Cell Host & Microbe
Short Article

T_H17-Based Vaccine Design for Prevention of *Streptococcus pneumoniae* Colonization

Kristin L. Moffitt,[1,5] Todd M. Gierahn,[2,5] Ying-jie Lu,[1] Paulo Gouveia,[2] Mark Alderson,[3] Jessica Baker Flechtner,[2] Darren E. Higgins,[4] and Richard Malley[1,*]
[1]Division of Infectious Diseases, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115, USA
[2]Genocea Biosciences, Cambridge, MA 02142, USA
[3]PATH, Seattle, WA 98109, USA
[4]Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115, USA
[5]These authors contributed equally to this work
*Correspondence: richard.malley@childrens.harvard.edu
DOI 10.1016/j.chom.2011.01.007

SUMMARY

***Streptococcus pneumoniae* is a leading cause of mortality in young children. While successful conjugate polysaccharide vaccines exist, a less expensive serotype-independent protein-based pneumococcal vaccine offers a major advancement for preventing life-threatening pneumococcal infections, particularly in developing nations. IL-17A-secreting CD4+ T cells (T_H17) mediate resistance to mucosal colonization by multiple pathogens including *S. pneumoniae*. Screening an expression library containing >96% of predicted pneumococcal proteins, we identified antigens recognized by T_H17 cells from mice immune to pneumococcal colonization. The identified antigens also elicited IL-17A secretion from colonized mouse splenocytes and human PBMCs suggesting that similar responses are primed during natural exposure. Immunization of two mouse strains with identified antigens provided protection from pneumococcal colonization that was significantly diminished in animals treated with blocking CD4 or IL-17A antibodies. This work demonstrates the potential of proteomic screening approaches to identify specific antigens for the design of subunit vaccines against mucosal pathogens via harnessingT_H17-mediated immunity.**

INTRODUCTION

Recent estimates indicate that *Streptococcus pneumoniae* causes 11% of mortality in children under the age of five worldwide (Huang et al., 2005; O'Brien et al., 2009). Current conjugated polysaccharide vaccines effectively prevent most invasive pneumococcal disease caused by vaccine-type strains, but their high manufacturing costs and increases in the rates of disease caused by pneumococci-expressing capsules not covered by current vaccines (Huang et al., 2005; Singleton et al., 2007) have made creating an inexpensive vaccine based on conserved pneumococcal protein antigens a high global health priority. A vaccine based on noncapsular protein antigens that are well-conserved amongst the >90 known pneumococcal serotypes would prevent immunologic escape through serotype replacement and would significantly lower the cost of vaccine manufacture.

Expanded availability of pneumococcal genomic information has facilitated development of genome-based approaches for protein antigen identification. Efforts thus far have focused on identifying surface-exposed proteins that can be bound by circulating antibody and thereby direct clearance of the pathogen through similar mechanisms to polysaccharide-based vaccines (Giefing et al., 2008; Wizemann et al., 2001). Although several proteins have been evaluated in Phase I clinical trials (Briles et al., 2000; Nabors et al., 2000; Nagy, 2010), it is currently unknown whether antibodies elicited against pneumococcal protein antigens will be as effective as anticapsular antibodies in providing protective immunity against pneumococcus in humans.

During childhood, the incidence of pneumococcal disease caused by a broad range of serotypes declines years before natural acquisition of anticapsular antibodies (Lipsitch et al., 2005), suggesting other mechanisms provide natural immunity to pneumococcus. Studies in mice have shown that acquired immunity to pneumococcal colonization either after mucosal exposure to live bacteria (Trzcinski et al., 2005) or elicited by intranasal immunization with killed unencapsulated pneumococcal whole-cell antigen (WCA) (Malley et al., 2005) is antibody independent and CD4+ T cell dependent. This immunity was unchanged in mice that genetically lacked antibodies, IFNγ, or IL-4, but was completely abrogated in mice treated with neutralizing anti-CD4 or anti-IL-17A antibody or in mice genetically lacking the IL-17A receptor, thus identifying the likely effector cells as IL-17A-producing CD4+ T_H17 cells. A similar role for IL-17 signaling in pathogen clearance has been observed in mouse models of infection for at least 12 other mucosal pathogens (Curtis and Way, 2009; O'Connor et al., 2010), indicating this pathway plays a general role in clearance of pathogens at mucosal surfaces. Furthermore, humans lacking T_H17 cells because of genetic mutation are highly susceptible to mucosal infections by pathogens such as *Staphylococcus aureus, Haemophilus influenzae* and *S. pneumoniae* (Milner et al., 2008), indicating that T_H17 cells may also be playing a role in natural immunity to important mucosal pathogens of humans.

Here, we report a comprehensive proteomic screening approach to identify pneumococcal T cell antigens that activate T_H17 cells isolated from immune mice. We show that the identified antigens are effective mucosal immunogens that protect mice from nasopharyngeal colonization in a CD4+ T cell- and IL-17A-dependent manner. The identified antigens stimulate IL-17A secretion from splenocytes isolated from mice previously exposed to live pneumococcus, indicating that the antigens are effectively presented during mucosal colonization. Similarly, human peripheral blood mononuclear cells (PBMCs) secrete IL-17A when stimulated with the antigens, indicating similar T_H17 responses are primed during natural exposure to pneumococcus. The identified antigens represent strong candidates for a protein subunit vaccine designed to prevent colonization by *S. pneumoniae*. This work additionally describes the use of a powerful proteomic screening method for vaccine development against pathogens where infection of the human host begins with mucosal colonization.

RESULTS

Generation of a *S. pneumoniae* Expression Library

An expression library containing 1458 full-length ORFs from the *S. pneumoniae* genome acquired from the Pathogen Functional Genomic Resource Center (PFGRC) was cloned into an inducible expression vector that fuses the in-frame H2-K^k CD4+ T cell epitope (DEVSGLEQLESIINFEKL) from ovalbumin ($OVA_{247–264}$) to the 3′ end of each insert. Seven hundred forty-nine additional ORFs not represented in the PFGRC library were PCR amplified and cloned from TIGR4 genomic DNA, yielding a library that contained 2207 of the predicted 2233 ORFs in the TIGR4 genome. The protein expression of each clone was determined by assaying for the presence of the C-terminal OVA epitope tag fused to each protein. KZO T cell hybridoma cells, which are specific for the $OVA_{247–264}$ epitope (Sanderson et al., 1995), were added to cultures of H2-K^k macrophages that had been pulsed with each induced clone in the library. Upon activation, KZO cells upregulate production of β-galactosidase, which we measured by using the colorimetric substrate chlorophenol red-β-D-galactopyranoside (CPRG). Activation of KZO cells by macrophages pulsed with a clone indicates the clone was expressed to full length and was successfully delivered to the MHC class II presentation pathway. Ninety-three percent (2048/2207) of the clones yielded detectable KZO activation (Figure S1, available online). To further increase proteomic coverage of the library, we recloned ORFs that were not successfully expressed as overlapping fragments. Forty-six percent (155/340) of the gene fragments induced KZO activation, bringing the estimated final coverage of the expression library to 95% of the total proteome sequence of *S. pneumoniae.*

Identification of Antigens Recognized by T_H17 cells Isolated from WCA-Immunized Mice

The validated library was used to screen CD4+ T cells isolated from C57BL/6 and BALB/c mice that were previously immunized intranasally with WCA with cholera toxin, a preparation known to elicit broad, antibody-independent, IL-17A- and CD4+ T cell-dependent protection against pneumococcal colonization in mice (Lu et al., 2008; Malley et al., 2001), thereby overcoming the possibility of incomplete protection from colonization that has been demonstrated after exposure to live pneumococci (Malley et al., 2005). Pools of four library clones were pulsed onto thioglycollate-induced peritoneal macrophages seeded onto a 96-well plate. After incubation, CD4+ T cells isolated from WCA-immunized mice were added. The amount of IL-17A present in the supernatants of each well after 3 days of culture was determined to identify pools that activated T_H17 cells. Three screens of CD4+ T cells isolated from independent cohorts of WCA-immunized BALB/c or C57BL/6 mice were completed with the pooled library. A sample T cell screen is shown in Figure S2A. Interestingly, a comparison of the IFN-γ concentration to the IL-17 content in the same supernatants demonstrated that pools eliciting the highest IL-17 responses were not the same as those eliciting the highest IFN-γ responses (Figures S2B and S2C), indicating that using IL-17 as the screen readout highlights different antigens from when a more traditional cytokine like IFN-γ is used. A total of 127 pools induced an IL-17A response greater than a threshold of 2 median absolute deviations (MAD) above the median of all data points (positive response) in at least two of three replicates of either mouse strain. All clones present in pools inducing a positive response in at least one replicate were screened individually against CD4+ T cells isolated from WCA-immunized mice. In these secondary screens, 100 clones induced a response >2 MAD above the median in secondary screens of either BALB/c or C57BL/6 CD4+ T cells. A screen of CD4+ T cells isolated from naive mice yielded negligible responses (maximum 9.2 pg/ml, median 0.84 pg/ml) indicating these responses were specific to immunized mice. The insert of each positive clone was sequence verified and submitted for bioinformatic analysis.

Prioritization of Antigens through Bioinformatic Analysis

The sequence of each putative antigen was analyzed by using a set of bioinformatic filters to identify the most promising vaccine candidates. Top candidates were required to have a homolog of >90% amino acid sequence identity in all 22 of the sequenced *S. pneumoniae* genomes, to have no homology to human proteins, and to be >100 amino acids in length. Low-sequence conservation (<40% identity) with other sequenced bacterial genera was also favored to minimize the chance of immunologic cross-reactivity with other bacterial species. Seventeen antigens met all bioinformatic filters (Table 1). Five antigens prioritized based on expression levels in *Escherichia coli*, SP0148, SP2108, SP0882, SP1634, and SP0314.1, were successfully produced recombinantly with a C-terminal His_6-tag and purified through Ni^{2+} affinity purification.

Selected Antigens Are Presented during the Course of Pneumococcal Exposure

To assess whether the identified antigens are well presented during pneumococcal exposure, we evaluated IL-17A responses of experimentally colonized mice in vitro. The purified antigens were used to stimulate splenocytes isolated from C57BL/6, BALB/c, and CD1 mice that had been intranasally inoculated three times with a clinical type 6B *S. pneumoniae* strain. In all mouse strains, splenocytes secreted significant amounts of

Table 1. Top T_H17 Cell Antigens Selected by Bioinformatic Filters

TIGR4 ORF Locus	Annotated Function	Positive Screens (n=6)	Size (aa)	SPN Sequence Identity (%)	Bacterial Sequence Identity (%)	Human Homolog
SP0148	ABC transporter	5	276	99	32	N
SP0314.1	Hyaluronidase	3	1066	99	31	N
SP1919	ABC transporter	3	273	97	38	N
SP0335	Cell division protein FtsL	2	105	100	34	N
SP0562	Conserved hypothetical	2	444	97	36	N
SP0662	Sensor histidine kinase	2	563	99	29	N
SP1533	Conserved domain protein	2	170	98	0	N
SP1634	Hypothetical protein	2	357	97	33	N
SP1652	Putative membrane protein	2	924	98	32	N
SP1712	Hypothetical protein	2	389	97	29	N
SP1988	Putative immunity protein	2	677	98	21	N
SP2108	Maltose-binding protein	2	423	99	32	N
SP0790	Conserved domain protein	1	271	99	29	N
SP0882	Conserved hypothetical	1	274	99*	38	N
SP1754	Conserved hypothetical	1	317	99	33	N
SP1858	Transcriptional regulator	1	178	99	27	N
SP2002	Conserved hypothetical	1	245	94	36	N

The antigens identified in the screens of T_H17 cells from WCA-immunized mice that met all bioinformatic filters are listed with their annotated function, size, the number of times the antigen pool was above the positive-response threshold, the percent sequence identity in 22 sequenced *S. pneumoniae* strains, the percent sequence identity to the closest known homolog outside the *Streptococcus* genus and whether a human homolog of the protein was detected by BLAST analysis.

* Data for this protein are based on first N-terminal 130 amino acids.

IL-17A when stimulated with SP2108 or SP0148 compared to medium alone (Figure 1A). Stimulation with SP0882 induced statistically significant IL-17A secretion in both BALB/c and CD1 mice, but not in C57BL/6 mice. In contrast, stimulation with recombinant pneumolysoid (PdT), a known pneumococcal antibody target (Paton et al., 1991), did not induce IL-17A in any of the mouse genetic backgrounds examined. Splenocytes isolated from C57BL/6 mice after a single intranasal exposure with type 6B secreted significant amounts of IL-17A when stimulated with SP2108 or SP0148 (Figure 1B) suggesting these antigens are readily presented and processed in the course of mucosal exposure.

The purified antigens were then used to stimulate human PBMCs from healthy adult donors to determine whether humans prime T_H17 cells specifically for the selected antigens during the course of natural exposure to *S. pneumoniae*. After 6 days in culture, 81% (9/11) of donors had a demonstrable IL-17A response (>50 pg/ml) after WCA stimulation compared to the DMEM control (Figure 1C); these donors were used in the subsequent analyses of the purified antigens. All 11 donors had an IL-17A response to phytohemagglutinin (minimum 349 pg/ml, maximum 21,111 pg/ml, median 1860 pg/ml) indicating appropriate T cell activation in the assay. To adjust for nonspecific responses, we compared the IL-17A response of each donor to recombinant green fluorescence protein (GFP), similarly purified from *E. coli* as with the pneumococcal proteins, to the responses of each pneumococcal protein stimulus. The IL-17A responses to SP2108 and SP0148 in the population were significantly higher than the GFP stimulations ($p < 0.05$, Wilcoxon rank sum test) (Figure 1C). The responses to SP0314.1 and SP0882 were not statistically different from the responses to GFP in the population sampled, though some individuals may be responding. Taken together, these findings suggest that SP2108 and SP0148 are accessible to antigen-presenting cells during the course of mucosal colonization and effectively processed for presentation to CD4+ T cells and that these responses are not MHC haplotype specific.

Antigens Identified in the Murine T_H17 Cell Screens Protect Mice from Colonization by *S. pneumoniae*

The protective efficacy of immunization with each antigen was next determined in a mouse model of pneumococcal nasopharyngeal colonization. Groups of C57BL/6 mice were immunized twice intranasally with the recombinant antigens and cholera toxin as an adjuvant. Four weeks after the final immunization, the animals were challenged with a live type 6B clinical *S. pneumoniae* strain. One week after challenge, mice immunized with SP2108, SP0148, and SP0882 had significantly lower levels of pneumococcus in nasal washes compared to animals immunized with the nonspecific ICP47 protein from herpes simplex virus 2 (Figure 2A). Mice immunized with SP0314.1, SP1634, or the single-repeat PASTA domain of serine/threonine kinase (StkP-R), a protective antibody target (Giefing et al., 2008), had bacterial burdens not significantly different from the nonspecific ICP47 group (Figure 2A, data not shown for SP1634). Additionally, four other pneumococcal proteins that did not meet criteria for selection as T_H17 cell antigens from the screens were expressed and purified and used as mucosal vaccines with CT in C57BL/6 mice; these were not protective against colonization (data not shown).

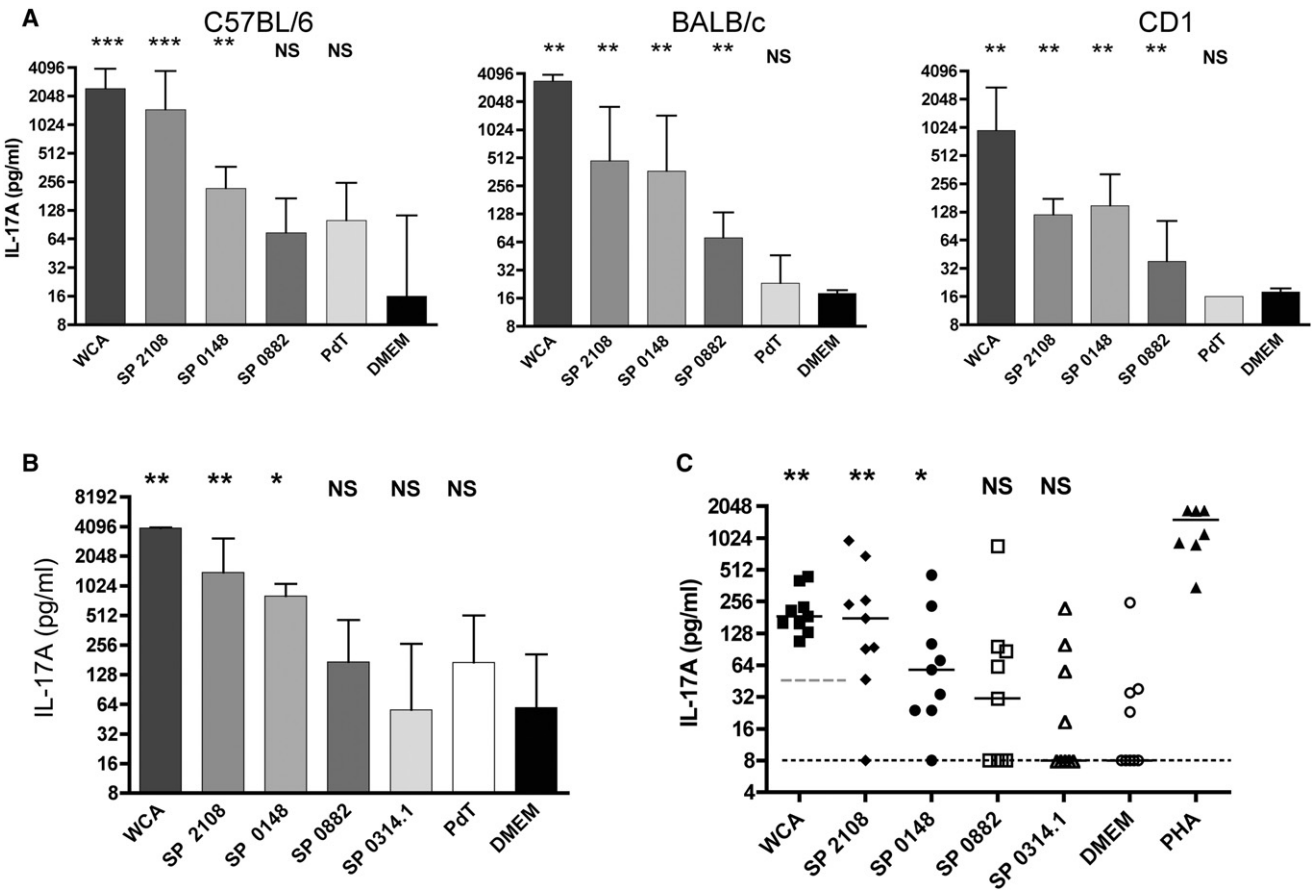

Figure 1. SP2108 and SP0148 Are Recognized by T_H17 Cells of Mice Colonized with Pneumococcus and Adult Human Volunteers

(A) Antigen-specific IL-17A secretion by splenocytes isolated from mice after intranasal exposure to live pneumococcus three times at 1 week intervals. Bars represent median IL-17A values with interquartile range. *p < 0.05, **p < 0.01, ***p < 0.001 by Mann-Whitney test when compared with secretion of IL-17A in response to medium (DMEM) alone. NS = not statistically different.

(B) Antigen-specific IL-17A secretion by splenocytes of C57BL/6J mice 10 days after a single intranasal exposure to live pneumococcus.

(C) The amount of IL-17A secreted by human PBMC stimulated with the indicated antigen. Nine of eleven donors had a response to WCA above the 50 pg/ml IL-17A cutoff (dashed gray line). Only WCA responders are shown here and were included in analysis. Each symbol represents the IL-17A value from a single donor with the GFP-induced IL-17A response subtracted. Median GFP value was 52 pg/ml. Bars represent median values. *p < 0.05, **p < 0.01 by paired Wilcoxon rank sum test when compared with IL-17A secretion after stimulation with GFP for the protein antigens or DMEM alone (median DMEM value at limit of detection; dotted black line) for WCA. NS = not statistically different.

The lack of protection from SP0314.1 or StkP-R immunization was not due to poor T cell priming by these antigens. Whole blood cells isolated 1 week prior to challenge from mice immunized with SP0882, SP0314.1, and StkP-R secreted similar amounts of IL-17A when stimulated with the immunizing protein (Figure 2B), but of the three antigens only immunization with SP0882 induced protection suggesting that presentation of the antigen in the context of natural colonization by *S. pneumoniae* is critical for protective efficacy of the vaccination. Interestingly, the IL-17A response elicited by WCA stimulation of the whole blood samples strongly correlated with the protective efficacy of the immunogen (Figure 2C, Spearman ρ coefficient = -0.6120 with $p < 0.0001$), further suggesting that only antigens that successfully compete for the antigen processing and presentation machinery in the context of the entire bacterium can induce protective immunity. Immunization with SP0148 and SP2108 also induced significant protection from pneumococcal colonization in BALB/c mice (Figure S3) indicating protection stimulated by the antigens is not MHC haplotype restricted.

Protection from Colonization by *S. pneumoniae* Is CD4+ T Cell and IL-17A Dependent

The mechanism of protection elicited by the immunizations was analyzed by treating C57BL/6 mice previously immunized with a combination of SP2108, SP0148, and cholera toxin with anti-CD4 or anti-IL-17A antibody to deplete CD4+ cells or inhibit the IL-17A signaling pathway, respectively. Both CD4+ T cell depletion and neutralization of IL-17A eliminated the protection elicited by immunization with the combination of proteins (Figure 3A). Animals similarly immunized and treated with an isotype control antibody were well protected against colonization. Flow cytometric analysis of splenocytes from antibody-treated mice confirmed systemic CD4+ T cell depletion in the anti-CD4 group

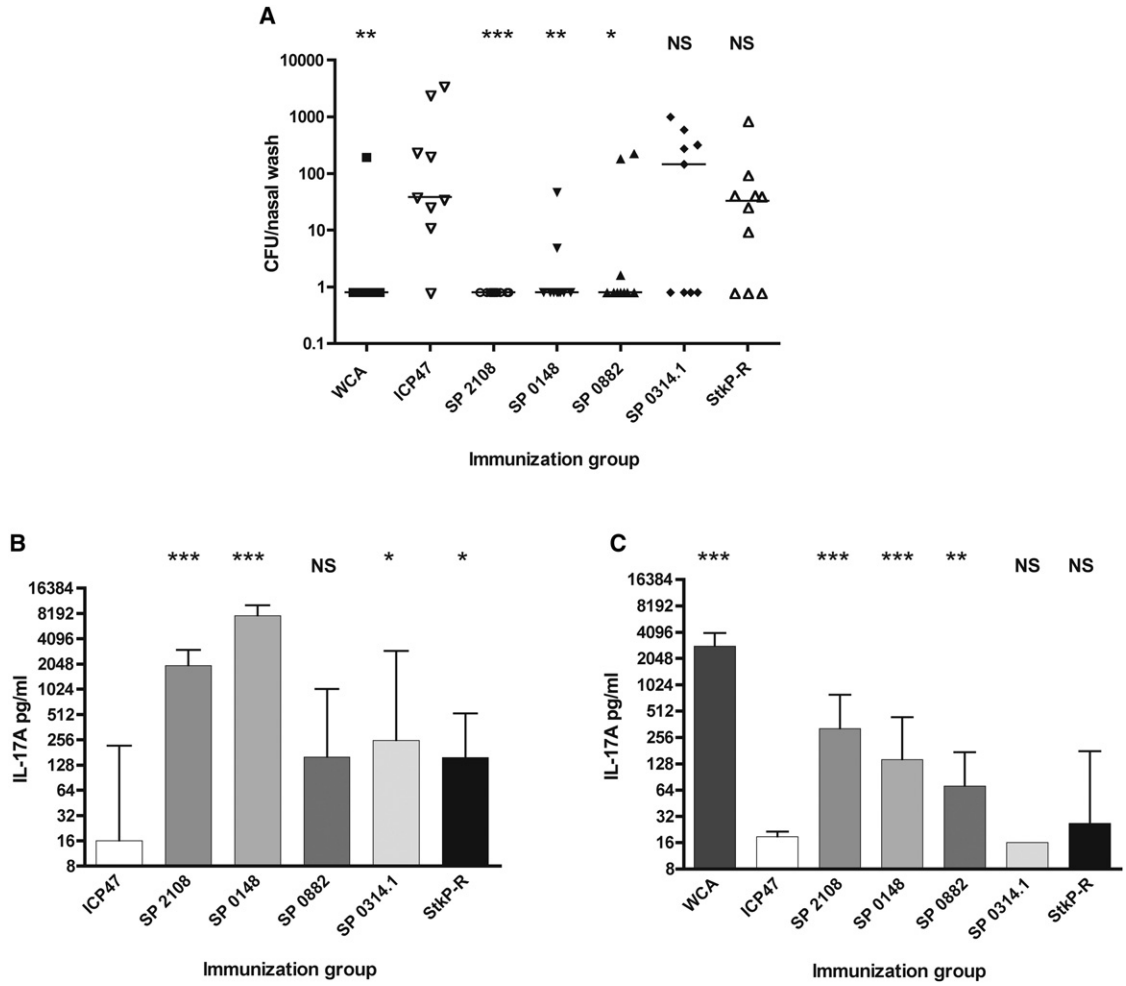

Figure 2. Intranasal Immunization with SP2108, SP0148, and SP0882 Induces T_H17 Responses and Protection against Pneumococcal Colonization

(A) Enumeration of pneumococcus in nasal washes 7 days after intranasal challenge by pneumococcus in C57BL/6 mice that were previously immunized with the indicated antigen and cholera toxin. Bars represent median CFU per immunization group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with ICP47-immunized group by Mann-Whitney test.

(B and C) IL-17A secretion by whole blood cells isolated from the same immunized mice prior to challenge when stimulated with (B) the immunizing protein or (C) WCA. Bars represent median IL-17A values with interquartile range. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Mann-Whitney test when compared with ICP47-immunized group. NS = not statistically different.

and maintenance of CD4+ cells in the anti-IL-17A group (Figure 3B). These data demonstrate that protection elicited by the immunizations is dependent on CD4+ T cells and IL-17A secretion.

DISCUSSION

The successes of the pneumococcal conjugate vaccine trials in The Gambia and in South Africa, as well as the remarkable reduction in pneumococcal invasive disease observed after implementation of the conjugate vaccine in the United States, gave rise to the possibility that with expanded serotype coverage, worldwide pneumococcal disease could be significantly prevented or controlled. However, the issues of cost, complexity of manufacturing, and the emergence of nonvaccine serotypes in several countries have tempered this view. While current efforts are promoting the use of appropriately broadened pneumococcal conjugate vaccines in developed and developing countries, the need for alternative approaches to vaccination against pneumococcus remains urgent.

Several groups have evaluated candidate vaccines based on antigens that were selected on the basis of protection against invasive pneumococcal disease in animal models (Alexander et al., 1994; Briles et al., 2003; Giefing et al., 2008; Glover et al., 2008; Ogunniyi et al., 2000). In general, while these antigens have demonstrated protection against a variety of strains in different mouse models of invasive disease, their protective efficacy against mucosal colonization and/or infection has been marginal or has not been evaluated. Here, we show the use of a proteomic screening approach to identify several previously unknown protein vaccine candidates for *S. pneumoniae* that provide near complete protection from pneumococcal

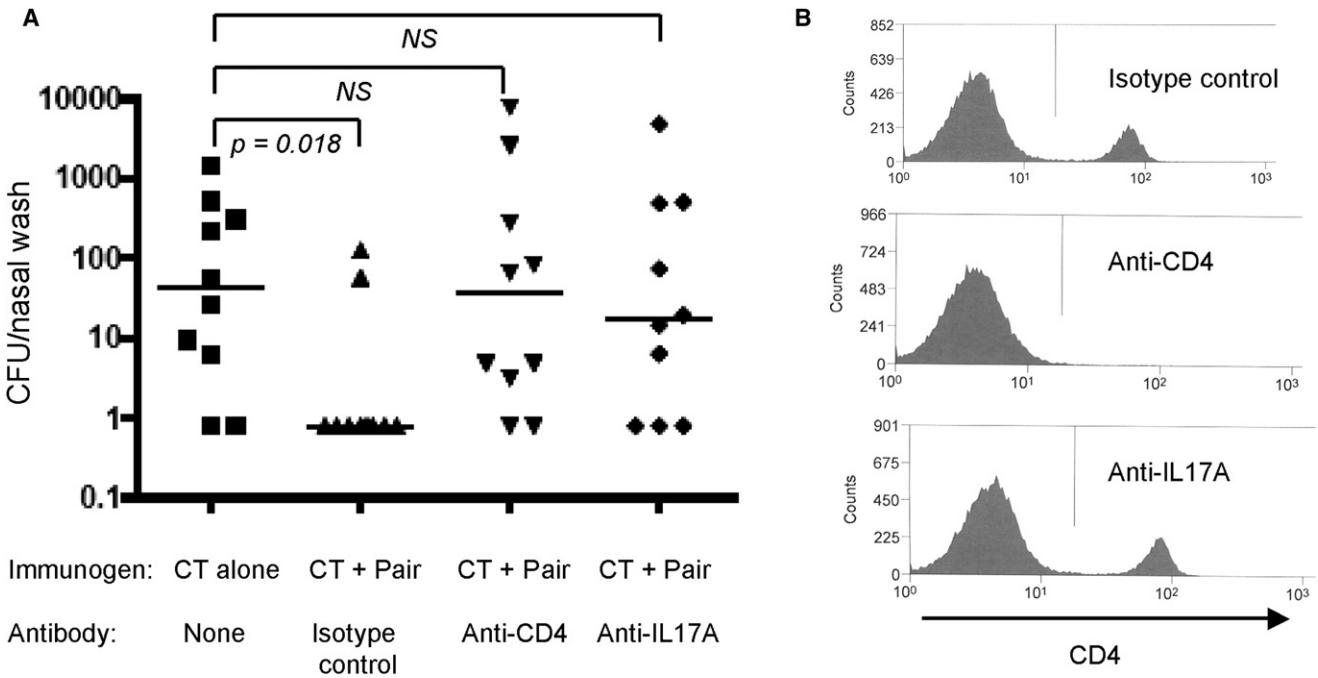

Figure 3. SP2108 and SP0148 Protect Mice from Pneumococcal Colonization in a CD4+ T cell- and IL-17A-Dependent Fashion

(A) Enumeration of pneumococcus in nasal washes 7 days after intranasal challenge of C57BL/6 mice that were previously immunized with a mixture of SP2108, SP0148 (Pair), and cholera toxin (CT) and then treated with anti-CD4, anti-IL-17A, or an isotype control antibody. Bars represent median CFU per group. p values for bracketed groups are indicated. NS = not statistically different.

(B) Flow cytometric analysis of CD4+ cells in spleens of immunized mice treated with anti-CD4, anti-IL-17A, or an isotype control antibody.

colonization through a T_H17-mediated mechanism. Furthermore, we propose that this approach can be applied towards the identification of vaccine candidates for other mucosal pathogens. With respect to pneumococcus, both in vitro stimulation experiments and in vivo protection studies indicate that protective T_H17 antigens may differ significantly from traditional antibody targets (Table 1). Many well-characterized pneumococcal virulence factors were not identified in our screens, including pneumolysin, pneumococcal surface adhesin A (PsaA), and StkP, among others; pneumococcal surface protein A (PspA) was identified in one C57BL/6 screen only. Furthermore, StkP-R and the pneumolysin toxoid PdT induced poor T_H17-mediated protection or T_H17 responses in immunized or exposed mice, respectively. These results, obtained by using targets of antibody-mediated protection, suggest that antigens recognized by T_H17 cells may not be the same as those recognized by protective antibodies. Supporting this view, two of the protective T_H17 antigens, SP0148 and SP0882, have not to our knowledge been previously described as antibody targets giving further validity to our approach of identifying antigens recognized directly by protective T_H17 cells.

Several of the identified pneumococcal antigens did not induce immunologic protection from colonization despite the presence of a T_H17 response to the antigen, indicating that the subsequent selection of appropriate antigens is essential for the development of an effective vaccine designed to prevent colonization. As demonstrated by the in vitro IL-17A responses of splenocytes from mice after brief exposure to live pneumococcus (Figure 1B), there is a clear hierarchy in the IL-17A responses with protective antigens (SP2108 and SP0148) eliciting demonstrably higher IL-17A than antigens that were identified but not protective (SP0314.1). This suggests that effective presentation of the antigen to CD4+ T cells in the context of the entire bacterium in vivo is an important determinant of which antigens induce protective T_H17 cell responses.

The efficacy and safety of vaccines designed to prevent pathogen colonization and subsequent disease through T_H17-mediated immunity remain to be demonstrated in humans and may present additional challenges in vaccine design, such as the route of delivery and appropriate adjuvant selection. As recent work has shown that CT adjuvant favors a T_H17-mediated response to bystander immunogens (Datta et al., 2010; Lee et al., 2009), these proteins will need to be tested with clinically appropriate adjuvants and by other routes. Additionally, an ideal pneumococcal vaccine candidate would combine protection against colonization of mucosal surfaces as well as invasive disease. A vaccine consisting of a combination of antigens that promote protective IL-17A responses and elicit protective antibodies would have significant advantages. Given the well-documented safety and efficacy of pneumococcal polysaccharide conjugates, one could envision a vaccine combining polysaccharide conjugates and T cell antigens, or even conjugates in which the protein carriers are the T cell antigens of interest. Looking ahead, the clinical evaluation of such a combination vaccine could entail a comparison with existing pneumococcal conjugate vaccines, which would have significant advantages for the design of an efficacy trial in children in developed or developing countries.

More generally, the approach we present here may open avenues for the rational design of vaccines for other mucosa-colonizing pathogens. As noted above, there are many pathogens for which a role for IL-17A-mediated protection is suggested in humans (as in the case of patients with Job's syndrome and increased susceptibility to *S. aureus* infections; Milner et al., 2008) or demonstrated in animal models (such as *Mycobacterium tuberculosis*, *Bordetella pertussis*, *Listeria monocytogenes*, among others; Aujla et al., 2007; O'Connor et al., 2010). These pathogens are major causes of morbidity and mortality worldwide and have thus far eluded traditional antibody-driven vaccinology approaches. The antigen-screening tool we describe here offers the possibility to identify and evaluate proteins that may confer significant protection against colonization and/or disease, and thus accelerate the development of vaccines directed against these important pathogens.

EXPERIMENTAL PROCEDURES

Media and Reagents

cRPMI contains RPMI-1640 media supplemented with 10% FBS (Hyclone), 1 mM sodium pyruvate, 2 mM L-glutamine, 50 mM β-mercaptoethanol, 200 U/ml penicillin, and 200 g/ml streptomycin. cDMEM/F12 was composed of DMEM/F12 media supplemented with 10% FBS, 2 mM L-glutamine, 50 mM β-mercaptoethanol, and 10 mg/ml ciprofloxacin.

Expression Library Generation

The expression plasmid pDESTSL4.8 was derived from pDEST17 (Invitrogen) by adding the OVA epitope tag (GTGCTGTTGCCTGATGAAGTCTCAGGC CTTGAGCAGCTTGAGAGTA TAATCAACTTTGAAAAACTG) after the 3′ end of the $attR_2$ DNA sequence in pDESTS17 by sequentially amplifying pDEST17 with the primer sets pDEST17-1 and pDEST17-2 (Table S1) and then ligating the SacI and NheI digested PCR product to the annealed oligo set pDESTSL4.8 oligo1 and pDESTSL4.8 oligo2 (Table S1). A library of 1458 DONR clones containing genes of the *S. pneumoniae* TIGR4 genome acquired from the NIAID/PFGRC was transferred into pDESTSL4.8 by using Gateway LR clonase (Invitrogen). ORFs missing in the NIAID/PFGRC library (http://pfgrc.jcvi.org/index.php/home.html) or overlapping fragments of nonexpressing genes were amplified from TIGR4 genomic DNA with the primers listed in Table S1. Each PCR product was cloned into pDONR211 (Invitrogen) and then transferred to pDESTSL4.8 by using Gateway clonases. The library was expressed in the BL21RAIL *E. coli* strain. BL21RAIL was derived from BL21AI by genetic deletion of *lacZ-lacA* and *recA* by using the BL21RAIL primer sets in Table S1 and the lambda red system according to previously described protocols (Datsenko and Wanner, 2000). Expression of each clone was induced in BL21RAIL through arabinose induction. Bacteria were fixed with 0.5% paraformaldehyde, washed, resuspended in cRPMI at a concentration of 2×10^8 bacteria/ml, and frozen at −80°C.

Expression Library Validation

Peritoneal macrophages were isolated from C3H/HeNCrl mice (Charles River) by thioglycollate elicitation as described (Zhang et al., 2008). An aliquot of the library was added to the macrophages (5×10^4 cells/well in a 96-well plate). Purified ovalbumin was added to two wells on each plate to a final concentration of 10 μg/ml. The cells were incubated for 2 hr and then fixed with 1% paraformaldehyde. After extensive washing, 1×10^5 KZO cells was added to each well. After 18 hr of culture incubation, the amount of β-galactosidase produced was measured as described (Sanderson and Shastri, 1994). Positive expression was defined as a KZO response that was >10% of the response induced by recombinant OVA.

Primary T Cell Screening

CD4+ T cells were isolated by magnetic sorting (Miltenyi Biotec) from splenocytes pooled from five WCA-immunized mice. Pools of four library clones (4×10^7 bacteria/well) were pulsed onto thioglycollate-elicited macrophages (5×10^4 cells/well). After incubation, macrophage fixation, and washing, 1×10^5 T cells was added to each well in cDMEM/F12. After 3 days of incubation, the amount of IL-17A in the supernatant from each well was measured by ELISA (R&D Systems). The absorbance values from the ELISA were normalized by subtracting the median of all data points and then dividing by the MAD of the screen. Pools with normalized values >2 were defined as positive for the screen. The screens of individual clones were conducted with the same protocol except individual clones from the library rather than pools were pulsed directly onto the macrophages.

Bacterial Strains, Immunogens, and Animal Studies

S. pneumoniae strain 0603 is a serotype 6B clinical strain described previously (Malley et al., 2001). The WCA was derived from Rx1AL-, a capsule- and autolysin-negative mutant and was prepared as described (Malley et al., 2001). Recombinant antigens were expressed in BL21 (DE3) from the pET24b expression vector and then purified through Ni^{2+} affinity chromatography as described by the manufacturer (Qiagen). SP2108, SP0148, SP0314.1, StkP-R, ICP47, PdT, and GFP were purified under native conditions. SP1634 and SP0882 were solubilized from inclusion bodies with 8 M urea and were refolded in TBS media. LPS was depleted from the purified proteins through repeated extractions with Triton X114 (Adam et al., 1995) until LPS concentration was <1 EU/μg protein. Purified proteins were used as immunogens with 4 μg of each protein and 1 μg CT (List Biological Laboratories) per 20 μl intranasal vaccine dose. The animal model of immunization followed by colonization has been described previously (Malley et al., 2001). Colonizing exposure was performed similarly to challenge, except animals were exposed three times intranasally at 1 week intervals. Stimulation of mouse splenocytes and whole blood and IL-17A measurements were performed as described (Lu et al., 2008). All animal work was performed in accordance with institutional guidelines approved by the IACUC of Children's Hospital Boston and Harvard Medical School.

Human PBMC Stimulation

Human PBMCs were isolated by Ficoll™ density gradient centrifugation from blood collected from blinded, adult platelet aphaeresis donors at Children's Hospital Boston. 1×10^6 PBMCs were plated per well of a 12-well plate suspended in 1 ml of cDMEM/F12. Purified proteins were used for stimulation at a final concentration of 1 μg/well. After 5 days of incubation, supernatants were collected and stored at −20°C until analyzed by ELISA for IL-17A content (eBiosciences, San Diego, CA).

Anti-CD4+ and Anti-17A Treatment and Flow Cytometry for CD4+ T Cell Populations

Animals were injected intraperitoneally with 100 μg of functional-grade purified anti-mouse IL-17A or mouse IgG1 isotype control antibody (eBiosciences, San Diego, CA) in 500 μl volume at day −1 and day +3 relative to colonization challenge. CD4+ T cell depletion was accomplished as described (Malley et al., 2005). Splenocytes from each treated group were stained with FITC-labeled anti-mouse CD4 antibody (BD Biosciences) and analyzed on a Cytomation MoFlo (Beckman Coulter).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one table and can be found with this article online at doi:10.1016/j.chom.2011.01.007.

ACKNOWLEDGMENTS

The authors gratefully acknowledge PATH for supporting these studies. The clonal TIGR4 library was provided by NIAID/PFGRC. K.L.M. is a recipient of a Pediatric Infectious Diseases Society Fellowship Award funded by Sanofi Pasteur, Inc. T.M.G., P.G., and J.B.F. are employees of Genocea Biosciences. D.E.H. is the scientific cofounder of Genocea Biosciences and R.M. is a member of the scientific advisory board of Genocea Biosciences. R.M. also acknowledges support from the National Institutes of Health (R01 AI066013).

Received: August 20, 2010
Revised: November 19, 2010
Accepted: December 20, 2010
Published: February 16, 2011

REFERENCES

Adam, O., Vercellone, A., Paul, F., Monsan, P.F., and Puzo, G. (1995). A non-degradative route for the removal of endotoxin from exopolysaccharides. Anal. Biochem. *225*, 321–327.

Alexander, J.E., Lock, R.A., Peeters, C.C., Poolman, J.T., Andrew, P.W., Mitchell, T.J., Hansman, D., and Paton, J.C. (1994). Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of Streptococcus pneumoniae. Infect. Immun. *62*, 5683–5688.

Aujla, S.J., Dubin, P.J., and Kolls, J.K. (2007). Th17 cells and mucosal host defense. Semin. Immunol. *19*, 377–382.

Briles, D.E., Hollingshead, S.K., King, J., Swift, A., Braun, P.A., Park, M.K., Ferguson, L.M., Nahm, M.H., and Nabors, G.S. (2000). Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with Streptococcus pneumoniae bearing heterologous PspA. J. Infect. Dis. *182*, 1694–1701.

Briles, D.E., Hollingshead, S.K., Paton, J.C., Ades, E.W., Novak, L., van Ginkel, F.W., and Benjamin, W.H., Jr. (2003). Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with Streptococcus pneumoniae. J. Infect. Dis. *188*, 339–348.

Curtis, M.M., and Way, S.S. (2009). Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens. Immunology *126*, 177–185.

Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. USA *97*, 6640–6645.

Datta, S.K., Sabet, M., Nguyen, K.P., Valdez, P.A., Gonzalez-Navajas, J.M., Islam, S., Mihajlov, I., Fierer, J., Insel, P.A., Webster, N.J., et al. (2010). Mucosal adjuvant activity of cholera toxin requires Th17 cells and protects against inhalation anthrax. Proc. Natl. Acad. Sci. USA *107*, 10638–10643.

Giefing, C., Meinke, A.L., Hanner, M., Henics, T., Bui, M.D., Gelbmann, D., Lundberg, U., Senn, B.M., Schunn, M., Habel, A., et al. (2008). Discovery of a novel class of highly conserved vaccine antigens using genomic scale antigenic fingerprinting of pneumococcus with human antibodies. J. Exp. Med. *205*, 117–131.

Glover, D.T., Hollingshead, S.K., and Briles, D.E. (2008). Streptococcus pneumoniae surface protein PcpA elicits protection against lung infection and fatal sepsis. Infect. Immun. *76*, 2767–2776.

Huang, S.S., Platt, R., Rifas-Shiman, S.L., Pelton, S.I., Goldmann, D., and Finkelstein, J.A. (2005). Post-PCV7 changes in colonizing pneumococcal serotypes in 16 Massachusetts communities, 2001 and 2004. Pediatrics *116*, e408–e413.

Lee, J.B., Jang, J.E., Song, M.K., and Chang, J. (2009). Intranasal delivery of cholera toxin induces th17-dominated T-cell response to bystander antigens. PLoS ONE *4*, e5190.

Lipsitch, M., Whitney, C.G., Zell, E., Kaijalainen, T., Dagan, R., and Malley, R. (2005). Are anticapsular antibodies the primary mechanism of protection against invasive pneumococcal disease? PLoS Med. *2*, e15.

Lu, Y.J., Gross, J., Bogaert, D., Finn, A., Bagrade, L., Zhang, Q., Kolls, J.K., Srivastava, A., Lundgren, A., Forte, S., et al. (2008). Interleukin-17A mediates acquired immunity to pneumococcal colonization. PLoS Pathog. *4*, e1000159.

Malley, R., Lipsitch, M., Stack, A., Saladino, R., Fleisher, G., Pelton, S., Thompson, C., Briles, D., and Anderson, P. (2001). Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. Infect. Immun. *69*, 4870–4873.

Malley, R., Trzcinski, K., Srivastava, A., Thompson, C.M., Anderson, P.W., and Lipsitch, M. (2005). CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. Proc. Natl. Acad. Sci. USA *102*, 4848–4853.

Milner, J.D., Brenchley, J.M., Laurence, A., Freeman, A.F., Hill, B.J., Elias, K.M., Kanno, Y., Spalding, C., Elloumi, H.Z., Paulson, M.L., et al. (2008). Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. Nature *452*, 773–776.

Nabors, G.S., Braun, P.A., Herrmann, D.J., Heise, M.L., Pyle, D.J., Gravenstein, S., Schilling, M., Ferguson, L.M., Hollingshead, S.K., Briles, D.E., and Becker, R.S. (2000). Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. Vaccine *18*, 1743–1754.

Nagy, E. (2010). IC47, a novel protein-based pneumococcal vaccine: from bench to the clinic. 7th International Symposium on Pneumococci and Pneumococcal Diseases. Tel Aviv, Israel. March 14–18, 2010.

O'Brien, K.L., Wolfson, L.J., Watt, J.P., Henkle, E., Deloria-Knoll, M., McCall, N., Lee, E., Mulholland, K., Levine, O.S., and Cherian, T.; Hib and Pneumococcal Global Burden of Disease Study Team. (2009). Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. Lancet *374*, 893–902.

O'Connor, W., Jr., Zenewicz, L.A., and Flavell, R.A. (2010). The dual nature of T(H)17 cells: shifting the focus to function. Nat. Immunol. *11*, 471–476.

Ogunniyi, A.D., Folland, R.L., Briles, D.E., Hollingshead, S.K., and Paton, J.C. (2000). Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with Streptococcus pneumoniae. Infect. Immun. *68*, 3028–3033.

Paton, J.C., Lock, R.A., Lee, C.J., Li, J.P., Berry, A.M., Mitchell, T.J., Andrew, P.W., Hansman, D., and Boulnois, G.J. (1991). Purification and immunogenicity of genetically obtained pneumolysin toxoids and their conjugation to Streptococcus pneumoniae type 19F polysaccharide. Infect. Immun. *59*, 2297–2304.

Sanderson, S., and Shastri, N. (1994). LacZ inducible, antigen/MHC-specific T cell hybrids. Int. Immunol. *6*, 369–376.

Sanderson, S., Frauwirth, K., and Shastri, N. (1995). Expression of endogenous peptide-major histocompatibility complex class II complexes derived from invariant chain-antigen fusion proteins. Proc. Natl. Acad. Sci. USA *92*, 7217–7221.

Singleton, R.J., Hennessy, T.W., Bulkow, L.R., Hammitt, L.L., Zulz, T., Hurlburt, D.A., Butler, J.C., Rudolph, K., and Parkinson, A. (2007). Invasive pneumococcal disease caused by nonvaccine serotypes among alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. JAMA *297*, 1784–1792.

Trzcinski, K., Thompson, C., Malley, R., and Lipsitch, M. (2005). Antibodies to conserved pneumococcal antigens correlate with, but are not required for, protection against pneumococcal colonization induced by prior exposure in a mouse model. Infect. Immun. *73*, 7043–7046.

Wizemann, T.M., Heinrichs, J.H., Adamou, J.E., Erwin, A.L., Kunsch, C., Choi, G.H., Barash, S.C., Rosen, C.A., Masure, H.R., Tuomanen, E., et al. (2001). Use of a whole genome approach to identify vaccine molecules affording protection against Streptococcus pneumoniae infection. Infect. Immun. *69*, 1593–1598.

Zhang, X., Goncalves, R., and Mosser, D.M. (2008). The isolation and characterization of murine macrophages. Curr. Protoc. Immunol., *Chapter 14*, Unit 14.1.