

Next generation protein based *Streptococcus pneumoniae* vaccines

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All currently available *Streptococcus pneumoniae* (Spn) vaccines have limitations due to their capsular serotype composition. Both the 23-valent Spn polysaccharide vaccine (PPV) and 7, 10, or 13-valent Spn conjugate vaccines (PCV-7, 10, -13) are serotype-based vaccines and therefore they elicit only serotype-specific immunity. Emergence of replacement Spn strains expressing other serotypes has consistently occurred following introduction of capsular serotype based Spn vaccines. Furthermore, capsular polysaccharide vaccines are less effective in protection against non-bacteremic pneumonia and acute otitis media (AOM) than against invasive pneumococcal disease (IPD). These shortcomings of capsular polysaccharide-based Spn vaccines have created high interest in development of non-serotype specific protein-based vaccines that could be effective in preventing both IPD and non-IPD infections. This review discusses the progress to date on development of Spn protein vaccine candidates that are highly conserved by all Spn strains, are highly conserved, exhibit maximal antigenicity and minimal reactogenicity to replace or complement the current capsule-based vaccines. Key to development of a protein based Spn vaccine is an understanding of Spn pathogenesis. Based on pathogenesis, a protein-based Spn vaccine should include one or more ingredients that reduce NP colonization below a pathogenic inoculum. Elimination of all Spn colonization may not be achievable or even advisable. The level of expression of a target protein antigen during pathogenesis is another key to the success of protein based vaccines. As with virtually all currently licensed vaccines, production of a serum antibody response in response to protein based vaccines is anticipated to

provide protection from Spn infections. A significant advantage that protein vaccine formulations can offer over capsule based vaccination is their potential benefits associated with natural priming and boosting to all strains of Spn. One of the most universal and comprehensive approaches of identifying novel vaccine candidates is the investigation of human sera from different disease stages of natural infections. Antigens that are robustly reactive in preliminary human serum screening constitute a pathogen-specific antigenome. This strategy has identified a number of Spn protein vaccine candidates that are moving forward in human clinical trials.

Introduction

Streptococcus pneumoniae (Spn), an encapsulated gram-positive diplococcus, is responsible for a wide spectrum of infections in children and adults including invasive (meningitis, bacteremia and bacteremic pneumonia) and non-invasive infections (non-bacteremic pneumonia, otitis media, sinusitis and conjunctivitis).¹ Invasive Spn infections (IPD) are a major cause of morbidity and mortality.^{2–5} It was estimated in 2008 that 333000–529000 children aged < 5 years died of Spn infections worldwide.⁶

The capsular polysaccharide has been considered the most important virulence factor and target for vaccines to prevent Spn infections.⁷ To date, 94 distinct capsular serotypes have been identified based on polysaccharide composition, including most recently reported serotypes 11E, 20A/B.^{8–11} Two types of Spn vaccines are presently used for prevention of Spn infections: a 23-valent Spn polysaccharide vaccine (PPV-23) and 7, 10, or 13-valent Spn conjugate vaccines

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(PCV-7, 10, -13). PPV-23 includes serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9F, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F. PPV-23 is widely recommended as a single dose for person ≥ 65 years old and for persons aged 2–65 years with high risk for Spn diseases. However, because PPV-23 is poorly immunogenic in children < 2 years of age, fails at any age to generate an immune memory anamnestic response upon revaccination, and does not generate herd immunity, alternative capsular-protein conjugate vaccines were introduced in year 2000.⁶ PCV-7, PCV-10 and PCV-13 are currently licensed for prevention of pneumococcal infections caused by the serotypes included in the vaccines in children from 6 weeks to 5 years of age. PCV-7 includes serotypes 4, 6B, 9V, 14, 18C, 19F, 23F. PCV-10 includes serotypes PCV7+ 1, 5, 7F, 23F and PCV-13 includes PCV10 + 3, 6A, 19A.¹² PCV-7 is gradually being removed from the market in developed countries.^{1,6}

All currently available Spn vaccines have limitations due to their capsular serotype composition (Table 1). Both PPV and PCVs are serotype-based vaccines and therefore they elicit only serotype-specific immunity. Emergence of replacement Spn strains expressing other serotypes has consistently occurred following introduction of capsular serotype based Spn vaccines.^{9,13,14} For instance, after introduction of PCV-7, IPD and acute otitis media (AOM) cases caused by serotype 19A dramatically increased, including emergence of a strain that was resistant to all antibiotics approved for children.^{9,15,16} Furthermore, capsular polysaccharide vaccines are less effective in protection against non-bacteremic pneumonia and AOM than against IPD.^{3,17} There has been only a marginal decline in overall AOM rate in child populations since introduction of PCV13. However serotype

specific reductions in AOM caused by Spn strains expressing the 6 new serotypes included in PCV13 have not been demonstrated. Such a study is ongoing with results expected in 2016 (Pichichero et al, unpublished). These shortcomings of capsular polysaccharide-based Spn vaccines has created high interest in development of non-serotype specific protein-based vaccines that could be effective in preventing both IPD and non-IPD infections.^{3,5,18} Therefore, research scientists from academia and industry have been seeking Spn protein vaccine candidates that are universally expressed by all *Spn* strains, are highly conserved, exhibit maximal antigenicity and minimal reactogenicity to replace or complement the current capsule-based vaccines.^{3,5,19,20} This review discusses the progress to date.

Key to Development of a Protein Based Spn Vaccine is an Understanding of Spn Pathogenesis

Spn is a commensal of the nasopharynx (NP) and only becomes pathogenic when changes in the NP, typically caused by viral upper respiratory infections, allow the organism to achieve a pathogenic inoculum that overcomes innate and adaptive host defense.^{21–23} Spn carriage is most prevalent in vulnerable populations such as young children, immunocompromised adults, and the elderly.^{24,25} In children under 5 years of age, Spn can be detected in the NP from 10% up to 90% of children with the variation mostly attributable to the frequency of NP sampling, and study population.²⁶ The burden of non-invasive and invasive Spn disease correlates with the NP carriage rate.^{24,27,28} Children in developing countries generally have a higher

carriage rate and density of organisms than in developed countries. Spn colonization is an immunizing event that leads to the generation of antigen specific antibodies and cellular immunity, and is therefore beneficial for the host.^{29–33} However, colonization is also a prerequisite for Spn diseases.²⁴ The strategy currently guiding vaccination with polysaccharide-based conjugate vaccines seeks to prevent Spn disease by completely eliminating colonization.^{34–36} This has been an overwhelming success with respect to the Spn serotypes included in the vaccines.

Based on pathogenesis, a protein-based Spn vaccine should include one or more ingredients that reduce NP colonization below a pathogenic inoculum. Elimination of all Spn colonization may not be achievable or even advisable. Therefore, the challenge before vaccinologists is to define the NP carriage threshold below which perturbations by viral co-infections will not enable Spn to make the transition to invasive pathogen.

Expression of Proteins During Pathogenesis

The level of expression of a target protein antigen during pathogenesis is key to the success of protein based vaccines. It is therefore imperative to have a detailed expression analysis of candidate vaccine antigenic components during both healthy and disease conditions. Unlike constitutively-expressed capsular antigens, the expression of any given Spn protein may vary considerably during colonization compared to when the organism is locally or systemically invasive. An adequate and functional mucosal immune response to protein antigens requires expression of the target antigen by Spn in order for the response to be protective.

Table 1. Limitations of current licensed pneumococcal vaccines

Coverage
Limited serotype coverage
Increase of non-vaccine <i>Spn</i> IPD
Emergence of serotype replacement
High costs
Cumbersome manufacturing approach results in high cost
Efficacy
Effective in invasive pneumococcal diseases (IPD) Less effective in non-IPD

Host Inflammatory and Bacterial Virulence Factors

From the standpoint of modern vaccination strategies it is imperative to comprehensively understand the host inflammatory and bacterial virulence factors in the polymicrobial NP

environment. Spn synergistically or antagonistically interacts with other upper respiratory bacteria and viruses and the commensal flora in the NP environment. The dynamics of this interaction modulates the transition of Spn from a commensal to a disease phenotype.³⁷⁻⁴¹

Bacterial-viral interaction plays a critical role in pathogenesis of upper and lower respiratory infections. Spn infection almost always follows a viral upper respiratory infection (URI). Therefore animal co-infection models have been used to study co-pathogenesis of Spn with viruses such as human influenza virus,⁴²⁻⁴⁷ respiratory syncytial virus,⁴⁸ adenovirus,^{49,50} metapneumovirus,⁵¹ parainfluenza virus.⁵²

The most well-investigated bacterial-viral interaction is the synergism between influenza virus and Spn²³ and the interaction has been shown to be strongly associated with Spn dissemination from the NP and subsequent local or systemic invasion.^{22,53,54} Influenza dampens host innate immunity, compromising innate immune surveillance and allows Spn to rapidly increase in inoculum in the NP, a prerequisite for invasion.⁵⁴⁻⁵⁷ The prolonged low inflammatory environment during a viral URI permits a subsequent rapid increase in the Spn population, followed by pneumolysin-mediated tissue damage and a robust pro-inflammatory response that sets the stage for pathogenesis (Fig. 1).

In the human, apart from well described Spn-viral pathogenesis synergy, other potential determinants of Spn carriage transition to pathogen include host genetics,⁵⁸ environmental exposures, e. g. tobacco smoke and air particulates,^{59,60} antibiotic use, and variation in the microbiome.⁶¹ Spn has an antagonistic relationship with *Staphylococcus aureus* in the NP.⁶² Vaccination induced decreases in Spn colonization have been associated with an increased incidence of *S. aureus*-induced carriage in children.⁶³ An interaction of Spn with *Haemophilus influenza* (Hi) has also been shown in humans whereby the latter organism outcompetes Spn except when Spn expresses the 19A

FluA, Secondary pneumococcal infections and pneumolysin

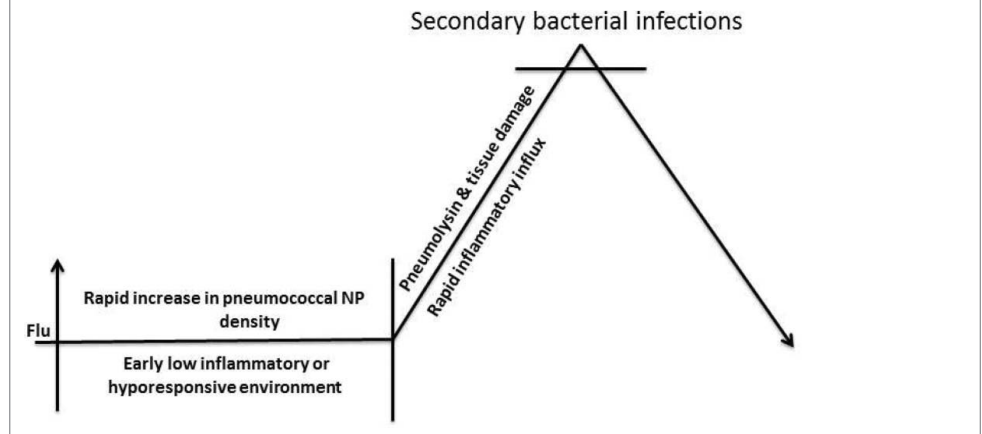


Figure 1. Upper respiratory bacterial-viral coinfection and Spn pathogenesis.

serotype capsule.⁶⁴ Also, co-colonization with Spn and Hi enhances serum antibody responses against Spn antigens, modulating pathogen specific adaptive immunity. In contrast, the presence of Spn does not change Hi specific immunity.³⁸ These data suggest an antagonistic relationship in humans between Spn and Hi in the NP. Introduction of Spn protein vaccines therefore will require a comprehensive examination of alterations in the balance among Spn, Hi, *S. aureus* and likely *Moraxella catarrhalis*.

Innate Immunity Especially in Early Life

The fundamental role for the innate immune system in sensing vaccines and adjuvants and in programming protective adaptive immune responses has been increasingly recognized.⁶⁵ It is now clear that activation of innate immunity by particular adjuvants plays a major role in determining the magnitude and quality of the adaptive immune response following vaccination.⁶⁵ Data from mouse models suggest that infant mice are more susceptible to Spn colonization than adult mice, as evidenced by higher densities and longer durations of colonization.⁶⁶ Macrophages are an indispensable innate effector cell subset for carriage clearance, and neonatal macrophages have been shown to produce significantly less cytokines like

IL-8/KC, IL-1 α , TNF- α and IFN- γ .⁶⁶ These are important inflammatory mediators involved in immune defense against Spn. In contrast, neonatal macrophages have been found to produce elevated levels of the anti-inflammatory cytokine IL-10 compared to adults.⁶⁶

In humans, a number of studies have highlighted that compromised TLR dependent signaling leading to low pro-inflammatory responses in early life. The overall pattern of response deficiency has been reported among infants (Table 4). Neonatal innate immune cells including monocytes, plasmacytoid dendritic cells, and conventional dendritic cells produce less interleukin (IL)-12p70 and type I interferon, and similar or higher levels of IL-1 β , IL-6, IL-23, and IL-10 than adult cells when stimulated by the same pattern recognition receptor (PRR) stimulants.⁶⁷ In addition to compromised cytokine responses, neonatal cells have also been shown to exhibit relatively limited ability to produce multiple cytokines in polyfunctional responses to PRR stimulation.⁶⁸ Perinatal IL-10 production decreases in concert with increasing pro-inflammatory responses (such as TNF- α and IL-1 β) over the first few years of life while neonatal antiviral cytokine responses (type I IFN) reach adult levels within the first month after birth.⁶⁹ Underlying patterns of immune ontogeny lead to windows of vulnerability to different types of infection during different stages of

development. Human genetic and environmental factors may also have a profound impact on the magnitude and quality of these responses.⁷⁰ Innate immune ontogeny in early life could significantly be influenced by environment, overall microbial burden and genetic factors like polymorphisms in key innate effector genes. Knowledge of innate immunity in different age groups and different populations informs on questions of anticipating different results following Spn protein based vaccination. Populations in developing countries, elderly and young children with poor innate responses may demonstrate a less robust adaptive response than populations in developed countries and older children and adults. Alternative adjuvants may be needed.⁷¹

Adaptive Immunity: Assessment of Antigen Specific Functional Immune Response

As with virtually all currently licensed vaccines, production of a serum antibody response in response to protein based vaccines is anticipated to provide protection from Spn infections.⁷² Moreover, a correlate of protection in IgG antibody levels should be sought that can provide a signature of immunity. An independent mucosal IgA response to systemic vaccination can occur but usually sustained immune memory at the mucosal level is not observed. Protection from Spn infections following protein based vaccination therefore likely will be mediated by serum antibody with transudation of IgG to mucosal sites such as the middle ear, sinuses, eye and lung. However, antigen specific CD4 T cells can also contribute to Spn clearance in an antibody independent manner⁷³ so those cellular responses will also require characterization.

It has been well documented that specific anti-Spn antibody levels induced by polysaccharide-based vaccines correlate well with opsonophagocytic/bactericidal antibodies.^{74,75} Anti-capsular opsonophagocytic assay (OPA) constitutes the main tool for evaluating the functional immune response as a correlate of protection against Spn diseases.⁷⁶ Unlike capsular opsonophagocytic antibodies, protein

based vaccines are hypothesized to have different protective mechanisms against Spn carriage and invasiveness. Anti-adhesin antibodies are expected to reduce carriage density in the NP. We would not expect that blocking one Spn adhesin would eliminate carriage entirely since Spn expresses multiple adhesins and the surface expression of adhesins occupies far less physical space on the surface of Spn compared to the polysaccharide capsule. A multi-epitope approach involving a number of adhesins in one formulation could potentially block NP carriage to a greater extent. We endorse this particular approach as a next generation vaccination strategy.

Protein based Spn vaccines will surely target infants an especially vulnerable population. Though human neonates are capable of mounting T-cell responses, CD4 T-cell responses in early life have a predisposition to T helper-2 (Th2).^{77,78} Th1 responses are less well developed and reach adult levels only after about 2 years of age^{77,78} (Table 4). Epigenetic programming is potentially an important factor in the differentiation of naïve and memory T cells, and a precise understanding of the involvement of epigenetic factors in immune ontogeny will have lasting implications for health and disease.^{68,79} A higher proportion and homing of Tregs in neonatal lymph nodes also forms an important immunosuppressive response leading to the persistence of microbial flora in mucosal areas.⁸⁰ This is a strategy the host adapts in order to retain commensal bacteria at mucosal surfaces in order to prevent the colonization and persistence of unwanted bacteria in these environments. It also helps the host to prevent inflammation-induced pathology. Although these immune effector and regulatory responses are developed in early life, their balance is continually altered with age.⁸¹

A growing body of evidence in mouse models of NP colonization suggests an indispensable contribution of Th17 cells in host defense. Prominently, B cell deficient mice were able to clear bacterial colonization, demonstrating the importance of CD4 T cell subsets in mouse colonization model.^{73,82} In addition to defense against colonization, substantial evidence

of their involvement in lung defense has also been found in both experimental mouse models and human studies in the context of Spn.^{32,82-84} In our ongoing work, we found infant mice elicit a less robust natural Th17 response after Spn colonization, and this deficiency potentially reduces their ability to fight subsequent Spn lung infection (unpublished data). Therefore, the Th17 CD4+ T cell subset has emerged as a crucial component of the host response against Spn and will need to be studied as part of Spn protein-based vaccines.

Genocea (Cambridge, MA, USA) is developing a Spn whole cell-based vaccine that targets stimulation of Th17 cells. Th17 cells are characterized by the production of the signature cytokines IL-17A and IL-22.⁸⁵ While IL-17A enhances the recruitment and function of innate effector cells, IL-22 is an important mediator for epithelial proliferation, repair and integrity at mucosal lining.^{83,86,87} The balance of IL-17A⁺/IL-22⁺ could be instrumental in an infection or immunization setting as IL-17A⁺/IL-22⁺ double positive cells have been found to be of more profound biological significance than IL-17A⁺ single positive cells.⁸⁸ While IL-17A+ cells form an important protective host component, IL-17A-induced immunopathology has been reported in the context of infection and autoimmunity.⁸⁹ Therefore, immunization strategies aiming at eliciting Th17 responses involving both IL-17A and IL-22 could be of more clinical significance. Ontogenic factors in early life that are involved in the differentiation of antigen specific Th17 subsets are required to be better understood in order to design next-generation Th17 based vaccination strategies against microbial pathogens including Spn.

With regard to infant vaccination, in our prospective studies we have seen little or no antigen specific or global CD4 T cell IL-17A response in children during the first year of life and only moderate responses in children age 1–2 years. A number of previous reports using mouse cells have demonstrated that although neonatal cells can have an apparently normal innate inflammatory microenvironment that supports Th17

differentiation, they still produce less IL-17A.^{90–92} It is therefore plausible that intrinsic early life CD4 T cell defects may limit acquisition of the Th17 phenotype. In a recent study, PBMCs from Bangladesh young children were found to produce levels of IL-17A equivalent to adults. More interestingly, their overall IL-17A responses were higher than the response from Swedish children, and, furthermore, overall IL-17A responses from Swedish children were lower than in Swedish adults.⁹³ These observations suggest that young children in a developing country (Bangladesh) differ from young children in a developed country (Sweden) and such differences may impact successful vaccination. It may be that different microbial burdens and divergent immune priming in different geographies leads to differential Th17 responses.^{78,94} Understanding the epigenetic changes in these developing and developed country populations driving these responses could help develop adjuvants to enhance immune priming in early life.

Importance of Natural Priming and Boosting in Different Populations/Geographies

Spn colonization in the NP is an immunizing event that elicits serum and mucosal antibody responses and cellular immunity that contributes to host protection against subsequent colonization episodes.^{52,53} However, the immunity induced by natural colonization may not persist as long as vaccine induced immunity. PPV and PCV vaccination strategies are based on complete elimination of carriage in the NP and subsequent low cross reactivity of vaccine serotypes with non-vaccine serotypes.⁹⁵

A significant advantage that protein vaccine formulations can offer over capsule based vaccination is their potential benefits associated with natural priming

and boosting to all strains of Spn. Protein based vaccines are not expected to eliminate NP carriage; rather protein-based vaccines will prevent increased in Spn carriage density below disease threshold. Thus, protein-based vaccines which may elicit stronger priming may also elicit stronger boosting of vaccine induced memory responses to help maintain stable protective immunity over the long term. However, this effect may not manifest the same way in resource poor countries with a high Spn bacterial burden. It has been reported that persistently high antigenic exposure might lead to immune hyporesponsiveness resulting in low vaccine efficacy, since antigen specific T cells can lose their function and become anergic in response to persistently high antigenic exposure.⁹⁶ Protein based antigens are based on eliciting antigen specific CD4 T cell responses that help B cells to differentiate into antibody secreting cells (Fig. 2). A loss of T cell function would therefore lead to poor B cell responses. Vaccine development and deployment strategies will therefore require a more comprehensive understanding of various factors that might influence vaccine responses in a particular population/geography.

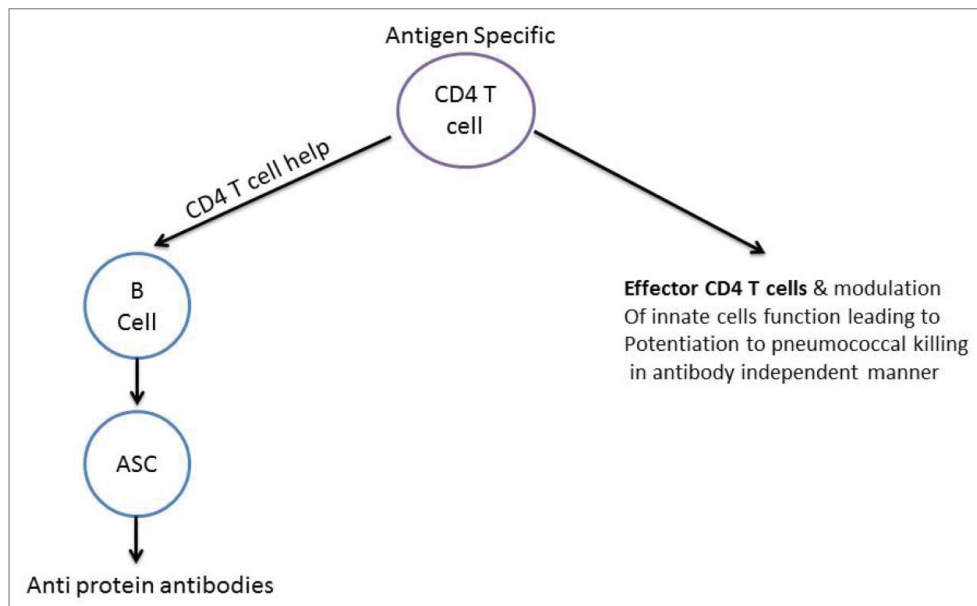


Figure 2. Protein antigen specific CD4 T cells and Spn clearance.

Animal Models

Animals including mouse,^{97–100} rat,¹⁰¹ chinchilla,¹⁰² rabbit,¹⁰³ monkeys,¹⁰⁴ ferret,¹⁰⁵ and gerbil¹⁰⁶ have been employed for studying pathogenesis of Spn diseases, assessing host response to Spn carriage and infections, identifying virulence genes, and testing the efficacy of vaccines against Spn disease.^{107,108} Due to low cost, ease of genetic manipulation and availability of ex vivo reagents for mechanism studies, the mouse has become the model of choice for IPD.¹⁰⁸ The chinchilla was historically the preferred model for otitis media (OM) due to its large bullae which makes it easier for both inoculation of Spn and collection of middle ear fluid (MEF) samples, however, in recent years, the mouse has become the preferred model for OM research.^{108,109}

Antigenome Approach to Identify Protein Vaccine Candidates

One of the most universal and comprehensive approaches of identifying novel vaccine candidates is the investigation of human sera from different disease stages of natural infections.

Antigens that are robustly reactive in preliminary human serum screening constitute a pathogen-specific antigenome.¹¹⁰ The antigens are subsequently characterized, individually cloned and purified. The immunogenicity and protective efficacy of these antigens is tested in a number of animal disease models in order to ascertain the possibility of using them as potential vaccine candidates. This is especially important since a number of microbial antigens could be reactive in human sera due to their phylogenetic differences. In addition, a number of these antigens might not be surface expressed, which is necessary for vaccine candidates apart from secreted toxins like pneumolysin. The protein antigens that are currently being investigated have the potential to address the needs of both children and the elderly by being exceptionally conserved among all serotypes and highly immunogenic.^{31,111-114}

Spn Protein Vaccine Components

In a 10 year long prospective study in children age 6–36 months old, we have made a comprehensive investigation of 5 Spn proteins considered potential vaccine candidates. Three of the antigens have transitioned to clinical trials in single- and multi-component vaccine formulations.

Histidine triad proteins PhtD and PhtE belong to a well-conserved Pht protein family expressed by Spn.¹¹¹ They are surface exposed proteins characterized mainly by a histidine triad motif.¹¹¹ In animal models, both of these proteins have been shown to elicit protection against sepsis, pneumonia and colonization.^{113,115,116} We have demonstrated their role as Spn adhesins that are required for attachment to NP and lung epithelial cells.¹¹² Spn attachment to NP epithelium is a first step in establishing carriage and potential for pathogenesis and attachment to lung epithelia must occur for Spn to cause pneumonia.²⁴ Among the Pht family proteins (PhtD, PhtE, PhtA, PhtB), PhtD has been shown to display the least variability, and is expressed by all Spn strains screened thus far, while PhtE has been PhtD is expressed by 100% of

Table 2. Characteristic components of Spn Protein Vaccine Candidates

PhtD	Surface protein with 5–6 × Histidine triad motif Expressed in 100% of Spn strains, 97–100% identity across Spn strains Elicits protective antibody responses against pneumonia, sepsis, NP colonization in animal model Safe in clinical trials
PcpA	Surface protein Expressed in > 97% of Spn strains Regulated by manganese (Mn++) Elicits protective antibody responses against pneumonia, sepsis, NP colonization in animal model Safe in clinical trials
Ply	Pore-forming toxin, produced by all strains A putative virulence factor Causes injury to lung endothelial cells Recognition by TLR-4 induces pro- inflammatory cytokines Elicits neutralizing antibody response, and protection against IPD Safe in clinical trials

Spn strains studied to date whereas has been found to be expressed by 97% of strains¹¹¹ (Table 2). All 5 Pht proteins elicit serum antibody that is cross-reactive to some degree.

PcpA is a highly prevalent protein antigen of Spn. In mouse models we and others have demonstrated a predominant role for PcpA in lung defense with very little or no involvement in NP colonization.¹¹⁷ Higher manganese levels in the NP environment of the mouse have been discussed as a negative regulator of PcpA expression.¹¹⁷ However, our clinical studies have shown that PcpA elicits robust serum antibody responses during asymptomatic colonization in young children^{31,114} and that during a viral URI the concentration of manganese drops to undetectable in many children due to dilution occurring with rhinorrhea (Kaur and Pichichero, unpublished results (Table 2).

Pneumolysin (Ply), a 53 kDa toxin, is a key virulence factor of Spn that is expressed by virtually all clinically relevant serotypes.¹¹⁸ Its detoxified derivative is a leading component of Spn protein based vaccines currently being developed. GlaxoSmithKline has detoxified Ply chemically and Sanofi Pasteur has detoxified Ply by genetic substitution.^{119,120} At sublytic concentrations, Ply activates host innate immune responses including TLR4 and the NLRP3 inflammasome.¹²¹⁻¹²³ However, at higher concentrations Ply is toxic to host cells and is associated with

immunopathology.¹²⁴ Therefore, increased expression of Ply in the host is considered to be associated with Spn pathogenesis¹²⁵ (Table 2). This supports the observation that an increase in density of Spn in the NP is a precursor to disease. Therefore, vaccination strategies that do not completely eliminate carriage but reduce its density to sub optimal level should carefully orchestrate the inflammatory environment in NP such that it would favor the host and harness the beneficial aspects of Ply.

We contend that candidate vaccine antigens should be evaluated for elicitation of a naturally-induced immune response in the target population(s) intended for vaccination. Proof that a vaccine candidate stimulates serum and mucosal antibody and B and T cell memory responses in young children would indicate the potential for natural priming of the immune system for a vaccine response and natural boosting of vaccine-induced responses. Therefore in a prospective clinical study we evaluated PhtD, PhtE, PcpA, LytB and Ply for their potential to stimulate naturally induced antibodies in 6–30 month old children. We found NP colonization was an immunizing event for PhtD, PhtE, PcpA, and Ply but much less so for LytB.³¹ Elicited serum antibodies were higher in children that were colonized with Spn but rose in all children most likely indicating that NP colonization events occurred but were not

Table 3. Pneumococcal protein vaccine candidate evaluated in clinical trials

Immunogen	Institute	Phase
Clinical Trials – Proteins		
PspA	Sanofi-Pasteur	Phase I complete
PspA and PsaA	Sanofi-Pasteur/CDC	Phase I complete
PhpA and PhtB	GSK	Phase II complete
PsaA, PcsB and StkP	InterCell AG/Novartis/PATH	
PhtD/PcpA and PlyD1	Sanofi-Pasteur	Phase I complete
PhtD/dPly w/or w/o PCV10	GSK	Phase I complete
PhtD/dPly/w/PCV10	GSK	Phase II complete
w/DTPa-HBV-IPV/Hib	GSK	Phase II complete
SP0148,1912, 2108	Genocea	Phase IIa complete
Non-encapsulated killed whole cell	Boston Children's Hospital/Instituto Butantan/PATH	Phase I complete

detected due to the program of every 3 month prospective samplings.¹²⁶ PcpA IgG serum titers were the highest followed by PhtD and PhtE titers were equivalent followed by Ply and lastly LytB.¹²⁶ Subsequently we tested the 5 antigens for their capacity to stimulate antigen specific memory CD4 T and B cell responses and found the same rank-order of potency (PcpA > PhtD = PhtE > Ply > LytB).¹²⁷

Based in the results generated by our group and other considerations Sanofi Pasteur transitioned to clinical trials in humans with 3 protein candidates–PhtD, PcpA and PlyD1 (genetically detoxified Ply) for monovalent, bivalent and trivalent

protein based vaccines. GlaxoSmithKline has ongoing clinical trials in humans using vaccines containing PhtD and Ply (chemically detoxified).

Mucosal vs. Serum Antibody; Transudate in the NP and MEF

Spn colonization is a necessary prerequisite for *Spn* infections. NP carriage of *Spn* is mostly asymptomatic.¹⁷ *Spn* carriage as an immunizing event elicits serum and mucosal antibody response to *Spn* protein vaccine candidates. A robust systemic immune response against protein antigens results in transudation of

serum antibodies to mucosal sites such as NP and middle ear, which constitutes a major defense component. We have recently shown a correlation of higher mucosal antibody titers against PhtD, PcpA and PlyD1 with a reduction in acute otitis media caused by *Spn*.¹²⁸ In addition to serum transudate antibodies at mucosal sites, active upper respiratory mucosal tissues such as tonsils and adenoids are mucosal inductive secondary lymphoid sites and contributors to local antibody production in the NP. A number of *Spn* protein antigens have been shown to elicit robust mucosal responses in an infection or ex-vivo stimulation setting.¹²⁹

Table 4 Vulnerable populations, risk factors for *Spn* infections and immune system

Risk Factors & <i>Spn</i> diseases	
Environmental factors (dust, smoke, quality of air)	
Nutrition	
Microbiome	
<i>Spn</i> -viral coinfections	
Genetic polymorphisms in immune mediators	
Immune System	
Immune cells and mediators	Shortcomings
Th1 responses	Unable to mount, Th2 skewed
Treg development	Strongly favored
Th17 responses	Less well developed
B cell signaling and responses	ompromised, leading to attenuated antigen specific responses
Germinal centers and secondary lymphoid tissues	Underdeveloped, increased Treg homing leading to diminished antigen specific responses
TLR signaling and innate inflammatory mediators	Suboptimal
Dendritic cells	Immature with low HLA-DR, B7 molecules
Protein Based <i>Spn</i> Vaccines and Expectations	
CD4 T cell dependent antigens	
Ability to ameliorate/overcome early immune shortcoming	
Sustained CD4 T cell dependent B cell help	
Robust antigen specific memory and persistence	
Reduce carriage to tolerogenic threshold leading to increase vaccine immune priming	
Less boosters required	
Broad spectrum serotype independent protection	
Cost effective, universal coverage, easy availability in developing world	

Numerous previous studies show serum antibodies to Spn proteins rise following Spn carriage,^{31,38,130–132} however the naturally acquired antibody levels in serum are not directly associated with reduced subsequent carriage.¹³² Holmlund E et al (2006)¹³⁰ reported an increase in serum antibody concentrations to Spn surface adhesin A and pneumolysin in infants who were colonized with *Spn*. Simell B et al showed that children with prior positive NP cultures for *Spn* had significantly higher serum anti-CbpA and anti-PhtD IgG.¹³¹ Prevaes SM et al reported that colonization with *Spn* induced serum IgG against 14 Spn proteins.¹³² Our group reported that colonization elicits serum IgG and IgA responses to PhtD, PcpA, and PlyD1.^{38,133} More recently we established a correlation between sera antibody titers to these proteins and protection of occurrence of *Spn* AOM (Unpublished data).

Mucosal immunity plays a critical role in control of *Spn* locally invasive infections such as pneumonia, AOM and sinusitis.¹³⁴ *Spn* colonization stimulates mucosal antibody levels in nasal secretions to PhtD, PcpA and PlyD1 proteins.^{38,135} We have shown that higher mucosal antibody levels in the NP to PhtD, PcpA and PlyD1 correlates with reduced risk of AOM in young children caused by *Spn*.¹³⁵ We also showed that antibody levels in the NP correlated with serum antibody levels.¹³⁵

For a vaccine to prevent AOM, we contend that special attention should be given to studies in the infection prone child who has an immune response that is generally deficient compared to non-otitis prone children. We have shown that children who are prone to otitis media have lower antibody levels to PhtD, PhtE, PcpA, LytB and Ply after *Spn* colonization and AOM. However, among the 5 proteins PcpA stood out as more immunogenic than the others.^{38,135,136}

Functional Antibodies to Spn Protein Candidates

Protein based Spn vaccines currently under evaluation lack a robustly validated functional assay as a correlate of their

efficacy. In the context of protein-based vaccines, there is a need for a reliable assay to assess the efficacy of antibodies.

We have applied a flow cytometry based assay to evaluate the functional efficacy of human anti-adhesin PhtD, PhtE and PcpA antibodies in reducing Spn adherence in vitro.^{112,137} Unlike traditional CFU-based methods, this assay is highly sensitive and robustly reproducible. We are further evaluating the role of aforementioned antigen specific antibodies for their ability to induce opsonophagocytic/bactericidal response and developing assays that can be used as surrogate functionality assays in conjunction with the established adherence assay.

Animal Studies

In order to support our clinical findings, we have also tested the efficacy of PhtD, PhtE, PcpA and Ply vaccine candidates in a number of mouse infection models. Our mouse studies have highlighted the role of PhtD and PhtE in Spn NP colonization.¹¹³ In our laboratory we have been studying the effect of vaccination with adhesion proteins of Spn in an Spn-Flu A coinfection model and we have established a carriage density threshold in the NP that is required for local and systemic invasion. Moreover, we found that vaccination with PhtD reduces the density of Spn carriage sufficiently to provide protection in an invasive Spn-Influenza A coinfection model (*Khan and Pichichero: unpublished data*).

In a mouse co-infection model, co-colonization with *H. influenzae* results in a rapid depletion of Spn from the NP within 3 days post-inoculation and complete clearance by 2 weeks.⁵⁷ This is in contrast to Spn colonization without *H. influenzae* that remains stable for 3–4 weeks.⁵⁷ Based on our ongoing work involving the role of PhtD in a complex Spn-influenza A co-infection model, it is plausible that even PhtD-induced reduction in Spn carriage inocula in the NP would confer indirect benefits by bringing down the level of Ply in the local NP environment (*Khan and Pichichero, unpublished data*). Because young children are at the greatest risk of Spn infections and a

primary target population for vaccination,²⁰ a preferred animal model for vaccine development should be an infant mouse as it more closely mimics the maturing immunological state of young children. Therefore recently an infant murine vaccination model has been developed⁹⁸ and used to evaluate protection from pneumonia by pneumococcal protein vaccine candidates PhtD, PcpA and Ply.^{97,98,100}

Protein Vaccine Studies in Special Populations/Geographies

In 2012, Sanofi Pasteur commenced (with International Center for Diarrheal Research, Bangladesh) a phase I clinical trial of a vaccine containing three Spn proteins (PcpA, PhtD, PlyD1) in different dosage forms (NCT01764126). This was an observer-blind, randomized, vaccine controlled, vaccine trial to determine the safety and immunogenicity of the vaccine in an age down approach beginning with adults, then toddlers and infants, with data safety review at each step before stepping down to the next age group. In a separate phase I study conducted in Switzerland, promising safety profiles and immunogenicity of monovalent (PhtD or PcpA) and bivalent (PhtD and PcpA) protein vaccine candidates were demonstrated in an adult population.¹³⁸ In another clinical trial, GSK is working with PATH, the Medical Research Council of The Gambia, and London School of Tropical Medicine and Hygiene in a phase II clinical study in the Gambia of GSK's protein-plus-conjugate vaccine candidate^{139,140} (Table 3).

There is empirical evidence to suggest that different populations in different geographical regions might exhibit differences in response to vaccinations. For instance, efficacy of BCG in the prevention of pulmonary TB ranges between 0 and 80%; nearly half of this variability can be explained by population/geographical differences among study sites. For example, clinical trials show good protection against adult pulmonary TB in the UK, but little protection in Malawi.^{141–143} A recent study compared the ontogeny of TLR induced immune responses from study

populations of different population/geographical regions. A significant divergence in innate immune response was noted.^{68,70} Composition of the NP microflora is a key factor in the transition of Spn from commensal to pathogen, and a substantial difference in the microflora between different populations/geographical regions has been reported.⁶¹ Outside first world countries, routine and over-arching immunization programs against Flu A, Spn, and *Haemophilus influenzae* are not performed. This increased microbial burden in the NP along with a number of other crucial factors including malnutrition, poor environmental air quality, and genetics not only predisposes some populations to increased susceptibility for infections but may also lead to compromised vaccine efficacy or vaccine hyporesponsiveness (Table 4). Therefore, it is plausible that as explained above in the case of BCG, Spn protein vaccines might have different efficacies in diverse geographical regions having differential upper respiratory polymicrobial niche in response to routine vaccinations.

Conclusions

The cost of Spn conjugate vaccination is a major global concern. PCV formulations are expensive to produce because the product is given as a single shot but consists of 7, 10 or 13 vaccines. A protein-based vaccination strategy likely will require less or no boosting because natural boosting should continue to occur since Spn will not be eliminated as a colonizing bacteria. However, it should be noted that a vaccine inducing a moderate immune response might be sufficient for invasive but not non-invasive infections.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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