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# A Bivalent Vaccine to Protect against *Streptococcus pneumoniae* and *Salmonella typhi*

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## Abstract

Pneumococcal and *Salmonella typhi* infections are two major diseases for children in developing countries. For typhoid fever, licensed Vi polysaccharide vaccines are ineffective in children <2 year-old. While investigational Vi conjugate vaccines have been shown effective in clinical trials, they are currently only available to restricted areas. Pneumococcal capsular polysaccharide conjugate vaccines are highly effective in children, but suffer from some limitations including cost and limited serotype coverage. We have previously shown that a fusion conjugate vaccine, consisting of pneumococcal fusion protein PsaA and pneumolysoid (PdT) conjugated to a polysaccharide, results in enhanced antibody and CD4+ Th17 cell responses as well as protection against pneumococcal colonization and disease in mice. Here we applied this approach to develop a bivalent vaccine against pneumococcus and *S. typhi.* Two species-conserved pneumococcal antigens (SP1572 or SP2070) were fused to the nonhemolytic pneumolysoid PdT. SP1572-PdT was then conjugated to Vi polysaccharide and SP2070-PdT was conjugated to the pneumococcal cell wall polysaccharide (CWPS; also conserved). Mice immunized with this bivalent conjugate were protected against pneumococcal colonization and sepsis challenges, and made anti-Vi antibody concentrations higher by 40 fold compared to mice that received equimolar mixtures of the antigens. An enhanced killing of Vi-bearing *Salmonellae in vitro* was demonstrated from plasma of mice that received the fusion conjugate but not the mixture of antigens. Our results support further evaluation of this bivalent immunogen for the prevention of pneumococcal colonization and disease, and of typhoid fever.

### Keywords

*Streptococcus pneumoniae*; *Salmonella typhi*; vaccine; Vi polysaccharide



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## INTRODUCTION

Infections by *Salmonella enterica serovar typhi* (*S. typhi*) and *Streptococcus pneumoniae* (pneumococcus) are major causes of morbidity and mortality in childhood, especially in developing countries. Typhoid fever [caused by *S. typhi*] is a major threat to children, particularly in southeast and central Asia, Latin America and Africa. More than 20 million cases are estimated each year, accounting for over 200,000 deaths, primarily in children under 5 years age [1–3]. With respect to pneumococcus, estimates suggest 14.5 million cases of invasive disease occur worldwide in children under 5 years of age and over 820,000 deaths [4]. Both of these pathogens afflict children in the same epidemiologic context and there remains an unmet need to develop an affordable and effective vaccine for use in developing countries. A bivalent vaccine that targets both pathogens could represent an economical solution to this problem.

The capsular polysaccharide Vi of *S. typhi* is an important virulence factor and also a protective antigen [5–7]. Both pure polysaccharide and conjugated Vi had been tested in clinical trials and shown to be protective against typhoid fever [8, 9]. Vi is a linear polymer composed of (α1-4)-2-deoxy-2-*N*-acetyl galacturonic acid moieties and is a thymus-independent antigen. Children under 2 years of age do not respond to immunization with Vi. In contrast, immunization with Vi conjugated to the nontoxic recombinant exotoxin A of *Pseudomonas aeruginosa* (Vi-rEPA) produces protective levels of serum anti-Vi IgG in infants and young children [9, 10]. Vi-protein conjugates using other carrier proteins such as $CRM_{197}$, tetanus toxoid, diphtheria toxoid, cholera toxins, the B subunit of the heat labile toxin of *Escherichia coli*, recombinant outer membrane protein of *Klebsiella pneumoniae* and ion-regulated outer-membrane proteins of *S. typhi* have been evaluated in preclinical studies, and some have been tested in humans [11–18]. A conjugate typhoid vaccine is licensed but only available in restricted areas and not widely used[19]. Another vaccine approach is oral immunization with the attenuated Ty21a strain, which provides comparable protection to the Vi polysaccharide vaccine [20, 21] but likewise is not approved for young children.

Current pneumococcal vaccines target the polysaccharide capsule by the inclusion of individual polysaccharide-protein conjugates for some of the more common capsular types associated with invasive disease. To date, there have been three licensed conjugate vaccines, comprising valencies of 7, 10 or 13 [22–24]. While these vaccines are highly effective against strains bearing the included capsular serotypes, their high cost and complexity of manufacture represent an important hurdle for widespread use. Furthermore, particularly in developing countries, the prevailing serotypes are not always well covered by the existing vaccines [25]. Finally, the rapid emergence of serotypes not included in the vaccine has been observed in several countries, including the US and in Europe [26], potentially threatening the long-term efficacy of this approach.

Thus, alternative vaccine strategies are being sought. It has long been recognized that antibodies to noncapsular antigens conserved widely within the *S. pneumoniae* species could protect mice against pneumococcal invasive disease [27, 28]. Some of these vaccine candidates have progressed to clinical trials [29, 30]. More recently, work in mice has revealed the existence of an antibody-independent, CD4+ Th17-mediated mechanism of protection against pneumococcal colonization [31–33]. It has been argued that a protein-based vaccine that could confer antibody-mediated immunity to invasive disease and Th17-mediated protection against nasopharyngeal colonization, may represent an attractive alternative to pneumococcal conjugate vaccines, by providing a two-pronged mechanism of protection [34, 35].

We have described a fusion conjugate construct, consisting of a conserved pneumococcal cell wall polysaccharide (CWPS) conjugated to the fused pneumococcal surface adhesin A (PsaA) and the non-hemolytic pneumolysin mutant PdT (W433F, D385N, and C428G). This construct elicited both antibody and Th17 cell responses to proteins and conferred protection against both invasive disease and colonization [36]. Here we applied this approach to two conserved protective pneumococcal proteins and *S. typhi* Vi polysaccharide for the development of a vaccine candidate targeting both pneumococcus and *S. typhi.*

## RESULTS

### Selection of the pneumococcal protein components

Based on the observation that a killed *S. pneumoniae* whole cell preparation administered intranasally (SPWCV) protects mice against colonization in a CD4+ T cell dependent manner [32, 37, 38], we fractionated SPWCV to identify protein antigens that elicit the highest IL-17A responses from splenocytes of immunized animals (data not shown). Two antigens proved highly potent at eliciting IL-17A responses from SPWCV-immunized animals and are known to be conserved: SP1572 (pneumococcal protective protein A), a non-heme iron-containing ferritin previously evaluated in mouse models of colonization and disease [39, 40] and SP2070, a surface-exposed glucose-6-phosphate isomerase against which age-dependent increases in antibodies have been demonstrated in humans [41]. These two proteins were then tested in an intranasal immunization model using cholera toxin as an adjuvant, and they conferred protection against nasopharyngeal pneumococcal carriage (data not shown).

### Fusion conjugates consisting of Vi polysaccharide conjugated to the fusions of the two pneumococcal proteins to PdT confer protection against pneumococcal colonization but not against sepsis challenge

DNA fragments encoding SP1572 or SP2070 fused to PdT with a polylinker GSGGGGS were generated through PCR and cloned into pQE30 vector as described in the Materials and Methods section. After transformation, His-tagged proteins were purified from *E. coli* using a Ni-NTA column (Qiagen), and peak fractions were combined and run through a Sepharose S200 gel filtration column for further purification. Proteins were then conjugated to Vi using a modification of the method described in [15]. The Vi-conjugates were separated from free protein and Vi by passage through a Superose 6 column (Fig 1A and 1B). The Vi-conjugates SP1572-PdT-Vi and SP2070-PdT-Vi had protein:Vi ratios of 1:1.4 and 1:1.7 mg/mg , respectively, determined by the BCA assay of protein and concentration of O-acetyl of Vi [42].

For immunization, the two Vi-conjugates were mixed in a 1:1 ratio (based on protein content. Mice were immunized subcutaneously three times at two-week intervals with either alum alone, the combination of both Vi fusion conjugates adsorbed onto alum, or the equimolar mixture of all the uncoupled components of the fusion conjugates (referred to hereafter as mixture). Mice in the Vi-conjugate group had similar levels of anti-SP1572, SP2070 or SPWCA IgG antibodies in the plasma (Figure 2A). Whole blood from mice immunized with the combination of Vi-conjugates and stimulated with either individual protein or with SPWCA made significantly higher IL-17A than the whole blood from mice in the alum or mixture groups (Figure 2B). The concentration of Vi antibody was over 40 times higher in mice that received the combination of fusion conjugates than in mice that received the mixture of antigens with alum (data not shown).

Two different pneumococcal challenge models were used to evaluate the protective capacity of these vaccines: a) a colonization model using a serotype 6B clinical isolate strain 603 which causes asymptomatic carriage and does not cause bacteremic disease in mice [43] and

b) a lung inhalation model in which lightly anesthetized mice are given a nasal inoculum containing a serotype 3 strain which causes lung infection, bacteremia and sepsis [36]. As shown in Figure 2C, when these mice were intranasally challenged with serotype 6B strain 0603 in the colonization model, the pneumococcal colony-forming units (cfu) recovered in the nasal wash from mice of the Vi-conjugate group were significantly lower than from mice of the mixture group or those that received alum alone. However, the combination of two Vi fusion conjugates did not offer protection against death compared to either alum or the mixture (Figure 2D) in the lung inhalation model.

## Immunization with a combination of Vi- and CWPS-fusion conjugates confers protection against both nasopharyngeal colonization and aspiration pneumonia/ sepsis

We have demonstrated previously that immunization with a conjugate of the cell wall polysaccharide (CWPS) of *S. pneumoniae* to a fusion protein of PsaA and PdT confers protection against colonization and sepsis, in a Th17- and antibody-dependent fashion, respectively [36, 44], We therefore reasoned that the combination of both approaches may be necessary to develop a vaccine candidate that elicits protection against both colonization and invasive disease. We hypothesized that a combination of a Vi and CWPS conjugates using the protein antigens SP1572 and SP2070 would be protective against pneumococcal colonization and sepsis, while also eliciting antibody responses to the Vi component. To test this hypothesis, SP1572-PdT was conjugated to Vi as described above and SP2070-PdT was conjugated to CWPS as described before [36], using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP); both conjugates were separated on a Superose 6 column. SP1572-PdT-Vi had a protein:Vi ratio of 1:1.6 mg/mg and SP2070-PdT-CWPS had a ratio of 1:0.65 mg/mg.

Mice were subcutaneously immunized with the combination of the Vi conjugate SP1572-PdT-Vi and the CWPS-conjugate SP2070-PdT-CWPS with alum, the mixture of all components with alum or alum alone. As shown in Figure 3A, mice in the combined CWPS- and Vi-conjugate group had similar antibody level responses to SP1572 and SP2070 but significantly higher antibody response to SPWCA. Stimulation of whole blood from the CWPS- and Vi- conjugates group also resulted in significantly greater IL-17A responses to either of the protein antigens (SP1572 or SP2070) and to SPWCA than mice in the alum or the mixture groups, whose responses to these stimuli were barely detectable (Figure 3B).

As predicted by the IL-17A assay, mice in the combined conjugate group were significantly protected against colonization compared with mice in the alum group ($p=0.036$, Fig 3C), whereas mice in the mixture group were not significantly protected. Similarly, the mice in the combined conjugate group were fully protected against type 3 pneumococcal aspiration/ sepsis ($p=0.002$) whereas immunization with the mixture conferred no survival advantage when compared to immunization with alum alone (Fig 3D).

## Immunization with Vi and CWPS conjugates induces functional anti-Vi antibodies

Because *S. typhi* does not infect mice, immunogenicity and in vitro killing assays were used to determine the ability of the fusion conjugates to elicit functional antibodies against this organism. As shown in Figure 4A, and as expected for an uncoupled polysaccharide, immunization with the mixture of all antigens increased the anti-Vi antibody concentration only slightly (4-fold) compared with immunization with alum alone; in contrast, mice in the combined CWPS- and VI-conjugate group demonstrated over 40-fold increases in anti-Vi IgG plasma concentration than mice that received the mixture of antigens, a response that is similar to that induced by immunization with the two Vi conjugates (data not shown). This result suggests that substitution of the 2070-PdT-CWPS conjugate for the 2070-PdT-Vi conjugate did not significantly reduce or interfere with Vi antibody production.

Cell-binding and in vitro killing assays were then performed. *S. typhimurium* strains C5 and C5.507 (the C5 strain engineered to express the Vi polysaccharide, both kind gifts of Drs. Dougan and Hale, The Wellcome Sanger Trust Institute, Cambridge UK) [13] were incubated with a 1:50 dilution of plasma from mice immunized with the mixture or CWPS- and Vi-conjugate. As shown in the Western blot of Figure 4B, binding of mouse antibody could only be observed when the C5.507 strain was incubated with plasma originating from mice in the combined conjugate group. This suggests that antibody elicited by the combined conjugate vaccine binds to the Vi-polysaccharide present on a whole organism and that the response is specific for this polysaccharide.

A killing assay using differentiated HL-60 cells was then performed to test the neutralizing capacity of these antibodies. While there was no appreciable killing when the Vi-negative C5 strain was incubated with plasma from mice immunized with either the mixture or the combined conjugate vaccine (data not shown), rapid and effective killing (almost 2-log reduction) could be observed when the Vi-positive strain C5.507 was pre-incubated with plasma from combined conjugate-immunized mice (Figure 4C). Similar results were obtained when human blood neutrophils purified from healthy volunteers were used in place of the HL-60 cells (data not shown). Thus, these results demonstrate that immunization with the combination of CWPS and Vi fusion conjugates elicits functionally active antibodies directed against Vi.

## DISCUSSION

The data support the development of a parenteral bivalent vaccine against pneumococcus and *S. typhi*, with aluminum hydroxide as adjuvant. The ability of Vi conjugates to elicit functional antibodies against the Vi capsular polysaccharide has been established in both animal models and clinical studies, and the results presented here are consistent with these findings. Addition of the CWPS conjugate resulted in an effective immunogen against pneumococcal pneumonia/sepsis by an encapsulated strain of serotype 3. Additionally, by making use of the fusion conjugate approach, we were also able to elicit Th17 responses to two pneumococcal proteins that are associated with protection against nasopharyngeal colonization, as demonstrated here.

The efficacy of pneumococcal capsular polysaccharide conjugate vaccines in the prevention of invasive disease has been well established [45]. More recently, concern has been raised regarding the phenomenon of serotype replacement, whereby strains carrying capsular polysaccharides not included in the seven-valent conjugate vaccine increased in frequency following the introduction of the conjugate vaccine [26]. To a large extent, the emergence of these non-vaccine type strains is the direct consequence of the success of the vaccines in reducing nasopharyngeal colonization by vaccine-type strains. This impact on colonization also underlies the overall success of the conjugate vaccines in the U.S., where 2/3 of all prevented invasive disease cases are the consequence of an indirect effect, or herd protection [45].

Based on these data, we adopted the premise that for a noncapsular pneumococcal vaccine candidate to be viable, it should provide protection against disease but also against colonization [34]. Building on recent studies by our group and others [31, 34, 46] that have implicated a critical role of Th17 cells in the control of pneumococcal colonization, we identified pneumococcal protein antigens that confer protection against carriage by this mechanism. The two proteins we identified, SP1572 and SP2070, are conserved (over 90% amino acid sequence identity) in all the presently sequenced pneumococcal strains (n=42) and thus may be expected to provide protection against a wide range of pneumococci. Unlike capsular PS, CWPS of pneumococcus is highly conserved across all serotypes, with

either one or two phosphorylcholine moieties per repeat unit[47]. Antibodies directed against components of CWPS have been shown to be protective in some animal models [48–50] and intranasal vaccination with CWPS is protective in murine colonization and sepsis models [44]. Here we showed that a bivalent vaccine consisting of a combination of two fusion conjugates of SP1572-PdT-Vi and SP2070-PdT-CWPS elicits Th17 responses and provides protection against pneumococcal nasopharyngeal colonization whereas the uncoupled components adsorbed onto alum neither induced this response nor conferred protection.

This vaccine also could be expected to confer protection against typhoid fever in young children. Currently licensed typhoid vaccines include the live Ty21a strain given orally and the purified Vi polysaccharide administered parenterally. The oral vaccine is not approved for young children and the Vi polysaccharide is ineffective under two years of age. Investigational Vi conjugate vaccine demonstrated to be safe and efficacious in infants and young children [10, 51, 52], however, the licensed Vi-conjugate is only for local distribution [19]. As could be expected, our conjugate induces functional antibodies in mice, as evidenced by killing assays using genetically-engineered *S. typhimurium* strains. Although at the present time it is difficult to determine the cost of such a vaccine, it is reasonable to assume that a combination of Vi and CWPS conjugates would be significantly less expensive to produce than currently licensed 10- or 13-valent conjugate vaccines, with or without the addition of a Vi-conjugate.

In conclusion, we describe here a candidate vaccine consisting of a combination of antigens directed against *S. typhi* and *S. pneumoniae.* The modifications to the antigens include fusion of two conserved pneumococcal proteins to the pneumolysoid PdT and conjugation to polysaccharides, which assists in Th17 elicitation [36]. The inclusion of Vi as one of the two conjugated polysaccharides renders the combination an effective immunogen against *S. typhi.* The development of a vaccine that may confer protection against both pathogens offers the promise of an economical and effective vaccine directed against diseases that are associated with major mortality and morbidity in children in developing countries. Further preclinical studies to optimize the bivalent immunogen structurally and to confirm the findings presented here for additional pneumococcal strains are underway.

## MATERIALS AND METHODS

### Materials

Aluminum hydroxide (alum) was from Brenntag North America (2% Alhydrogel). Vi polysaccharide was purified as described previously [14]. Adipic acid dihydrazide (ADH), 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide Hydrochloride (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) were purchased from Pierce. All other reagents were obtained from Sigma. The SPWCA was made by Dr. Luciana Leite's group at Instituto Butantan (Sao Paulo, Brazil) as previously described [37].

### Construct fusion protein expression vectors

pQE30 vector with His-tagged PdT inside was modified by inserting four restriction sites: SphI, SacI, SacII and ClaI between His-tag and GSGGGS polylinker, to generate vector pQE-PdT. SP1572 and SP2070 were amplified using S. pneumoniae TIGR4 genomic DNA as template. SphI and SacII sites were introduced at the beginning and end of PCR products by primers. Double digested PCR products were ligated into pQE-PdT and transformed with *E. coli* Xl/blue strain. The correct nucleotide sequences were confirmed at the Children's Hospital Boston Molecular Genetics Core Facility.

### Protein Purification

*E. coli* transformants containing the relevant cloned proteins were grown to OD600=0.6, and protein expression was induced with 0.2 mM IPTG at 16°C overnight. Cells were spun down and pellets were resuspended in lysis buffer (20 mM Tris-HCl, 500 mM NaCl, pH8.0) and then lysed by sonication. The proteins of interest were purified from supernatant over a Ni-NTA column; proteins were eluted in imidazole buffer. Protein-containing elutions were combined, purified over a gel-filtration column and eluted in PBS buffer.

### Generation of the Vi and CWPS conjugates

Proteins were conjugated to Vi polysaccharide according to Szu et al. with some modifications. [15]. Briefly, Vi was resuspended to 5 mg/ml in buffer A (0.2 M MES, 150 mM NaCl, pH 5.9), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and NHS-Sulfo was added into solution as powder for 30 minutes at room temperature. Excess EDC was removed by dialysis against PBS three times. Proteins were then added to the reaction as 1mg/mg, protein/sugar. The reaction was carried out at 4°C overnight with rotation. Conjugates were separated by elution through a Superose-6 gel filtration column and collection of the void volume fractions. The CWPS conjugate was prepared as described previously [36]. Protein concentration was determined by the BCA method (Pierce), and Vi concentration was determined by measurement of O-acetyl groups using Vi as a standard[42]. CWPS concentration was determined using the Anthrone method [53].

### Antigen preparations

One day prior to immunization, vaccines were prepared as follows. Frozen aliquots were thawed or lyophilized vials were reconstituted with sterile water, diluted to the appropriate concentration, and mixed with aluminum hydroxide (alum) at the indicated concentration in a 15 ml conical tube, which was then tumbled overnight at 4°C to allow for adsorption.

### Immunization and challenge of mice

C57BL/6J mice were used in all the experiments. The age at time of first immunization was between 4–6 weeks. Gently restrained, nonanesthetized mice received 2 or 3 subcutaneous injections of 200 μl containing adjuvant with or without antigen in the lower part of the back at 2-week intervals. Blood was drawn 2 weeks after the last immunization, and assayed for antibody and for IL-17A production *in vitro* upon stimulation with WCA. Nasopharyngeal colonization with the clinical pneumococcal isolate 0603 (serotype 6B) and aspiration challenge with strain WU-2 were carried out as previously described [37]. All animal studies were approved by our local animal ethics committees.

### Enzyme-linked immunosorbent assay (ELISA) and IL-17A production in whole blood samples

Assays for murine antibodies for WCA, individual protein and CWPS and IL-17A production in whole blood were carried out as previously described [36]. Vi antibody was measured using the method described by Azze et al. [54].

### Binding of Vi antibody to Vi-expressing *Salmonella*

*Salmonella typhimurium* carrying an empty vector (strain C5, Vi-negative) or the genes necessary for expression of Vi polysaccharide (strain C5.507, Vi-positive) were grown into late log phase and collected by centrifugation[13]. Bacteria were washed with PBS, and mouse plasma was then added with 1:50 dilution and incubated at room temperature for 30 minutes. Samples were then washed with PBS for 3 times and treated with SDS buffer. The gel was transferred to a nitrocellulose membrane and then blocked with 5% skim milk in PBS/0.05% Tween. Donkey anti-mouse IgG HRP conjugate (Sigma) was added 1:10,000 in

1% skim milk in PBS/Tween. The membrane was developed following addition of ECL substrate.

### Bacterial killing by phagocytic cells

Killing assays were performed as described previously [13, 55]. Salmonella typhimurium carrying an empty vector (Strain C5) or expressing Vi polysaccharide on the surface (Strain C5.507) were grown into late log phase and frozen in LB/10% glycerol at −80°C. HL-60 cells were differentiated into phagocytic cells by the addition of 100 mM *N,N*-dimethylformamide for 5 days. On the day of the experiment, bacteria were thawed and diluted to $10^6$ cfu/mL. Bacteria were incubated with a 1:10 dilution of plasma for 20 minutes at room temperature, and cells were added at a 100:1 ratio. Samples were incubated on a rocker plate at 20 rpm at 37 °C and numbers of viable *Salmonella* determined after 60, 120, and 180 min by serial dilution on Luria Bertani agar. Human neutrophils were purified from the peripheral blood of human volunteers using a Histopaque 1077, 1119 gradient (Sigma-Aldrich, St. Louis, MO) according to the manufacturers instructions and used immediately. Killing was performed similarly except the cell:bacteria ratio was 300:1.

### Statistical analysis

Antibody and IL-17A concentrations and NP colonization densities were compared by the Mann-Whitney *U* test using PRISM (version 4.0a, GraphPad Software, Inc). Differences in survival were analyzed with the Kaplan-Meier test, using PRISM as well.

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## Highlights

- Fusion conjugates are protein-TLR agonist fusions coupled to polysaccharides (PS)
- Salmonella typhi Vi PS and pneumococcal wall PS were coupled to pneumococcal fusions
- High-titer Vi antibody opsonic to *S. typhi* was elicited in mice.
- The induced IL17 and antibody prevented murine pneumococcal colonization and sepsis
- This bivalent combination may immunize infants vs typhoid and pneumococcal diseases

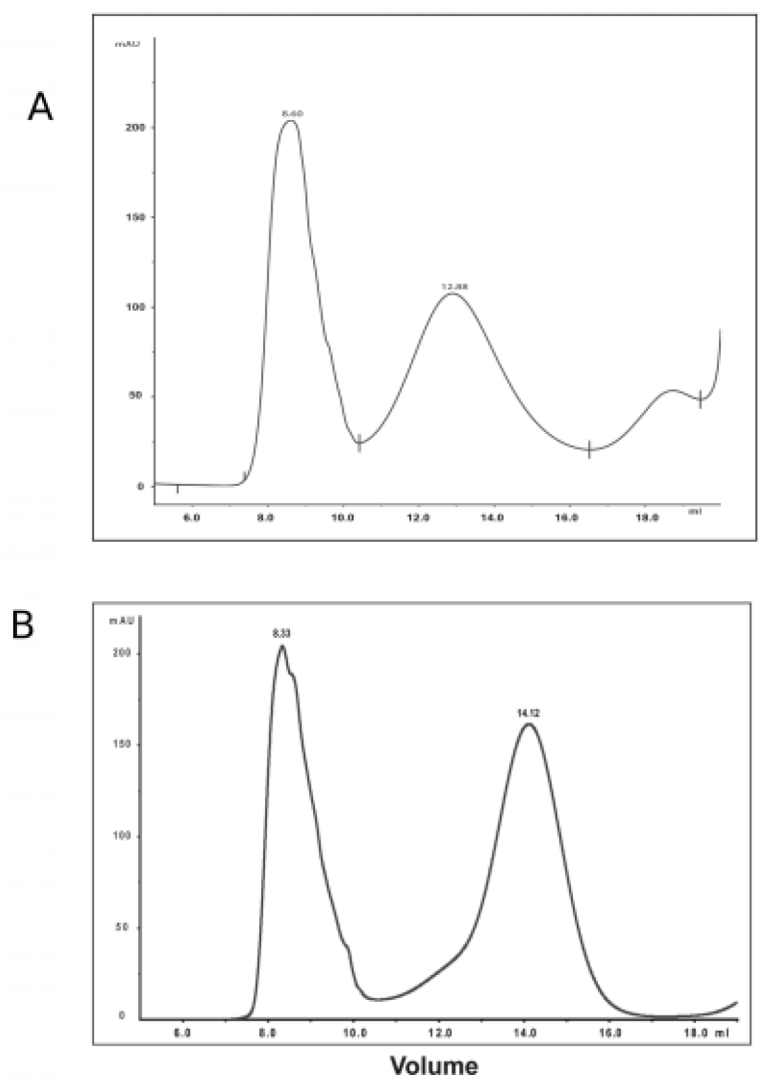


**Figure 1.**

Preparation of SP1572-PdT-Vi (A) and SP2070-PdT-Vi (B) conjugates. SP1572-PdT and SP2070-PdT were purified from *E. coli* and then conjugated to Vi as described in Materials and Methods. Products were run through a Superose 6 column and fractions from void volume (which contain conjugated material) were collected to separate conjugate from free proteins.

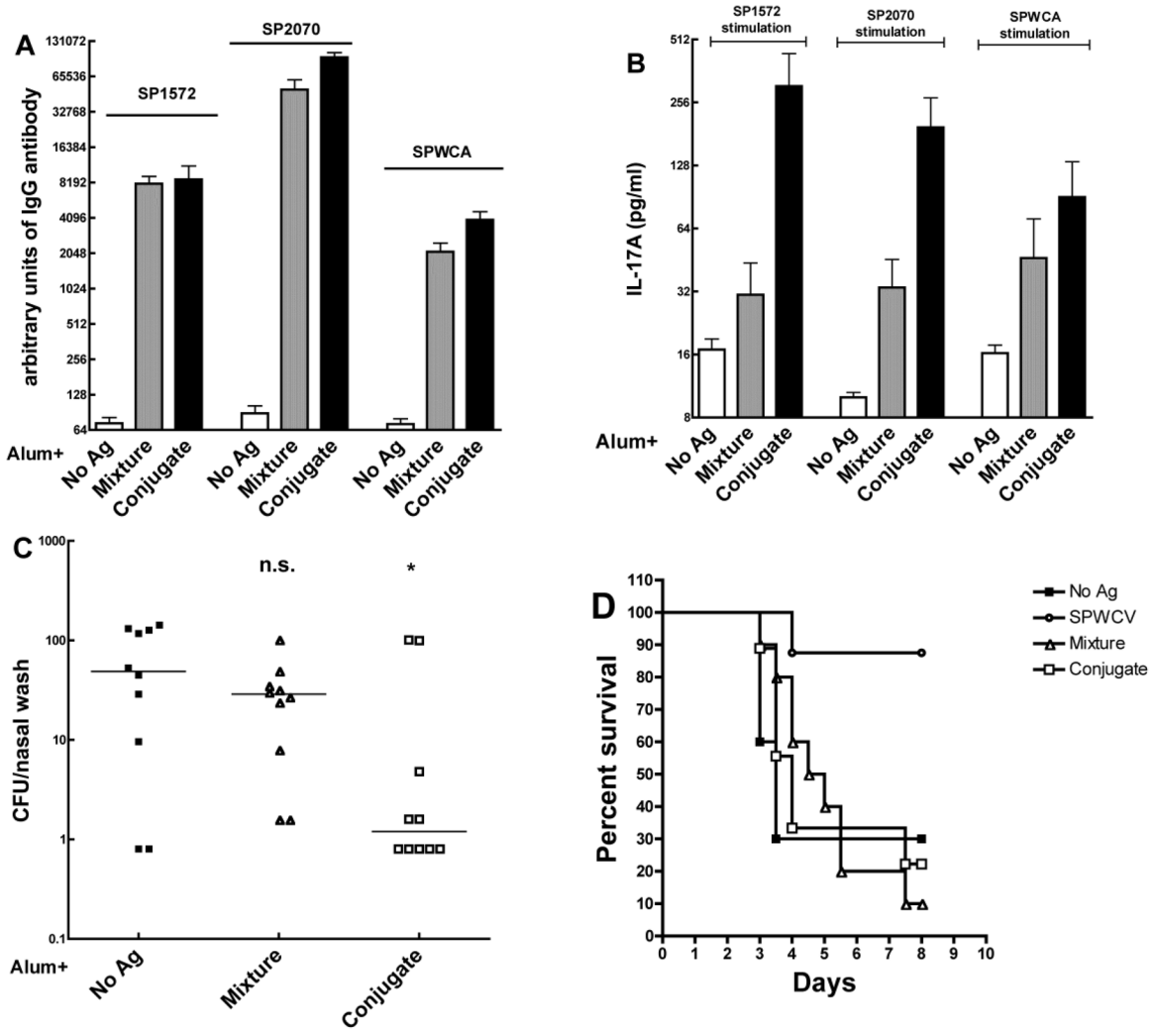


**Figure 2.**

Immunogenicity and evaluation of protection against pneumococcal colonization and aspiration/sepsis by SP1572-PdT-Vi and SP2070-PdT-Vi fusion conjugate. Mice were immunized subcutaneously three times two-weeks apart. Mice were bled two weeks after last immunization to analyze antibody levels in plasma and IL-17A responses in whole blood. (A). Antibody responses to SP1572, SP2070 and SPWCA in adjuvant alone (white bar), mixture (equimolar concentration, gray bar) or conjugate (combination of SP1572-PdT-Vi and SP2070-PdT-Vi, black bar) immunized mice. The antibody titers in mixture and conjugate groups were similar. (B). IL-17A responses following ex vivo stimulation with SP1572, SP2070 or SPWCA. Mice in the conjugate group had significantly higher IL-17A responses to all stimuli compared to mice in the mixture group. Bars represent mean value with SEM. (C). Protection against pneumococcal colonization challenge. Mice were challenged intranasally with the pneumococcal serotype 6B 0603 strain and the colonization density was determined 10 days later. Mice that received the combination of fusion conjugates had significantly lower densities of colonization than mice that received alum alone (P=0.043 by Mann-Whitney U analysis) whereas mice that received the mixture of all antigens were not significantly protected (P=0.28). Each symbol represents the density of colonization of an individual mouse and the horizontal line represents the median density of colonization. (D). Mice were challenged with $10^6$ cfu of WU2 strain by inducing aspiration

under light anesthesia and onset of illness/death monitored for 8 days. No significant protection by immunization with the Vi-conjugate or the mixture was observed, whereas mice that received the whole cell vaccine were significantly protected against illness/death compared to mice that received alum alone (P=0.01 by Kaplan-Meier analysis).

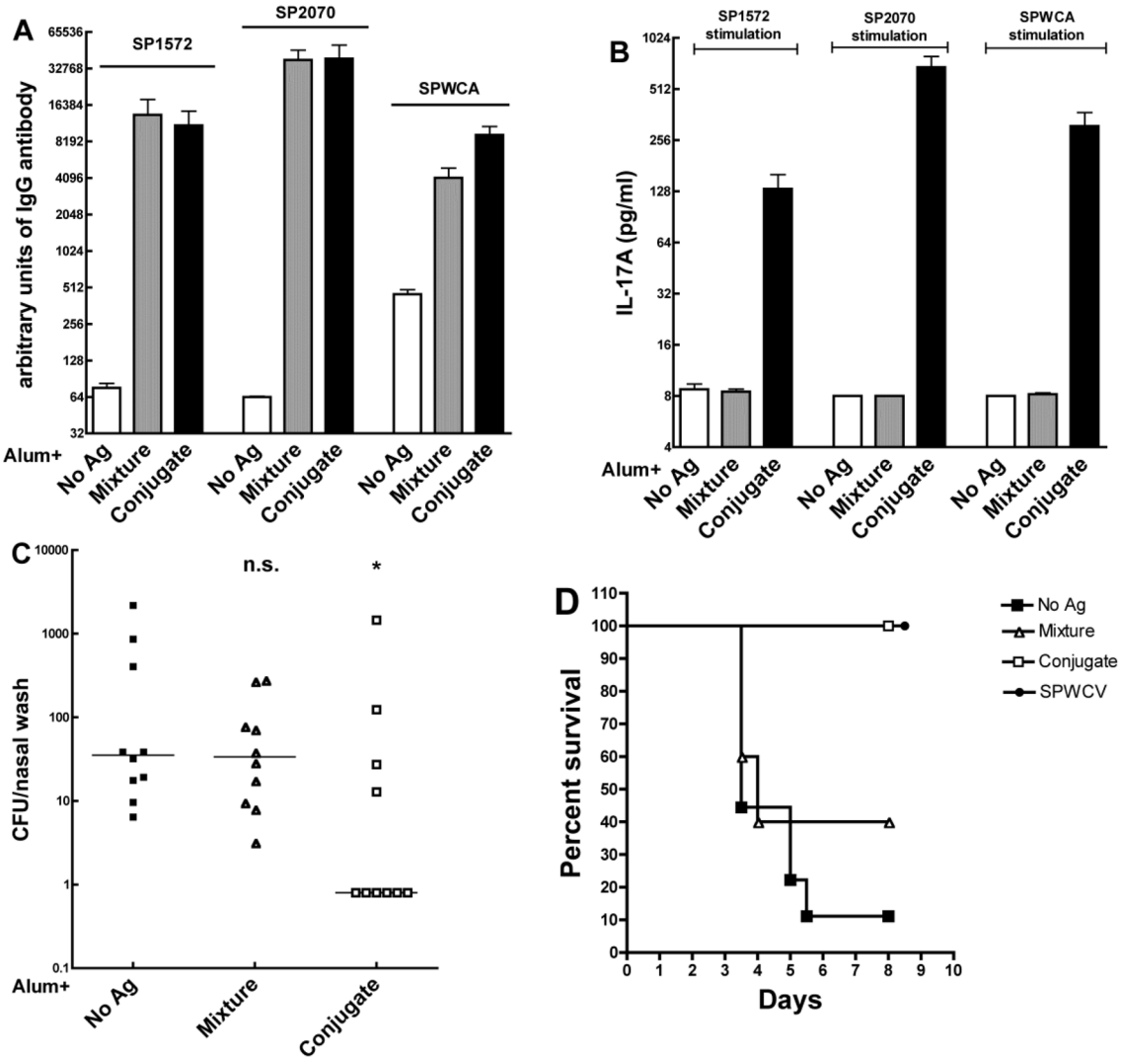


**Figure 3.**

Immunogenicity and evaluation of protection against pneumococcal colonization and aspiration/sepsis by the combination of SP1572-PdT-Vi and SP2070-PdT-CWPS fusion conjugates. (A). Antibody responses to SP1572, SP2070 and SPWCA in adjuvant alone (white bar), mixture (equimolar concentration, gray bar) or conjugate (combination of SP1572-PdT-Vi and SP2070-PdT-CWPS, black bar) immunized mice. The antibody responses to the proteins or SPWCA from mice in the mixture and conjugate group were similar. (B). IL-17A responses to SP1572, SP2070 or SPWCA. Mice in the conjugate group made significantly higher IL-17A in response to all stimuli than mice in the mixture group or mice immunized with alum alone. Bars represent mean value with SEM. (C). Protection against pneumococcal colonization. The same group of mice was challenged with strain 0603 intranasally and nasal washes collected 10 days later. Mice immunized with the combination of CWPS- and Vi-conjugates were significantly protected against colonization when compared to mice that received alum alone (P=0.036 by Mann-Whitney U) whereas mice that were immunized with the mixture of all components were not protected (P=0.63). (D). Mice immunized with either the SPWCV or the combination of CWPS and Vi fusion conjugates were significantly protected against aspiration pneumonia/sepsis due to strain

WU2 (P=0.002 by Kaplan Meier respectively vs. mice immunized with alum alone) whereas mice that received the mixture alone were not protected (P=0.29).

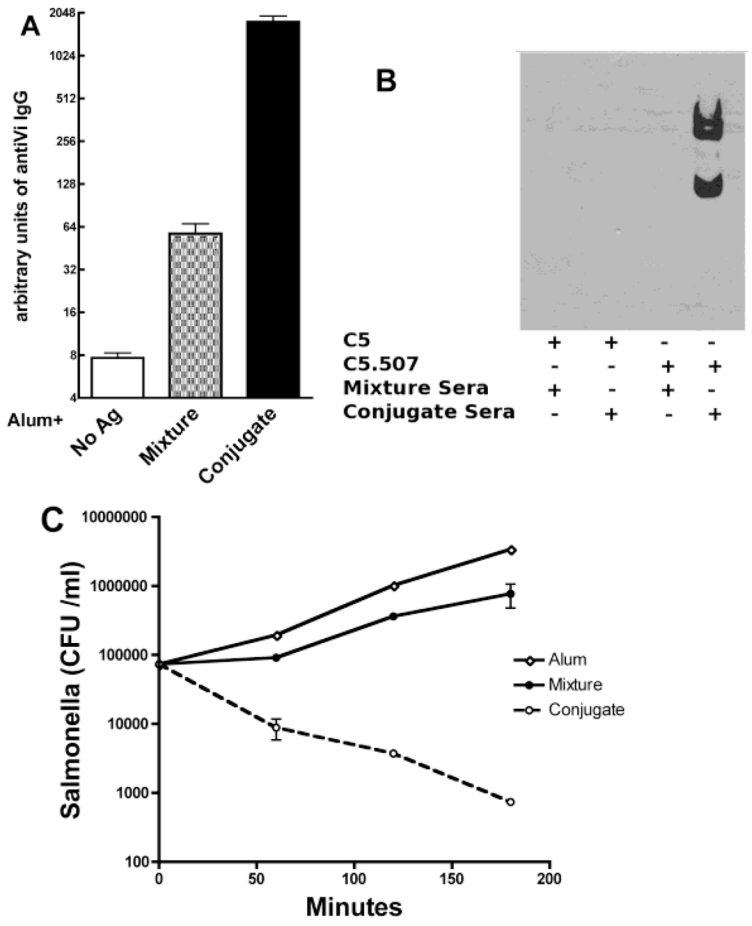


**Figure 4.**

Plasma samples from mice immunized with the combination of Vi and CWPS fusion conjugates bind to Vi and promote antibody-mediated killing of Vi-expressing *Salmonella* strain. (A). ELISA measured the plasma concentrations of anti-Vi IgG. Mice immunized with the combination of the two fusion conjugates had significantly higher plasma concentrations of anti-Vi IgG than mice immunized with the mixture of all antigens ($P<0.0001$ by Mann-Whitney U). (B) Western blotting analysis revealed that plasma from conjugate-immunized mice binds to Vi-bearing *S. typhimurium* (C5.507 strain) whereas no binding could be observed with plasma from mixture-immunized mice; no binding could be observed to the non Vi-bearing *Salmonella typhimurium* strain C5, confirming specificity of the immune response. (C) Sera from mice immunized with the CWPS and Vi fusion conjugates enhanced killing of Vi-expressing *Salmonella*. Plasma from mice immunized with the two fusion conjugates or with the mixture of all components were used in an killing assay using differentiated human HL-60 cells and strain C5.507. Bacterial growth was observed over a 3-hour period. C5.507 strain incubated with plasma from conjugate-immunized mice demonstrated a 2-log reduction in bacterial CFU, whereas no killing could be observed when plasma from mixture-immunized mice was used. The figure shows one representative result from 3 independent experiments.