for 30 min. Endpoint titers were defined as the highest reciprocal dilution exhibiting absorbance (405 nm) above 0.100 OD units above negative controls [285].

Statistical Analysis

Analyses of one-sample t-test, two-tailed Mann-Whitney and two-way ANOVA with Bonferroni post-test were performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, California).

Results

Spr0075- and Spr1345- Mutant Virulence

To determine potential contributions of the Spr0075 and Spr1345 genes to *S. pneumoniae* virulence, the genes were inactivated by the insertion-duplication strategy in which the plasmid DNA containing pEVP3 and the internal fragment of the target gene was inserted into the respective gene, thereby disrupting the gene. The mutants were selected by chloramphenicol selective plating and confirmed by PCR analysis. One PCR reaction used the gene-specific primers located beyond both the ends of the duplicated internal fragment (Table 3.1). An ~400-bp PCR product was obtained when using the D39 DNA as template (Fig. 3.1, lanes 1 and 5), however, no PCR products were obtained

when the Spr0075- or Spr1345- mutant DNA were used as templates, due to the inefficiency of the reaction to amplify the long DNA fragments containing the ~6-kb

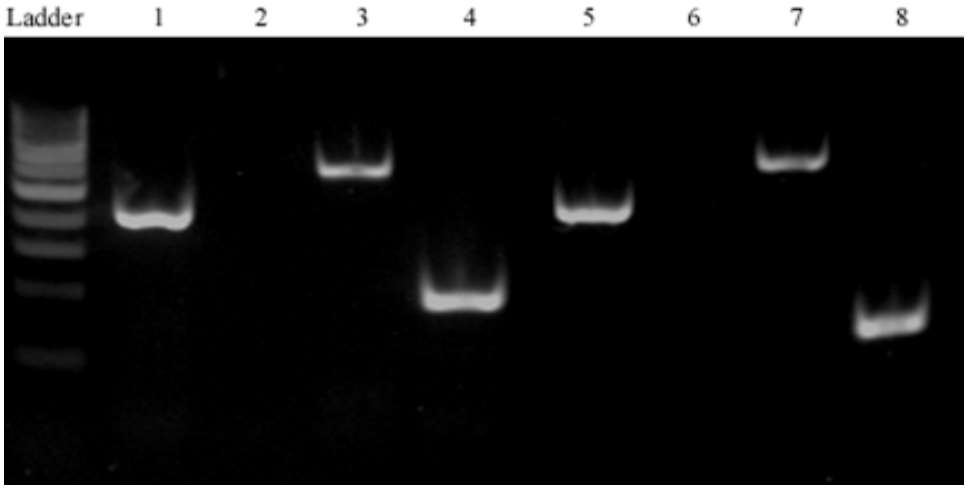

Figure 3.1. PCR Confirmation for Insertion-Duplication Mutagenesis of the *spr0075* and *spr1345* LPXTG-Bearing Pneumococcal Genes. Primers designed to flank the IDM targeting regions were used to amplify parental D39 and the Spr0075- and Spr1345- IDM mutants. Fragments of expected size were observed when using D39 as template (lanes 1 and 5) whereas plasmid insertion prevented amplification from Spr0075- (lane 2) and Spr1345- (lane 6) IDM mutants. An additional primer designed to anneal within the pEVP3 plasmid and allow amplification in conjunction with the reverse flanking region primers for Spr0075- (lane 3) and Spr1345- (lane 7) confirmed targeted plasmid insertions. An additional primer set amplifying a portion of the pneumococcal Ply gene was employed as positive control for DNA extractions (lanes 4 and 8).

suicide plasmids under the conditions employed (Fig. 3.1, lanes 2 and 6). In another PCR reaction, one of the primers used was vector-specific, and another gene-specific. In this case, expected ~700-bp PCR products were observed when the mutant DNA samples were used as template (Fig. 3.1, lanes 3 and 7), confirming the presence of the plasmid DNA. Primers amplifying a 200-bp fragment from the pneumococcal pneumolysin gene were also employed as positive controls for DNA extractions (Fig. 3.1, lanes 4 and 8).

These results confirm the interruption of the target genes in the Spr0075- and Spr1345- mutants.To determine whether the gene inactivation causes defects in growth, growth curves were measured for the Spr0075- and Spr1345- mutants. Both Spr0075- and Spr1345- displayed similar growth curves to the parental D39 strain reaching stationary phase between 12 and 14 hours (Fig. 3.2 A and B, respectively), indicating that both mutants have no *in vitro* growth defects. These results suggest that the Spr0075 and Spr1345 proteins do not participate in critical metabolic functions required for growth.

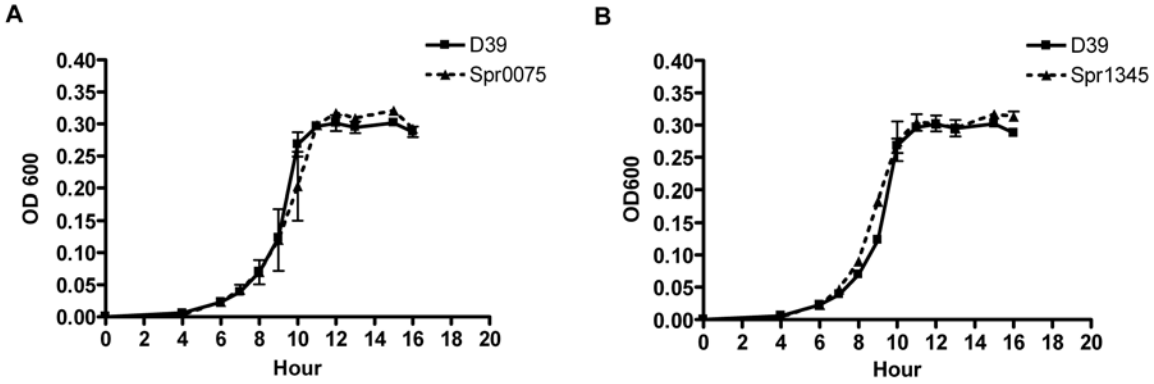

Figure 3.2. Growth Curves of Spr0075- and Spr1345- IDM Mutants and the D39 Parental Strain. Bacteria were grown in THY media supplemented, when appropriate, with 5 μg/ml chloramphenicol and growth assessed by spectrophotometer (OD 600).

Competitive Bacterial Growth in Mice
with and without Prior Influenza Infection

On day 6 following either influenza or sham infections, mice having received influenza displayed observable symptoms of infection including fur ruffling, a wasted appearance, clustering behavior and low activity. On day 6 following influenza or sham infection, both groups of mice were challenged with mixed IDM mutant/wildtype inocula and 24 h later, mice were euthanized and lungs harvested. Lungs of sham-infected mice

appeared healthy whereas those recovered from influenza-infected animals displayed hemorrhage and areas of red hepatization. Influenza-infected mice contained $6 \times 10^4 - 2 \times 10^5$ PFU in their lungs on day 7 after viral inoculation, whereas sham-infected mice revealed no detectable virus (Fig. 3.3).

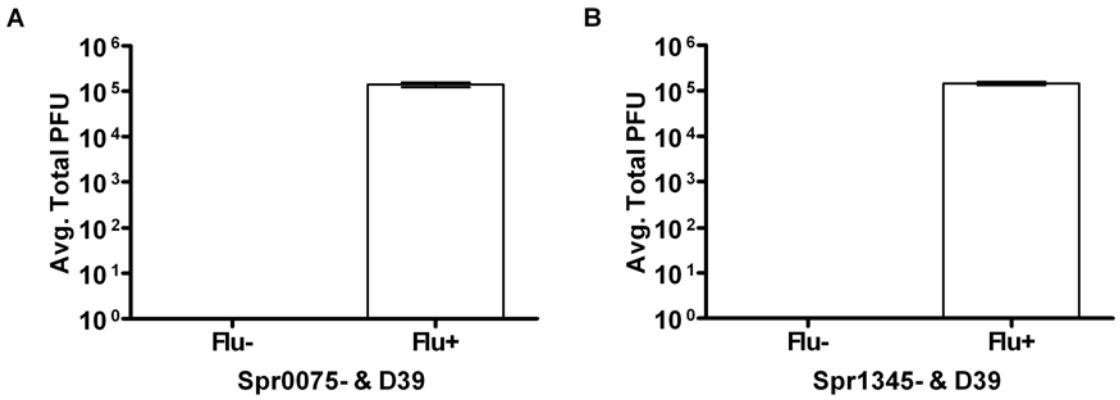

Figure 3.3. Average Total PFU Recovered from Mouse Lung 24 h following Spr0075- & D39 (A), or Spr1345- & D39 (B) Infection. Following inoculation with either a sham PBS infection (Flu-) or 400 PFU PR8 influenza (Flu+), mice received approximately 5×10^6 CFU of the D39 parental strain concurrently with approximately 5×10^6 CFU of Spr0075- or Spr1345- mutant strain (1×10^7 CFU total) day 6 post-influenza or sham treatment. Columns represent group means with error bars indicating SEM. Data presented were pooled from at least two independent replicates.

In agreement with previously reported observations of susceptibility to secondary pneumococcal infection following influenza infection, total bacteria (mutant and wildtype) recovered from influenza-infected mice were significantly higher than sham-infected controls for both the Spr0075- and D39 mixed inoculum and the Spr1345- and D39 mixed inoculum challenges. Total bacteria recovered from influenza-infected mice receiving a mixed Spr0075- and D39 inoculum displayed a median value 13,800-fold greater than that recovered from mice without prior influenza infection (Fig. 3.4 A).

Similarly, total bacteria recovered from influenza-infected mice having received a mixed Spr1345- and D39 inoculum exhibited a median value 360-fold greater than that recovered from mice without an antecedent influenza infection (Fig. 3.4 B).

Competitive growth is a commonly employed method for identifying fitness defects between co-administered bacterial strains [284, 286, 287]. To better resolve potential interactions of pneumococcal virulence factors with the increased susceptibility endowed by antecedent influenza infection, we chose this method to normalize lung deposition of mutant and wild type strains in influenza-infected mice given the synergistic nature of this superinfection can amplify small initial variation in bacteria numbers. We calculated CI values as a measure of mutant fitness and so where a CI value of 1 indicates both strains grew equally well, a CI value of 0.1 indicates a 10-fold reduction in mutant growth and/or survival relative to wild type.

Despite evident pneumococcal infection in mice receiving a primary mixed Spr0075- and D39 challenge (Fig. 3.4 A), selective plating failed to yield growth of IDM mutants lacking functional Spr0075 (Fig. 3.4 C). The Spr0075- mutant was recovered from the lungs of influenza-infected mice, however, this mutant displayed a greater than 4,100-fold reduction in growth relative to wildtype as evident in a median CI value of 0.00024. Mutants lacking functional Spr1345 were recovered from only 4 of 13 mice receiving a primary mixed Spr1345- and D39 challenge and exhibited a severe attenuation as evident in a median CI value of 0.00015 (Fig. 3.4 D). This translates into a greater than 6,400-fold reduction in mutants recovered when compared to recovery of the wildtype D39 strain. Recovery of Spr1345- mutants from mice with an antecedent

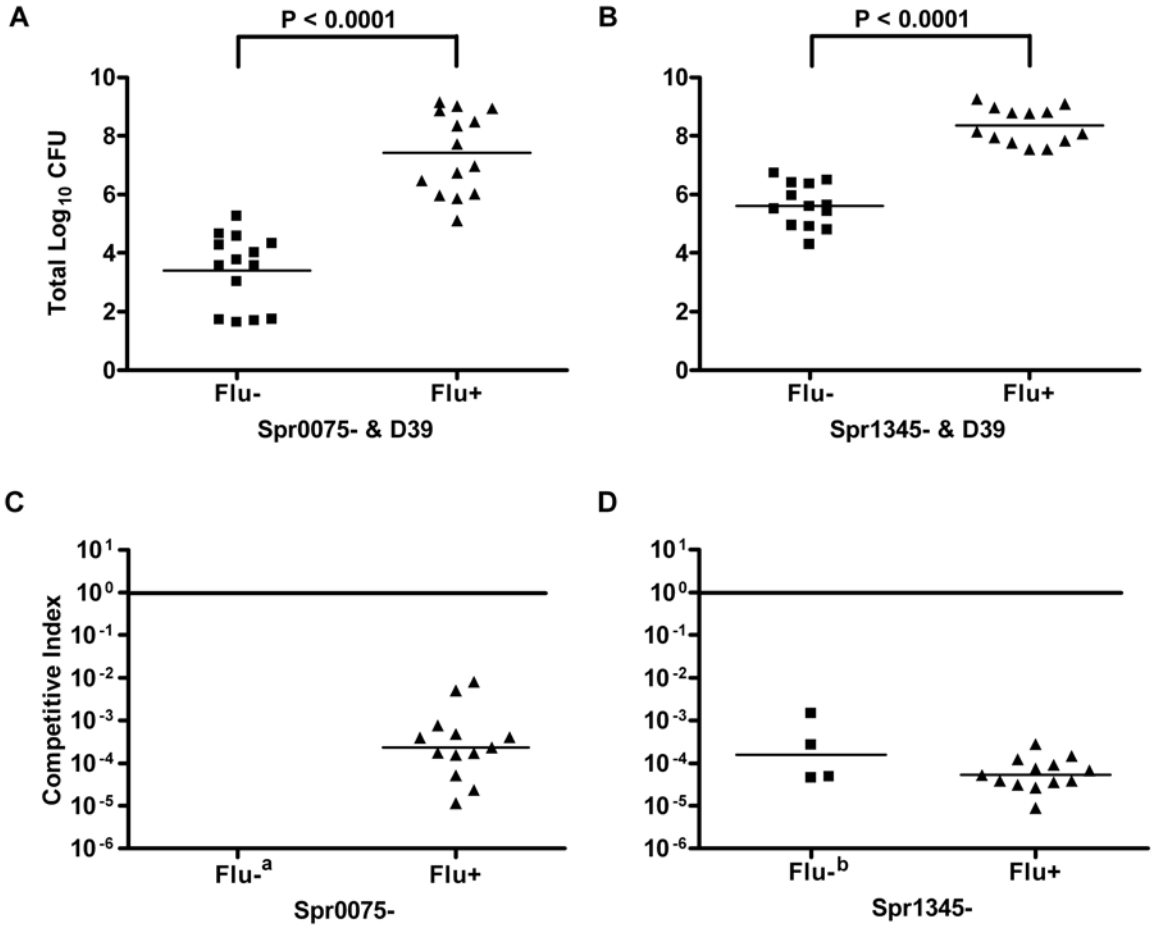

Figure 3.4. Total CFU and Competitive Indices of Spr0075- and Spr1345- Recovered from Mouse Lung 24 h following Pneumococcal Infection. Total CFU recovered from mouse lung 24 h following Spr0075- & D39 (A), or Spr1345- & D39 (B) infection. Mice received approximately 5×10^6 CFU of the D39 parental strain concurrently with approximately 5×10^6 CFU of Spr0075- or Spr1345- mutant strain (1×10^7 CFU total) day 6 post-influenza (▲) or sham treatment (■). Competitive indices of Spr0075- (C) or Spr1345- (D) growth from panels A and B, respectively, in mice with (▲) or without (■) prior influenza infection. Horizontal bars represent group medians. Data presented were pooled from at least two independent replicates and were analyzed with a two-tailed Mann-Whitney.

[a] - Selective plating failed to yield Spr0075- mutants from primary pneumococcal infections preventing CI calculations.

[b] - Selective plating yielded Spr1345- mutants from 4 of 13 samples.

influenza infection exhibited a greater than 18,000-fold reduction in numbers relative to wildtype as evident in a median CI value of 0.000053 (Fig. 3.4 D). However, comparison of median CI values for the mutants lacking functional Spr1345 between mice with and without prior influenza infection was not statistically significant. Together, these results indicate virulence contributions for the Spr0075 and Spr1345 proteins in both primary pneumococcal infection and secondary pneumococcal infection following influenza infection.

Effects of Intranasal Immunization with Spr0075 and Spr1345 on Primary and Secondary Pneumococcal Growth

In order to assess the ability of a host immune response targeting Spr0075 and Spr1345 to reduce primary and secondary pneumococcal lung burdens, mice were intranasally-immunized with adjuvant alone or in combination with purified recombinant Spr0075 and Spr1345 (Fig 3.5). Following immunization, adjuvant-only and adjuvant-recombinant protein groups were subdivided into groups which then received influenza or sham PBS infection. On the sixth day following influenza or sham infections, all mice were challenged intratracheally with type 2 D39 pneumococci. Mice previously infected with influenza again displayed behaviors and symptoms indicative of influenza infection on day 6 post-influenza infection and had $1 \times 10^5 – 1 \times 10^6$ PFU in their lung, whereas sham-infected mice appeared healthy and did not have detectable virus (Fig.3.6). In agreement with previous observations [297, 298], immunization with the Spr0075 and

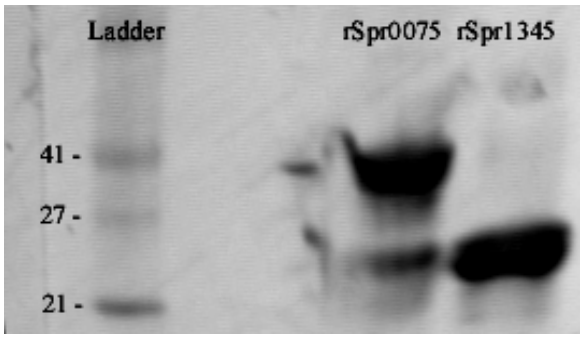

Figure 3.5. Purified Recombinant N-terminus His-tagged Spr0075 and Spr1345 Proteins Employed for Immunization. Proteins were separated via electrophoresis within an SDS-PAGE gel. Total protein was visualized by staining with Coomassie Blue. Dominant proteins bands are observed of the expected sizes for the Spr0075 (~36 kD) and Spr1345 (~23 kD) proteins.

Spr1345 proteins resulted in significant inductions of specific IgA (Fig. 3.7 A and C, respectively) and IgG (Fig. 3.7 B and D, respectively) responses whereas specific IgA and IgG responses were not detected in the adjuvant-only treated controls.

Mice infected with influenza exhibited highly significant increased bacterial burdens when compared to mice without prior influenza infection (Fig. 3.8 A and B). Intranasal immunization with Spr0075 resulted in a significant 19-fold average reduction in bacterial burdens recovered from mice receiving a primary pneumococcal challenge when compared to adjuvant-only controls (Fig. 3.8 A). Immunization with this protein was also found to significantly reduce bacterial burdens 10-fold in secondary pneumococcal challenges following influenza infection when compared to adjuvant-only controls (Fig. 3.8 A). However, this reduced secondary bacterial burden recovered from Spr0075 immunized mice remained significantly greater ($P < 0.001$) than that recovered

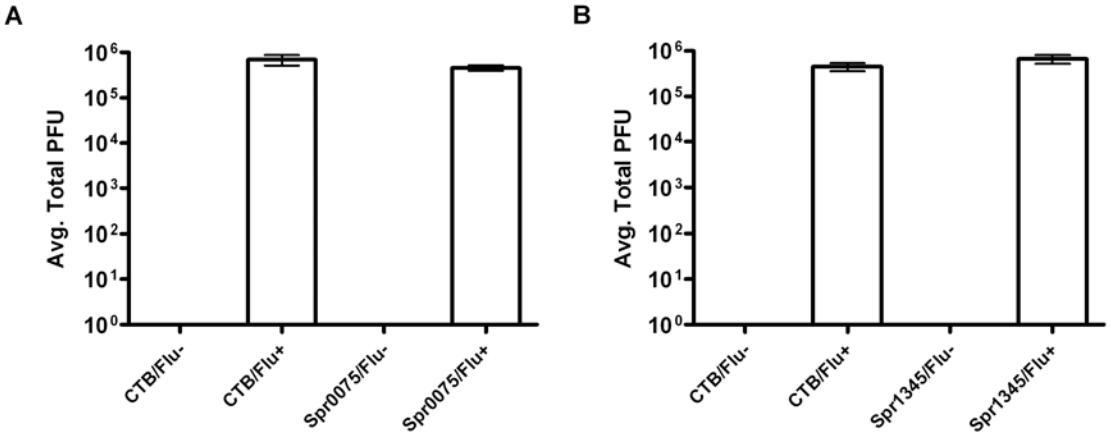

Figure 3.6. Average Total PFU Recovered 24 h Post-Pneumococcal Challenge from Lungs of Mice Immunized Intranasally with Spr0075 (A) or Spr1345 (B) Proteins. Following CTB-only (CTB) or CTB and Spr0075 (Spr0075) or CTB and Spr1345 (Spr1345) immunization, mice received either 400 PFU PR8 influenza (Flu+) or sham infection (Flu-). Six days post-influenza or sham infection, all mice received 1×10^6 CFU D39. Columns represent group means with error bars indicating SEM. Data presented were pooled from at least two independent replicates.

from adjuvant-only treated mice receiving a primary pneumococcal challenge absent a prior influenza infection. Intranasal immunization with Spr1345 resulted in a highly significant 80-fold reduction in bacterial burdens recovered from mice receiving a primary pneumococcal challenge when compared to adjuvant-only controls (Fig. 3.8 B). However, despite the observation that this surface protein is important for virulence in secondary pneumococcal infection following influenza, as indicated in reduced fitness of the Spr1345- mutant in the competitive growth experiments, the bacterial burdens

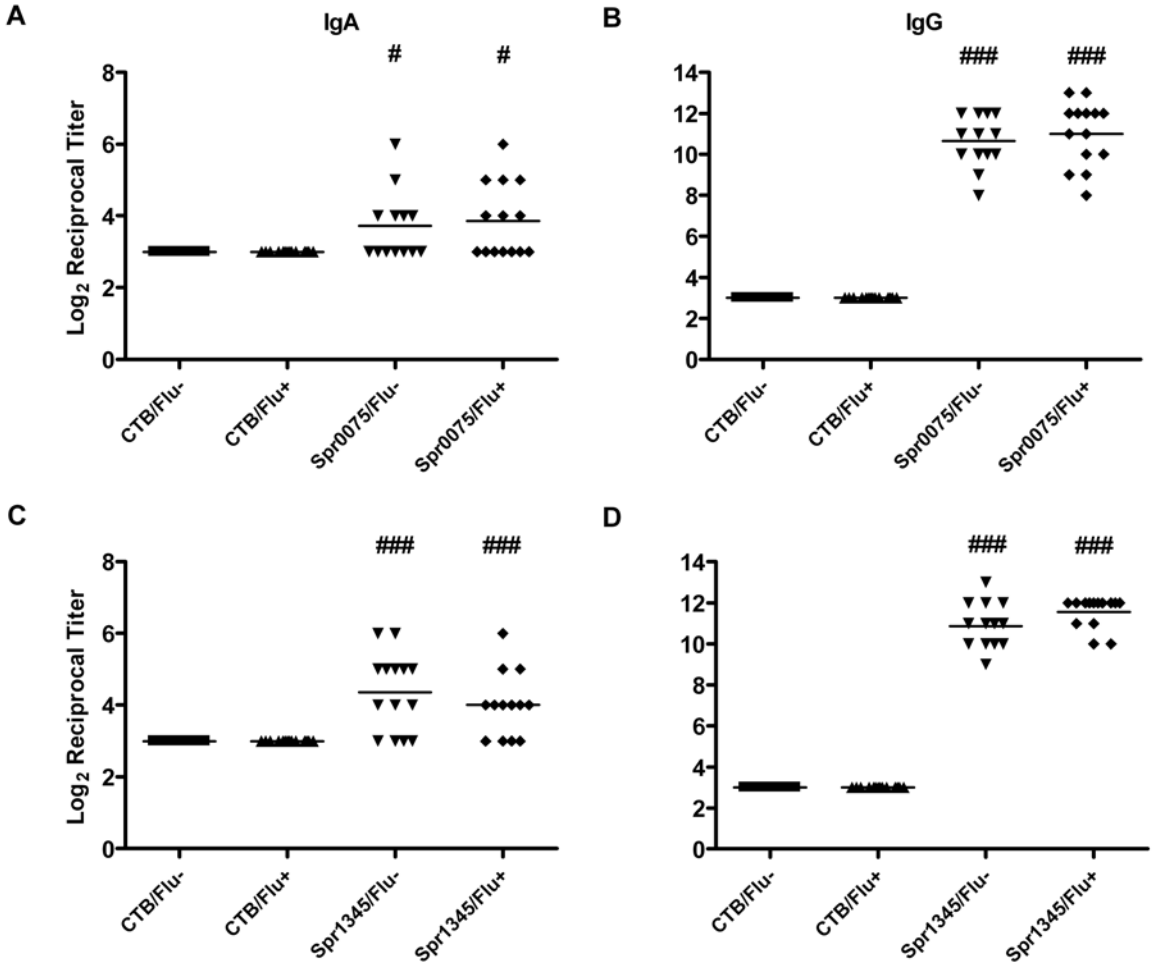

Figure 3.7. IgA and IgG Responses from Mice Receiving Intranasal Immunization with Spr0075 and Spr1345 N-terminus His-tagged Proteins as Determined by ELISA. Mice received intranasal immunizations with adjuvant-only (CTB) or in combination with recombinant Spr0075 (Spr0075) or Spr1345 (Spr1345) proteins. These treatment groups were then divided into subgroups receiving 400 PFU influenza (Flu+) or sham PBS (Flu-) infections. Six days post-influenza or sham infection, all mice received 1×10^6 CFU D39. Significance is as follows: Panel A: #, $P < 0.05$ comparing Spr0075-treated groups to CTB-treated groups; Panel B: ###, $P < 0.001$ comparing Spr0075-treated groups to CTB-treated groups. Panels C and D: ### $P < 0.001$ comparing Spr1345-treated groups to CTB-treated groups. Horizontal bars represent group means. Data presented were pooled from at least two independent replicates and were analyzed by two-way ANOVA with Bonferroni post test.

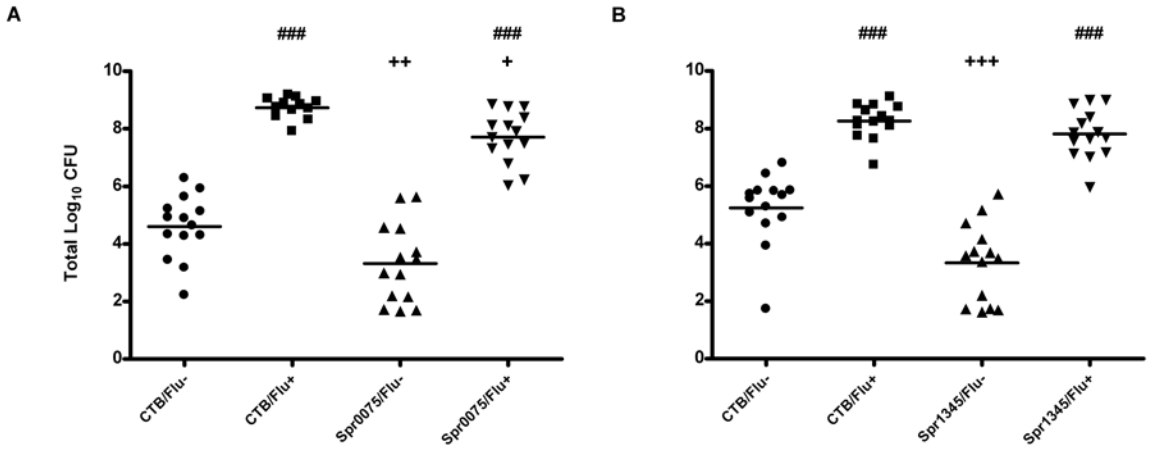

Figure 3.8. D39 Bacteria Titers Recovered 24 h Post-Pneumococcal Challenge from Lungs of Mice Immunized Intranasally with Spr0075 (A) or Spr1345 (B) Proteins. Following CTB-only (CTB) or CTB and Spr0075 (Spr0075) or CTB and Spr1345 (Spr1345) immunization, mice received either 400 PFU PR8 influenza (Flu+) or sham infection (Flu-). Six days post-influenza or sham infection, all mice received 1×10^6 CFU D39. Significance is as follows: Panel A: ###, $P < 0.001$ comparing influenza-infected groups to groups without prior influenza infection; ++, $P < 0.01$ comparing Spr0075/Flu- to CTB/Flu-; +, $P < 0.05$ comparing Spr0075/Flu+ to CTB/Flu+. Panel B: ###, $P < 0.001$ comparing influenza-infected groups to groups without prior influenza infection; +++, $P < 0.001$ comparing Spr1345/Flu- to CTB/Flu-. Horizontal bars represent group means. Data presented were pooled from two independent replicates and were analyzed by two-way ANOVA with Bonferroni post test.

recovered from immunized mice receiving secondary D39 challenges was not significantly different than burdens observed in the influenza-infected adjuvant-only controls (Fig. 3.8 B). Together, these results indicate that while immunization with either protein was capable of reducing primary pneumococcal burdens, only immunization with Spr0075 resulted in a significant reduction of a secondary D39 pneumococcal challenge following established influenza infection. However, immunization with this protein failed to mitigate influenza-induced susceptibility as evidenced in a still significantly

greater bacterial burden than that observed in adjuvant-only controls lacking influenza infection.

Discussion

S. pneumoniae is a major etiological source of disease capable of producing mild sinusitis or life-threatening pneumonia. Current vaccines are less than optimal in the highest risk age groups and have also resulted in a strong selection for non-targeted serotypes [293-295]. For these reasons, conserved pneumococcal proteins have received considerable attention as potential vaccine targets [230, 246, 303-305]. However, complicating this search is the significantly increased threat posed by pneumococcal infections secondary to influenza infection where the influenza-infected lung is known to be a far more conducive to *S. pneumoniae* colonization and growth. Using a similar approach employed in this present study, we recently reported a unique role for PspA in secondary infections following influenza infection and the ability of immunization with recombinant PspA to significantly reduce bacterial burdens in influenza-infected mice secondarily challenged with type 2, 3 and 4 pneumococci [300]. Here, we have examined both virulence contributions and the immunological protective capacity of Spr0075 and Spr1345 in primary pneumococcal infection but with an added focus on these functions in the context of a secondary pneumococcal challenge following influenza infection.

Spr0075 is an LPXTG-bearing protein revealed by the genomic sequence of the R6 nonencapsulated pneumococcal strain [134]. This protein was identified as an

immunogenic protein by screening a pneumococcal D39 genome phage display library with sera from immunized mice and that obtained from a patient hospitalized with pneumococcal pneumonia [297]. While the function of this protein is not yet known, these authors' analysis of the protein sequence predicts a mass of 123 kDa, a signal sequence (amino acids 1-40 with a putative cleavage site at aa 41), six contiguous 152 aa repeats and the aforementioned LPXTG cell-wall anchoring motif. They additionally report a high degree of preservation among numerous investigated serotypes (2, 4, 6B, 19F and 23F). Our results indicate a contribution for this protein to primary pneumococcal lung infection in that while wildtype bacteria were recovered from our competitive growth experiments, levels of the Spr0075- IDM mutant were below that of detection. Our results indicated a similar contribution of Spr0075 to secondary pneumococcal challenge where the mutant was recovered 4.1×10^3-fold below that of the wildtype. The role of influenza in increasing susceptibility to secondary pneumococcal infection is multi-faceted and includes a dramatic remodeling of the airway surface [10, 43, 252] as well as a suppression of various innate effector functions including phagocytosis and bactericidal capability [262, 267, 270]. Our observation of colonization of the influenza-infected lower respiratory tract by the Spr0075- mutant highlights this known increased susceptibility to prior influenza infection and indicates influenza-induced suppression of host defense can assist colonization, growth and/or survival of otherwise severely attenuated pneumococcal strains. However, how virus-induced changes in the host specifically contribute to this augmentation and the mechanistic nature of Spr0075 contribution to *S. pneumoniae* virulence in general remains to be

elucidated. In agreement with our competitive growth observations employing the Spr0075- mutant, immunization with recombinant Spr0075 protein significantly reduced both primary pneumococcal burdens and secondary burdens following established influenza infection when compared to controls. Together, these results indicate a contribution of Spr0075 to both primary and secondary pneumococcal infections in that targeting this protein through genetic manipulation or through a specific host immune response results in significantly reduced burdens after 24 h. However, despite the significant reduction of secondary pneumococcal burdens relative to controls, these numbers remained significantly greater than those observed in primary pneumococcal challenges and indicate that immunization with Spr0075 was incapable of overcoming the susceptibility induced by a prior influenza infection.

Spr1345 is a homolog of the Sp1492 mucin-binding protein identified from the TIGR4 genome and is both surface-exposed and immunogenic in mice [298]. We observed attenuation of the IDM mutant lacking an ability to functionally express Spr1345. This attenuation was severe in a primary pneumococcal infection with mutants recovered from only 4 of 13 mice and where these recovered numbers were greater than 6.1×10^3-fold below that of the wildtype D39. Furthermore, while this mutant was recovered from all influenza-infected mice receiving the mixed Spr1345- and D39 inoculum, the relative mutant fitness was 1.8×10^4-fold below that of wildtype. Again, that Spr1345- mutants were recovered from all of the mice with prior influenza infection, but not in mice challenged with pneumococci alone, indicates influenza-induced susceptibility to secondary pneumococcal infection can extend to pneumococcal strains

observed to be severely attenuated in primary infections. However, and as with the Spr0075- mutant, influenza-induced susceptibility to secondary infection was not sufficient to overcome the attenuation induced by an insertional disruption in the *spr1345* gene. In agreement with our observations of attenuation of the Spr1345- mutant in primary pneumococcal infection competitive growth experiments, immunization with Spr1345 protein significantly reduced bacterial burdens in mice challenged with D39 alone; however, we did not observe significant impact of this immunization upon bacterial burdens in influenza-infected mice. This was a surprise to us in that we had hypothesized that a host immune response targeting this protein would produce results analogous to those observed in secondary pneumococcal competitive growth experiments employing the Spr1345- mutant. Given this protein's described role as an adhesin [298], it is possible that our observations represent a temporally constrained application for this surface protein early in secondary pneumococcal lung colonization that becomes less critical in the progression to our 24 h endpoint. This could potentially explain the relative growth defect observed for the Spr1345- mutant in the secondary infection competitive growth experiments where initial colonization would be severely hampered by the absence of this adhesin but that a similar functional degree of early neutralization and clearance was not endowed by immunization with the Spr1345 protein. Investigation of *spr1345* expression throughout the first 24 h of primary pneumococcal infection and pneumococcal infection following influenza infection may help resolve this question.

To our knowledge, this is the first observations of virulence contributions attributable to the pneumococcal surface proteins Spr0075 and Spr1345. Our results

indicate that both of these cell-wall linked proteins contribute to pneumococcal virulence alone and within the far more dangerous scenario of secondary pneumococcal infection following antecedent influenza infection. However, we also observed that despite both proteins contributing to secondary pneumococcal infection, immunization with these proteins and their respective efficacies in reducing secondary pneumococcal infection were not equivalent. We believe these results illustrate a need for further exploration of current pneumococcal protein vaccine candidates with respect to their protective efficacy against secondary pneumococcal infections in that observation of a protein's virulence contribution and its ability via immunization to protect against primary pneumococcal infection doesn't necessarily correspond to a similar degree of immunological protection against secondary pneumococcal infection.

Given the historical periodicity of influenza pandemics, it is generally acknowledged that future influenza pandemics are inevitable. In light of this concern, and given the anticipated limited supplies of antibiotics during a pandemic, it has been previously suggested that improving vaccine coverage targeting common secondary bacterial pathogens could prove an effective and relatively easy measure in limiting the increased morbidity and mortality associated with secondary bacterial infections that would undoubtedly accompany an influenza pandemic [11, 306, 307]. We certainly agree and would add that development of a pneumococcal vaccine which has been specifically assessed for its protective efficacy against both primary and secondary pneumococcal infections would further serve to limit influenza pandemic associated mortality. Currently, PspA appears to be only other pneumococcal protein vaccine

candidate to have received this particular attention and while capable of significantly reducing secondary pneumococcal burdens, did not completely overcome influenza-induced susceptibility [300]. Increased protection against primary pneumococcal infection has been observed when immunizing with combinations of pneumococcal proteins [120, 230]. This multivalent strategy would likely also improve protection against secondary pneumococcal infections and in combination with PspA, the results presented here support further examination of Spr0075 for this application in the specific context of preventing secondary pneumococcal infections following influenza infection.

SUMMARY AND CONCLUSIONS

Influenza infection is known to increase susceptibility to secondary *S. pneumoniae* infection resulting in significantly increased morbidity and mortality. The mechanisms underlying this synergistic polymicrobial interaction have received considerable attention with respect to contributions originating with virus. Influenza infection is known to disrupt host defenses through two broad categories of action; influenza-induced denudation and modification of respiratory epithelium and influenza-induced suppression of innate cellular effector populations. Previous work in the Harmsen Lab has demonstrated significant contributions for both of these influenza-host interactions in increasing susceptibility to secondary pneumococcal infection [270]. We hypothesized that key to the success of secondary pneumococcal infection was the novel or enhanced application of one or more pneumococcal virulence factors in the context of the influenza-infected lung. The purpose of this present work was to assess contributions to the influenza-*S. pneumoniae* synergism originating with the bacterium, specifically, contributions from known and putative pneumococcal virulence factors.

Chapter two describes studies addressing contributions of the known pneumococcal virulence factors hyaluronidase (Hyl), neuraminidase (NanA) and pneumococcal surface protein A (PspA) to secondary *S. pneumoniae* infection following influenza infection. Using mutants with disruptions in the *hyl* (Hyl-), *nanA* (NanA-) and *pspA* (PspA-) genes in combination with the D39 parental wildtype strain, we employed a competitive growth model to assess mutant fitness in primary pneumococcal infection alone or as a secondary infection in the presence of an established influenza infection.

Whereas the Hyl- and NanA- mutants did not display attenuated growth in either primary or secondary pneumococcal infection, the PspA- mutant exhibited reduced fitness in primary infections where this attenuation was further exacerbated relative to wildtype in secondary infections. These results indicated a unique contribution of PspA to secondary pneumococcal infections presumably due to an enhanced role for PspA in inhibiting complement-mediated phagocytosis within the highly inflammatory environment of the influenza-infected lung.

To further characterize the contribution of PspA to secondary pneumococcal infections, we employed an intranasal immunization model in which mice received PBS-only, adjuvant-only or PspA with adjuvant. Mice subsequently received sham PBS or influenza infection followed six days later with primary or secondary pneumococcal challenges with the capsular serotype 2, type 25 PspA bearing D39 strain. PspA immunization with homologous type 25 PspA resulted in significantly reduced primary and secondary bacterial lung burdens after 24 h compared to PBS-only and adjuvant-only treated controls. Furthermore, when compared to PBS-only and adjuvant-only controls, mice receiving PspA immunization exhibited significantly reduced levels of lactate dehydrogenase (LDH) and albumin, two specific markers of pulmonary damage. Together, these results indicated a contribution of PspA to secondary pneumococcal infection in that targeting of the PspA protein via genetic interruption or a specific host immune response resulted in significant reductions of bacterial burdens.

Over ninety different *S. pneumoniae* capsular serotypes and at least 31 different PspA types have been described. The interaction of capsule and PspA type has been

shown to produce varying degrees of virulence and accordingly, immunization with PspA has been shown to provide variable degrees of protection with respect to different combinations of capsule and PspA type. To further assess the role of PspA immunization against secondary pneumococcal infections of different capsule and PspA types, we employed the PspA immunization model described above using the capsular serotype 3, PspA type 1 WU2 and capsular serotype 4, PspA type 20 TIGR4 strains. As with the homologous PspA-bearing D39 secondary challenges, immunization with type 25 PspA resulted in significantly reduced secondary bacterial burdens of the heterologous PspA-bearing serotype 3 WU2 and serotype 4 TIGR4 strains. Again, these reductions in bacterial lung burdens were associated with significantly reduced levels of pulmonary damage (i.e. LDH and albumin). Together, these results indicated that PspA immunization can provide protection against secondary pneumococcal infection with strains of different capsular serotype and different PspA type.

Chapter three describes studies addressing contributions of two cell-wall linked pneumococcal proteins, Spr0075 and Spr1345, to primary pneumococcal infection alone and secondary pneumococcal infection following established influenza infection. We constructed insertion-duplication mutants lacking the ability to functionally express the Spr0075 (Spr0075-) or Spr1345 (Spr1345-) proteins. Using similar methods to those described in chapter two, these mutants, in combination with the D39 parental wildtype strain, were then employed in competitive growth models of primary pneumococcal infection alone or secondary pneumococcal infection in influenza-infected mice. Competitive growth of the Spr0075- mutant in primary pneumococcal infection was

severely attenuated where recovery of this mutant was below the level of detection in all 14 animals assessed. A severe attenuation was also observed in secondary pneumococcal challenge where this mutant was recovered 4100-fold below that of the wildtype. Competitive growth of the Spr1345- mutant was also severely attenuated in primary pneumococcal infection with only 4 of 13 mice exhibiting mutants above the level of detection and with these exhibiting a 6400-fold reduction in numbers relative to wildtype. Similarly, Spr1345- mutants exhibited an 18,000-fold reduction in bacteria numbers relative to wildtype in influenza-infected mice. Together, these results represent the first reported observations of virulence contributions for both the Spr0075 and Spr1345 proteins to both primary pneumococcal infection and secondary pneumococcal infection following an established influenza infection.

To further characterize the virulence contributions of these proteins and to assess their ability as immunogens to protect against primary and secondary pneumococcal infection, purified recombinant N-terminus fragments of Spr0075 and Spr1345 were employed in an intranasal immunization model. Mice received adjuvant-only or Spr0075 or Spr1345 proteins with adjuvant. Mice then subsequently received sham PBS or influenza infection followed six days later with primary or secondary pneumococcal challenges with the D39 strain. Both Spr0075 and Spr1345 proteins induced specific serum IgA and IgG responses indicating both proteins were immunogenic. Immunization with Spr0075 resulted in significantly reduced bacterial burdens in mice receiving primary pneumococcal challenge alone or as a secondary pneumococcal challenge following established influenza infection. Immunization with Spr1345 resulted in

significantly reduced bacterial burdens in mice receiving primary pneumococcal challenges, but failed to exhibit a significant reduction in secondary bacterial burdens in influenza-infected mice. Together, these results indicated that targeting the Spr0075 protein through genetic interruption or via a specific host immune response significantly reduces primary and secondary infections. This is the first report of Spr0075 contributions to virulence and its ability as an antigen to protect against primary and secondary pneumococcal infections. Additionally, these results indicated that genetic interruption or a specific host immune response targeting the Spr1345 protein significantly reduces primary pneumococcal infections and again represent the first description of this protein as a virulence factor. However, these results also revealed that a protein's virulence contribution to secondary pneumococcal infection and a protective capacity against primary pneumococcal infection do not necessarily correlate to a similar degree of immunological protection against secondary pneumococcal infection.

While the result of Spr1345 failing to protect against secondary pneumococcal infection was disappointing, it does however illustrate a pervasive oversight in the development of pneumococcal protein vaccines. Pneumococcal disease is ubiquitous and deadly and is further complicated by the evolution of antibiotic resistance and a less than optimal protection afforded by current pneumococcal vaccine formulations. For these reasons, pneumococcal virulence factors have received considerable attention as pneumococcal protein vaccine candidates. Whereas these efforts have yielded candidates which will very likely protect against pneumococcal infection alone, there efficacy against the far more dangerous scenario of secondary pneumococcal infection following

influenza infection has remained largely unexplored. The studies presented here in chapters two and three represent to our knowledge the only work which has specifically assessed this critical concern. Furthermore, the work presented in chapter three illustrates that a pneumococcal protein vaccine candidate cannot be assumed to provide protection against secondary pneumococcal infection following influenza even though the protein in question may contribute to secondary infection virulence and/or may provide protection against a primary pneumococcal infection alone. This is an important consideration in that analysis of influenza pandemic mortality has attributed the vast majority of deaths to secondary bacterial pathogens, chiefly *S. pneumoniae*. It is accepted that, given its ability to rapidly evolve via antigenic drift and antigenic shift, future influenza pandemics are inevitable. It has further been suggested that vaccination against the principal secondary bacterial pathogens prior to a pandemic could represent a means to reduce the anticipated pandemic-associated mortality in light of limited antibiotic supplies or their reduced efficacies against resistant secondary pathogens. For these reasons, and with respect to the observations presented here (particularly those concerning Spr1345), pneumococcal protein vaccine candidates should be examined for their ability to protect against secondary infections following influenza in order to provide for the most robust application against pneumococcal disease. This need for an effective pneumococcal protein vaccine capable of protecting against secondary pneumococcal infections following influenza is highlighted in the fact that, even as these words are being typed, a novel H1N1 swine influenza comprised of swine, avian and human viral components

capable of sustained human-to-human transmission and prompting pandemic fears, has migrated out of Mexico City and spread around the globe [308, 309].

FUTURE STUDIES

The results presented in chapters two and three indicate virulence contributions of PspA, Spr0075 and Spr1345 to secondary pneumococcal infection following established influenza infection and all three proteins provided protection against primary pneumococcal infection. However, only immunization with the PspA and Spr0075 proteins provided protection against secondary pneumococcal infections. The inability of immunization with the pneumococcal Spr1345 protein to provide protection against a secondary *S. pneumoniae* challenge in influenza-infected mice illustrates that observations of virulence contributions and protection against a primary pneumococcal infection does not necessarily translate into protection against a secondary pneumococcal challenge. Given this observation, further exploration of known pneumococcal virulence proteins within the specific context of the influenza-infected respiratory tract is warranted.

As described in chapter one, the pneumococcal virulence proteins, pneumolysin, choline binding protein A and pneumococcal surface adhesin A have been shown to contribute to virulence and have been shown to provide protection against primary pneumococcal infections. Incorporating these proteins into the aforementioned immunization models described for PspA, Spr0075 and Spr1345 would provide an assessment of their protective capacity specifically in the context of secondary pneumococcal infection following established influenza infection. Furthermore, as additive protection has been demonstrated when pneumococcal protein vaccine candidates are used in combination; it would be of great interest to assess the PspA, Spr0075 and Spr1345 proteins in combination together and in combination with other

pneumococcal virulence proteins for their protective efficacy against secondary pneumococcal infections following influenza infection.

Also described in chapter one are a number of pneumococcal protein delivery methods which have been shown to provide protection against pneumococcal infection. Our use of direct intranasal immunization with protein fragments with an adjuvant, while effective, may represent a limitation in inducing an optimal specific immune response against secondary pneumococcal infection. Formulations of single or combined pneumococcal proteins delivered in live attenuated *Salmonella*-based or other immunostimulatory matrix platforms applied within our secondary pneumococcal infection model would provide insights toward optimizing an effective specific immune response. For instance, work in the Harmsen Lab has demonstrated an ability of nanoparticles to organize relevant pulmonary lymphoid tissues which in turn provide for a more rapid and effective response against pulmonary pathogens. It would be interesting to assess the ability of these nanoparticles in combination with pneumococcal protein vaccine candidates to facilitate protection against secondary pneumococcal infection following an established influenza infection.

Another factor to be considered among future immunization studies is the diversity of pneumococcal serotypes and their respective virulence and pathogenic attributes. As was assessed for the PspA protein in our secondary infection model, the ability of immunization with Spr0075 to protect against secondary pneumococcal infections with multiple serotypes would be necessary to ensure a broad and effective

application. This last point would also be warranted for any immunization study employing pneumococcal protein combinations and/or the various delivery platforms.

Chapter three describes to our knowledge the first analysis of virulence contributions for the Spr0075 and Spr1345 pneumococcal proteins. Whereas Spr1345 has been previously examined resulting in its description as a mucin-binding protein, the function of Spr0075 remains unresolved. This protein did not exhibit any recognizable domain architecture when subjected to BLAST searches. For use as an immunogen, an N-terminus fragment comprising amino acids 43-296 of the putative 1161 amino acid full length Spr0075 was expressed and purified; however, it would be useful to express larger fragments up to and including the full length protein. The resulting recombinant proteins could then be subjected to any number of experiments aimed at elucidating this protein's structure, molecular function and potential host interactors. Additionally, preliminary data suggests that expression of both Spr0075 and Spr1345 is regulated *in vivo* when comparing growth in the lungs of healthy mice or as a secondary infection following influenza infection. Further replication of these experiments in addition to analysis of gene expression within different host tissue compartments beyond the lung (e.g. nasopharynx, blood, etc) may also provide insight into the virulence function of pneumococcal Spr0075.

In addition to *S. pneumoniae*, influenza infection is known to predispose for secondary bacterial infection with staphylococci, streptococci, *Haemophilus influenzae* and *Klebsiella pneumoniae* among others. The competitive growth model employed in chapters two and three is amenable to studies investigating contributions of specific

bacterial virulence factors of these common secondary pathogens in the context of an influenza-infected respiratory tract. In collaboration with the Voyich-Kane lab of the Veterinary Molecular Biology department at Montana State University, preliminary data has been generated establishing a model of increased susceptibility to secondary infection with methicillin-resistant *Staphylcoccus aureus* (MRSA) following influenza infection. The Voyich-Kane lab has been exploring the role of the MRSA SaeR/S two-component regulatory system with respect to its ability to modulate virulence in cutaneous and intravenous models. As severe and fatal secondary infection with MRSA following influenza infection has been reported with increasing frequency [310-312] and as available effective therapeutics are severely restricted, application of this model to explore contributions of SaeR/S and other MRSA virulence factors to secondary infections following influenza could help identify novel and/or critical targets for vaccine and antibiotic development.

In conclusion, these studies would expand the relatively limited understanding of pneumococcal virulence contributions to secondary infection following influenza. Furthermore, they could refine the pool of pneumococcal protein vaccine candidates such that any resultant vaccine could be expected to provide protection against both primary pneumococcal infection and the far greater threat of a secondary pneumococcal infection following influenza. Additionally, an understanding of the molecular mechanism of the pneumococcal Spr0075 protein may provide insight into a potentially unique virulence function. And lastly, as future influenza pandemics are generally accepted to be inevitable, application of our model to explore the mechanisms by which other secondary

bacterial pathogens take advantage of influenza-induced susceptibility would provide a much needed knowledge base for the rational development of effective vaccines and pharmacologic interventions capable of reducing the increased morbidity and mortality associated with secondary bacterial infections following prior influenza infection.

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