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(54) Title: SYNERGISTIC IMMUNOGENIC FUSION PROTEIN-POLYSACCHARIDE CONJUGATE

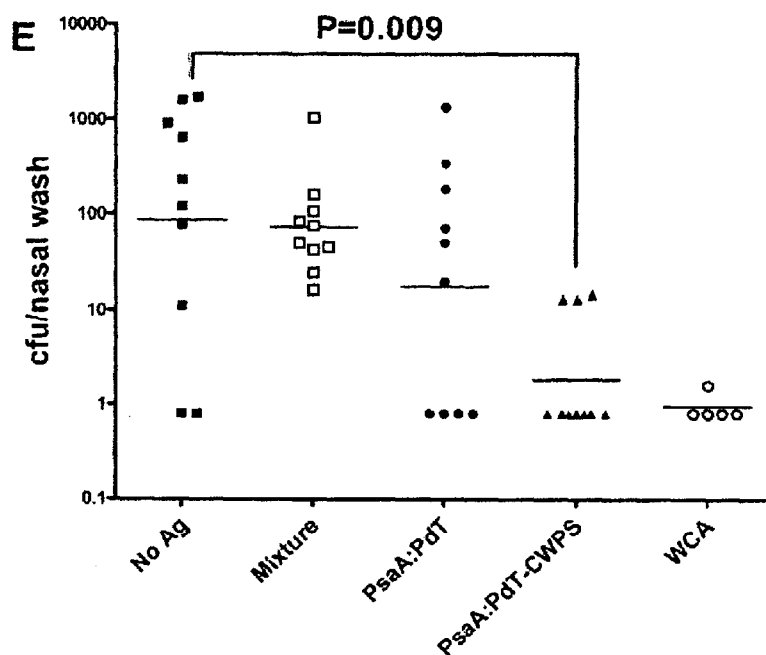


Figure 4E

(57) Abstract: An immunogenic composition comprising a fusion protein-polysaccharide conjugate consisting of a polysaccharide (PS) or pneumococcal bacterial cell wall polysaccharide (CWPS) conjugated to X:PdT where PdT represents the nonhemolytic variant of pneumolysin and X represents an antigenic peptide or protein of interest is provided. The immunity to X is synergistically enhanced by the fusion conjugate. Methods of protecting a subject from pneumococcal colonization or disease by administering an immunogenic composition including a fusion protein (PsaA:PdT) containing truncated PsaA and the nonhemolytic variant of pneumolysin (PdT) conjugated with CWPS (PsaA:PdT-CWPS) is also provided.



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SYNERGISTIC IMMUNOGENIC FUSION PROTEIN-POLYSACCHARIDE CONJUGATE

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RELATED APPLICATIONS

This application claims the benefit of U.S. Patent Applications Ser. No. 61/055,173, filed May 22, 2008; No. 61/156,973, filed March 3, 2009; and No. 61/164,605, filed March 30, 2009.

FIELD OF THE INVENTION

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The present invention relates to molecular genetics, immunology, and bacteriology. More specifically, an embodiment of the present invention provides for an immunogenic fusion conjugate comprising a fusion protein consisting of a protein or peptide of interest (for example, a truncated pneumococcal surface adhesin A protein) and a nonhemolytic pneumolysin protein, conjugated to a polysaccharide, such as dextran or a pneumococcal cell wall polysaccharide.

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This fusion conjugate confers a synergistic, immunogenic, humoral and cellular response, and in the case of pneumococci elicits synergistic, antibody- and cell- (including IL-17A)-mediated, protection against lethal infection and mucosal pneumococcal colonization.

BACKGROUND OF THE INVENTION

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Globally, infectious diseases cause over 13 million deaths each year, and cancers cause over 12 million deaths each year. For example, infection with *Streptococcus pneumoniae* is a major cause of morbidity and mortality worldwide and can lead to pneumonia, meningitis and bacteraemia, and some less severe infections such as otitis media. Almost one million children in the developing world die of infections due to *S. pneumoniae* (more commonly known as pneumococcus) each year. Antibiotics offer effective treatment for many cases of pneumococcal disease. The rapid emergence of multiple-drug-resistant strains of *S. pneumoniae*, however, has limited the effectiveness of antibiotics and stimulated renewed interest in the prevention of pneumococcal infections with vaccines.

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The success of passive immunization and polysaccharide-based vaccines for the prevention of colonization and/or disease has demonstrated the importance of capsular antibodies in controlling pneumococcal disease and colonization. Furthermore, studies in both

animals and humans demonstrate that these antibodies can protect against nasopharyngeal (NP) pneumococcal colonization, which precedes pneumococcal disease. The importance of this effect has become clear and has paralleled what was learned after universal immunization with *Haemophilus influenzae* type b vaccine: in the U.S., the pneumococcal conjugate vaccine has prevented more than twice as many cases of invasive pneumococcal disease through indirect effects on pneumococcal transmission (i.e., herd immunity) as through its direct effect of protecting vaccinated children.

Protection by anticapsular antibody is limited by its serotype specificity: The 7-valent pneumococcal conjugate vaccine (PCV7) has significantly reduced the incidence of invasive pneumococcal disease due to vaccine-type (VT) strains. Recent studies have shown, however, that non-VT serotypes in PCV7 are gradually replacing VT, potentially limiting the usefulness of the vaccine. This has led to the evaluation of whether pneumococcal colonization can be prevented by immunization with conserved antigens. In particular, several pneumococcal proteins have been evaluated as vaccine candidates in animal models of pneumococcal colonization. Mucosal immunization with some of these proteins has been shown to elicit systemic and mucosal antibodies and to confer protection against pneumococcal disease and colonization. There remains a need for an immunogenic composition, including pneumococcal polysaccharides and proteins, that raises antibodies and a robust cellular and humoral immune response to all serotypes.

The innate immune response provides rapid and usually effective defense against microbial pathogens. This response involves recognition of pathogen-associated molecules, triggering production and release of inflammatory mediators, recruitment of leukocytes, and activation of antimicrobial effectors. The Toll-like receptors (TLRs), of which at least eleven have been described for mammals, are capable of discriminating among a wide variety of pathogen-associated molecules and eliciting protective responses. TLR4 recognizes microbial products from organisms including gram-negative bacteria, the F protein of respiratory syncytial virus, and cholesterol-dependent cytolysins (CDC) of gram-positive bacteria. Hence, there remains a need to harness the TLR4 mechanism in eliciting an immune response against infections such as pneumococcal colonization and disease.

SUMMARY OF THE INVENTION

The present invention relates to immunogenic compositions comprising a fusion protein, X:PdT, where PdT represents a nonhemolytic variant of pneumolysin and X represents a peptide/protein of interest, conjugated to a polysaccharide (PS) such as dextran or pneumococcal

cell wall polysaccharide (CWPS) (X:PsT-PS), such that humoral and cellular/mucosal immunity to protein X is enhanced.

An embodiment of the present invention provides for an immunogenic conjugate comprising a fusion protein of a truncated pneumococcal PsA protein and a nonhemolytic pneumolysin protein, conjugated to a pneumococcal cell wall polysaccharide. This conjugate vaccine confers synergistic TLR4-dependent, IL-17A-mediated protection against pneumococcal colonization and also confers significant protection against lethal pneumonia. Importantly, CWPS, pneumolysin and pneumococcal surface adhesin A (PsA) are conserved in all serotypes and are important antigens for protection against *S. pneumoniae*. Additionally, as shown herein, protection against colonization in mice may be mediated by TH17 cells and correlates with IL-17A expression from whole blood cells stimulated with pneumococcal antigens. The present invention provides for enhanced systemic TH17 activity, increased antibody levels, and protection from pneumococcal lethal infection and colonization following immunization with the PsA:PsT-CWPS fusion conjugates of these antigens in mice.

In a particular embodiment, a fusion protein (PsA:PsT) containing truncated PsA (amino acid residues 22-309) and a nonhemolytic variant of pneumolysin (PsT) (W433F, D385N, and C428G) was constructed, grown in and purified from *E. coli*, then conjugated with CWPS. Analysis of this conjugate showed retention of the TLR4 stimulatory property of PsT. More specifically, mice were immunized intranasally, twice at a weekly interval with 1 µg cholera toxin (CT) as adjuvant. Mice were bled three weeks after the last immunization and challenged intranasally with a serotype 6B strain the following week; density of colonization was determined seven days later by plating dilutions of tracheal washes. Mice immunized with the fusion protein conjugate had significantly increased IL-17A expression following pneumococcal stimulation compared with mice that received a protein plus polysaccharide antigen mixture. Further, the fusion conjugate PsA:PsT-CWPS significantly protected mice against colonization compared with mice immunized with a simple mixture of the three antigens (mixture of PsA, PsT, and CWP at the same molar ratios as in the fusion conjugate), who were not protected. In contrast to C3H/HeOuJ mice (wild-type), C3H/HeJ (TLR4 mutant) mice were not protected by the PsA:PsT-CWPS conjugate, implying that TLR4 plays an important role in protection. The present invention thus provides for a new vaccine candidate that is antigenically common to all serotypes and confers TLR4-dependent protection against pneumococcal colonization.

Moreover, the efficacy of the conjugate PsA:PsT-CWPS mixed in alum vs. mixture in alum was tested in subcutaneous (s.c.) delivery to mice. Control groups included alum alone, WCV (given intranasally) and anticapsular antibodies given passively (to mimic the effect of a

conjugate vaccine). Mice were challenged subsequently either nasally (to evaluate the effect on colonization) or by aspiration (to evaluate protection against sepsis and pneumonia). The conjugate was significantly more immunogenic than a mixture when given s.c. There was a 90% reduction in density of colonization by s.c. immunization with conjugate in alum vs. alum alone.

- 5 Importantly, in aspiration challenge (sepsis) model, the conjugate was 100% protective against death, whereas mixture was non protective. Thus, the present invention provides for a conjugate vaccine that, with s.c. administration, is significantly more protective than an antigen mixture.

Additionally, the immunogenicity of one of the components of the fusion (PsaA) may be enhanced by combining it to CWPS and PdT. Therefore, other proteins of interest against which
 10 one wants to elicit an immune response may be incorporated as one part of the protein fusion. The invention thus relates to an immunogenic conjugate consisting of CWPS conjugated to X:PdT, where X represents any protein of interest, whether or not it is pneumococcal, so that an immune response against X is enhanced. In particular embodiments, X may be PsaA, StkPR, PcsB, or StkP. The data presented herein show that the immunogenicity of X is enhanced when
 15 X is presented as a synergistic fusion with PdT conjugated to CWPS, as opposed to when X is presented in a mixture of X, CWPS, and PdT antigens.

An embodiment of the present invention provides for an immunogenic composition comprising a fusion protein polysaccharide conjugate (X:PdT-PS) wherein X is an antigen, PdT is a nonhemolytic variant of pneumolysin, PS is a polysaccharide, wherein administration of said
 20 composition to a subject elicits enhanced humoral immunity, enhanced cellular immunity, and enhanced mucosal immunity. In one aspect of the embodiment, the PS has a molecular mass of <500 kDa. In another aspect of the embodiment, the PS has a molecular mass of <70 kDa.

Another embodiment of the present invention provides for an immunogenic composition comprising a fusion protein polysaccharide conjugate consisting of a polysaccharide conjugated
 25 to a X:PdT fusion protein where PdT is a nonhemolytic variant of pneumolysin and X is a target protein, such that immunity to X is enhanced. The polysaccharide may be dextran, Vi polysaccharide of *Salmonella typhi*, or pneumococcal cell wall polysaccharide (CWPS), or another polysaccharide of prokaryotic or eukaryotic origin.

In one aspect of the invention, X is an antigen derived from *Staphylococci*, *Streptococci*,
 30 *Brucella*, *Enterococci* species; *Listeria*, *Bacillus*, *Corynebacteria*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Moraxella*, typeable or nontypeable *Haemophilus*, *Haemophilus* nontypeable, *Pseudomonas*, *Salmonella*, *Shigella*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *E. coli*, *Clostridia*, *Bacteroides*, *Chlamydiaceae*, *Mycoplasma*, *Legionella*, *Treponemes*, *Borrelia*, *Candida* or other yeast or other fungi, *Plasmodium*, *Amoeba*, herpes viruses, cytomegalovirus,

Epstein-barr virus, varicella-zoster virus, influenza, adenoviruses, enteroviruses, or hemorrhagic viruses.

In another aspect of the invention, X is a truncated pneumococcal PsaA protein, pneumococcal serine/threonine protein kinase (StkP), pneumococcal serine/threonine protein kinase repeating unit (StkPR), pneumococcal PcsB protein, *Mycobacterium tuberculosis* mtb protein ESAT-6, *M. tuberculosis* cell wall core antigen, *Chlamydia* CT144, CT242 or CT812 polypeptides or fragments of these, *Chlamydia* DNA gyrase subunit B, *Chlamydia* sulfite synthesis/biphosphate phosphatase, *Chlamydia* cell division protein FtsY, *Chlamydia* methionyl-tRNA synthetase, *Chlamydia* DNA helicase (uvrD), *Chlamydia* ATP synthase subunit I (atpI), or *Chlamydia* metal dependent hydrolase.

In another embodiment of the present invention, X is a cancer antigen. In another embodiment of the invention, PS is a cancer antigen.

A particular embodiment of the present invention provides for an immunogenic composition comprising a fusion protein of a truncated pneumococcal PsaA protein and a nonhemolytic pneumolysin PdT protein, conjugated to a pneumococcal cell wall polysaccharide (CWPS).

Another embodiment of the present invention provides for a method of conferring TLR4-dependent protection against pneumococcal colonization comprising administering a composition comprising PsaA:PdT-PS or PsaA:PdT-CWPS.

Yet another embodiment provides for a composition consisting essentially of a fusion protein (PsaA:PdT) containing truncated PsaA and a nonhemolytic variant of pneumolysin (PdT) conjugated with CWPS.

DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B depict construction of a PsaA:PdT fusion protein. Figure 1A provides a diagrammatic description of a truncated PsaA (22-309) and a nonhemolytic variant of pneumolysin (PdT) (W433F, D385N, and C428G) connected together by a polylinker GSGGGGS (SEQ ID NO:1). Protein was cloned into pQE30 plasmid and then transformed *E. coli* for expression. Proteins were expressed in *E. coli* by IPTG induction and then purified using Ni-NTA beads. Figure 1B shows a SDS-PAGE characterization of the components. Lane 1, PdT; Lane 2, PsaA; Lane 3, PsaA:PdT.

Figure 2 shows the retention of TLR4 stimulatory activity of PdT in the conjugate and fusion constructs. Immortalized macrophage cell lines (WT or TLR4^{-/-}) were stimulated with 36 nM of each stimulus for 20 hours and the concentration of TNF- α in cell supernatant was measured by ELISA.

Figures 3A to 3E present responses to intranasal immunization with either two-component PdT-CWPS and PsaA-CWPS conjugates, or uncoupled antigens. Panels A-C: Mice were vaccinated weekly twice with 1 μ g of cholera toxin (CT) as adjuvant. Antigen dosages, coupled or not, were 8 μ g of PdT and 10 μ g of CWPS. Blood was taken three weeks after the second immunization. Serum IgG antibody responses were assayed to PdT (Figure 3A) and CWPS (Figure 3B). Mice immunized with the PdT-CWPS conjugate made significantly more anti-PdT and anti-CWPS antibodies than mice that received CT alone, and more anti-CWPS antibodies than mice that received the mixture of PdT and CWPS. (Figure 3C): The mice were challenged intranasally with the serotype 6B strain four weeks post-immunization, and the density of colonization was determined seven days later by plating dilutions of nasal washes. No protection against colonization was observed in any of the immunization groups. Panels 3D and 3E: Mice were immunized with CT with or without the addition of a conjugate of PsaA (5 μ g per dose) and CWPS (10 μ g per dose). Immunization with PsaA-CWPS resulted in significantly higher anti-PsaA antibodies than in control mice (Figure 3D), but no protection was observed following intranasal challenge (Figure 3E). For all panels, horizontal lines represent geometric means and statistical analysis was performed using the Kruskal-Wallis test with Dunn's correction or Mann-Whitney U. *P<0.05; **P<0.01.

Figures 4A to 4E reflect responses to intranasal immunization with the PdT:PsaA-CWPS fusion conjugate or controls. Immunizations were as in Figure 3. The conjugate contained 7.7 μ g of PdT, 5 μ g of PsaA, and 14 μ g of CWPS. The control antigens included a mixture of the individual components in dosage equal to the conjugate, the PdT:PsaA fusion protein in equal protein dosage, and WCA, the pneumococcal whole-cell antigen, about 10^8 cells = 100 μ g dry weight. Three weeks post-immunization, antibodies were measured against PdT (Figure 4A), CWPS (Figure 4B), and PsaA (Figure 4C). IL-17A production *in vitro* was measured in blood samples incubated six days with pneumococcal whole-cell antigen (Figure 4D). Four weeks post-immunization, the mice were challenged and nasopharyngeal colonization was determined as in Figure 3E. For all panels, horizontal lines represent geometric means and statistical analysis was performed using the Kruskal-Wallis test with Dunn's correction or Mann-Whitney U. *P<0.05; **P<0.01; ***P<0.001.

Figures 5A and 5B show protection conferred by fusion conjugate is dependent on TLR4. Figure 5A shows C3H/HeOuJ (wild type) and C3H/HeJ (TLR4 defective) mice immunized twice intranasally with CT or the fusion conjugate with CT then challenged with strain 0603. In contrast to HeOuJ mice (wild-type) that were well protected against colonization following immunization with the fusion conjugate, HeJ mice (TLR4-defective) were not protected. Figure 5B shows splenocytes from fusion conjugate-immunized HeOuJ and HeJ mice

that were stimulated with the antigens as shown, and the IL-17A concentration in the cell supernatant measured after three days. Splenocytes from HeOuJ mice made significantly more IL-17A in response to WCA and PdT than splenocytes from HeJ mice.

Figures 6A to 6D demonstrate the effect of subcutaneous vaccination on antibody production. Injections were given biweekly twice with 200 μ g of alum as adjuvant. The antigen contained 7.7 μ g of PdT, 14 μ g of CWPS, and 5 μ g of PsaA, given either as a mixture or as the fusion conjugate. The animals were bled two weeks after the last immunization, and serum IgG antibodies were measured against PdT (Figure 6A), CWPS (Figure 6B), PsaA (Figure 6C), or the pneumococcal whole cell antigen, WCA (Figure 6D). For all panels, horizontal lines represent geometric means and statistical analysis was performed using Mann-Whitney U test for comparison of titers in mice immunized with the fusion conjugate vs. the mixture. * $P < 0.05$, ** $P < 0.01$ by Kruskal-Wallis test with Dunn's correction.

Figures 7A and 7B show the effect of subcutaneous vaccination on priming for IL-17A production and on colonization by serotypes 19F and 6B. Mice were immunized as in Figure 6 except that one additional injection was given. Figure 7A: Three weeks post-immunization, IL-17A production in vitro was assayed in blood samples incubated 6 days with pneumococcal whole-cell antigen. *** $P < 0.0001$ determined by Mann-Whitney U test. Figure 7B: Four weeks post-immunization the animals were challenged intranasally with TIGR4 strain expressing serotype 19F or serotype 6B strain 0603 and nasopharyngeal colonization was determined as described as in Figure 3. Four mice were given IP injections of anti-CD4 antibodies just before challenge. Horizontal lines denote the geometric mean.

Figure 8 is a graph showing protection by the fusion conjugate administered subcutaneously in a model of fatal aspiration pneumonia. The conjugate or antigen mixture was injected with alum as in Figure 6. Three weeks post-immunization, a positive control group was given capsular antibody passively, then all animals were colonized by nontraumatic intranasal inoculation with 10^6 c.f.u. serotype 3 strain WU2. Three days later, mice were made to inhale WU2 into the lung and monitored twice daily. Deaths were noted, and sick animals were euthanized and their blood cultured. All the sick mice had pneumococci in the blood, and thus were counted as non-survivors. Differences in survival were compared using the Kaplan Meier test.

Figure 9 shows the IgG antibody response in s.c. immunization groups to mixtures of X, PdT and CWPS antigens, compared with X:PdT-CWPS conjugates of the same antigens, where X represents either StkpR, PcsB, or Stkp. *** $P < 0.0005$; ** $P < 0.01$.

Figure 10 shows antibody titers to PsaA in conjugates comparing CWPS with dextran. Mice were immunized three times, two weeks apart, with vaccine preparations as indicated

(alum control, PsaA:PdT-CWPS or PsaA:PdT-Dextran). Blood samples were obtained two weeks after the second (Figure 10A) and third immunizations (Figure 10B), and serum analyzed by ELISA for the titer of IgG antibodies directed against PsaA. Mice immunized only with alum control had essentially undetectable antibodies. Mice immunized with the dextran-containing conjugate did not have lower antibody titers ($P>0.2$) than mice immunized with the CWPS-containing conjugate after either two or three doses. Median and interquartile range are shown. N=10 per group.

Figure 11 shows T-cell responses following the immunization scheme and groups of Figure 10. Two weeks after second and third immunization, whole blood of mice was sampled and stimulated as indicated to measure T cell cytokine (IL-17A and IFN γ) responses. Mean and SEM are shown, N=10 mice per group. Figure 11A shows IL-17A responses after two or three doses for blood stimulated with whole cell pneumococcal antigen. Figure 11B shows IFN γ responses after three doses in blood stimulated with killed pneumococcus. Figure 11C shows IL-17A responses after three doses in blood stimulated with PsaA. Figure 11D shows IFN γ responses after three doses for blood stimulated with PsaA.

Figure 12 shows colonization testing using the conjugates of Figure 10. Two weeks after the last immunization, the mice were challenged intranasally with a clinical isolate of pneumococcus (strain 0603); colonization status was evaluated ten days later by determining density of bacteria in respiratory washes. Columns represent medians with interquartile range. P values by Mann Whitney test are shown.

DETAILED DESCRIPTION

The invention presented herein is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such may vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

As used herein and in the claims, the singular forms include the plural reference and vice versa unless the context clearly indicates otherwise. Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about."

All patents and other publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not

entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

5 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as those commonly understood to one of ordinary skill in the art to which this invention pertains. "Immunogenic" means a substance is capable of inducing an immune response in a subject. Although any known methods, devices, and materials may be used in the practice or testing of the invention, the methods, devices, and materials in this regard are described herein.

10 The present invention provides for enhanced immunogenicity of a composition consisting of a polysaccharide (e.g., dextran, CWPS) conjugated to pneumolysoid fused with a target protein that results in greatly enhanced immunity to the target protein and offers the possibility of providing protection against other pathogens. One embodiment provides for conjugate vaccine in which a fusion protein of PsaA and PdT was coupled to CWPS. This
15 conjugate was evaluated with respect to preservation of TLR4 activity and to immunogenicity. Serum antibodies, priming for IL-17A production and protection against nasopharyngeal colonization, in response to both intranasal (i.n.) and subcutaneous (s.c.) vaccination, were assayed, and the s.c. route was examined further in a fatal aspiration pneumonia model. The fusion conjugate was significantly more protective than a mixture of its components or than any
20 of the three combinations of two antigens. The fusion conjugate, when administered i.n., reduced nasopharyngeal colonization by a strain of serotype 6B, a major disease type in infancy. Although the conjugate elicited antibodies to all three of its component antigens, the protection against colonization appears more to depend upon priming for enhanced elicitation of IL-17A upon encounter with pneumococci. Lu et al., 77 Infect. Immun. 2076-83 (2009).

25 The present invention provides for the design of immunogenic conjugates or vaccines against many pathogens for which generating either antibody and/or cellular immunity is desirable. Over the past few years, there has been a growing list of pathogens for which the combination of the two forms of immune responses has been shown to play a role in defense against the pathogen. This is true for pneumococcus, *Staphylococcus aureus*, herpes viruses,
30 *Chlamydia trachomatis*, to name a few. Thus, the immunogenic conjugate of the present invention could be used to stimulate immunities to bacterial (including mycobacterial), fungal, parasitic, and viral pathogens.

Regarding bacterial pathogens, the antigenic X component of the present synergistic, immunogenic fusion conjugates may be derived from *Staphylococci* species, *Streptococci*
35 species (including Group A and B), *Enterococci* species; *Listeria*, *Bacillus* (including anthrax),

- Corynebacteria*, *Neisseria* (*meningitidis* and *gonorrhoeae*), *Moraxella*, *Haemophilus* (typeable and nontypeable), *Pseudomonas* (*aeruginosa* and others), *Salmonella* (*typhi* and nontyphi), *Shigella*, resistant gram-negative enteric bacteria (*Enterobacter*, *Citrobacter*, *Klebsiella*, *E. coli*, etc.), *Clostridium difficile* and other *Clostridia*, *Bacteroides* and other anaerobes,
- 5 *Chlamydiaceae* species (*C. trachomatis* and *C. pneumoniae*), *Mycoplasma* and *Legionella* as well as the *Treponemes* (*syphilis*, *leptospirosis*) and *Borrelia*.

Thus, in a particular embodiment of the present invention, PdT was genetically fused to pneumococcal PcsB protein (derived from *pcsB*, which encodes the protein required for cell separation in group B streptococci) (Giefing et al., 205 J. Exp. Med. 117-31 (2007)), and

10 conjugated to CWPS. This conjugate elicited more IgG against PcsB than did the corresponding mixture of antigens. In another particular embodiment, PdT was genetically fused to the pneumococcal serine/threonine protein kinase (StkP) (Giefing et al., 2007), and conjugated to CWPS. This conjugate elicited more IgG against StkP than did the corresponding mixture of antigens. The same synergistic response was observed in another anti-pneumococcal preparation

15 in which pneumococcal serine/threonine protein kinase repeating unit, StkPR, was genetically fused to PdT and conjugated to CWPS (Figure 9).

Another class of bacteria for which the antigenic X component of the present synergistic, immunogenic fusion conjugates may be derived include *Mycobacteria*, especially

Mycobacterium tuberculosis – thus for example the X is a protein from tuberculosis, such as *mtb*

20 protein ESAT-6, for example or cell wall core antigen (Kaplan et al., 5 P.N.A.S. USA 1917-21 (1988)) or a protein from *M. leprae*, e.g., PP-I (Chirmule et al., 86 Int'l Arch. Allergy Appl. Immun. 19-27 (1988))

As noted above, *Chlamydia* species may provide the target X antigen in the present invention. *Chlamydiaceae* (consisting of *Chlamydiae* and *Chlamydophila*), are obligate

25 intracellular gram-negative bacteria. *Chlamydia trachomatis* infections are among the most prevalent bacterial sexually transmitted infections, and perhaps 89 million new cases of genital *chlamydial* infection occur each year. The *Chlamydia* of the present invention include, for example, *C. trachomatis*, *Chlamydophila pneumoniae*, *C. muridarum*, *C. suis*, *Chlamydophila abortus*, *Chlamydophila psittaci*, *Chlamydophila caviae*, *Chlamydophila felis*, *Chlamydophila*

30 *pecorum*, and *C. pneumoniae*. Animal models of chlamydial infection have established that T-cells play a critical role both in the clearance of the initial infection and in protection from re-infection of susceptible hosts. Hence, the conjugates of the present invention may provide particular value by eliciting cellular immune responses against chlamydial infection.

More specifically, *Chlamydial* antigens useful in the present invention include DNA

35 gyrase subunit B, sulfite synthesis/biphosphate phosphatase, cell division protein FtsY,

methionyl-tRNA synthetase, DNA helicase (uvrD); ATP synthase subunit I (atpI) or a metal-dependent hydrolase (U.S. Patent Application Pub. No. 20090028891). Additional *Chlamydia trachomatis* antigens include CT144 polypeptide (SEQ ID NO:7), a peptide having amino acid residues 67-86 of CT144 (SEQ ID NO:8), a peptide having amino acid residues 77-96 of CT144 (SEQ ID NO:9), CT242 protein (SEQ ID NO:10), a peptide having amino acids 109-117 of CT242 (SEQ ID NO:11), a peptide having amino acids 112-120 of CT242 polypeptide (SEQ ID NO:12), CT812 protein (from the *pmpD* gene) (SEQ ID NO:13), a peptide having amino acid residues 103-111 (SEQ ID NO:14) of the CT812 protein; and several other antigenic peptides from *C. trachomatis*: NVTQDLTSSTAKLECTQDLI (SEQ ID NO:15), AKLECTQDLIAQGKLIVTNP (SEQ ID NO:16), SNLKRMQKI (SEQ ID NO:17), AALYSTEDL (SEQ ID NO:18), FQEKDADTL (SEQ ID NO:19), QSVNELVYV (SEQ ID NO:20), LEFASCSSL (SEQ ID NO:21), SQAEGQYRL (SEQ ID NO:22), GQSVNELVY (SEQ ID NO:23), and QAVLLLDQI (SEQ ID NO:24) (WO 2009/020553). Additionally, *Chlamydia pneumoniae* antigens including homologues of the foregoing polypeptides (see U.S. Patent No. 6,919,187), may be used as X in the X:PD-T-PS constructs of the present invention.

Fungal targets of the present synergistic, immunogenic conjugates include *Candida* species and other yeast; or other fungi (aspergillus, other environmental fungi). Regarding other parasites, malaria as well as worms and amoebae may provide the antigenic X for the synergistic, immunogenic conjugates of the invention.

Viral targets for the present synergistic, immunogenic conjugates include, in particular, herpes viruses including herpes simplex viruses, cytomegalovirus, Epstein-Barr virus, and varicella-zoster virus. Other common viral causes of disease in humans and animals may also be targeted, including influenza, adenoviruses, enteroviruses, and hemorrhagic viruses.

The X:PD-T-PS conjugate may also be constructed to provide for a synergistic immunogenic reaction to an antigenic sugar moiety. For example, the Vi polysaccharide of *Salmonella typhi* could be used. Vi capsular polysaccharide has been developed against bacterial enteric infections, such as typhoid fever (Robbins et al., 150(3) J. Infect. Dis. 436-49 (1984); Levine et al., 7 Baillieres Clin. Gastroenterol. 501-17 (1993)). Vi is a polymer of α -1 $\rightarrow$ 4-galacturonic acid with an N-acetyl at position C-2 and variable O-acetylation at C-3. The virulence of *S. typhi* correlates with the expression of this molecule (Sharma et al., 101 P.N.A.S. USA 17492-97 (2004)). The Vi polysaccharide vaccine of *S. typhi* has several advantages: Side effects are infrequent and mild, a single dose yields consistent immunogenicity and efficacy. Vi polysaccharide may be reliably standardized by physicochemical methods verified for other polysaccharide vaccines, Vi is stable at room temperature and it may be administered

simultaneously with other vaccines without affecting immunogenicity and tolerability (Azze et al., 21 Vaccine 2758–60 (2003)).

Thus, the Vi polysaccharide of *Salmonella typhi* may be conjugated to a fusion protein where the X is from the same or from another organism, such that the resulting vaccine confers immunity against one pathogen, or two different pathogens: if X confers protection against pneumococcus, a Vi-X:PD construct raises an immunogenic response against *S. typhi* and pneumococcus. Other examples include combining sugars from encapsulated bacteria (such as meningococcus, *S. aureus*, pneumococcus, etc.) and tuberculous protein, to provide a vaccine that protects against two different pathogens.

Other polysaccharide (PS) moieties that may be used in the present invention in alternative to dextran, CWPS, etc., include carbohydrate antigens of cancers. For example, the Tn antigen, an oligosaccharide expressed exclusively by cancer cells (Buskus et al., 44 Angew Chem. Int'l Ed. 5985-88 (2005)).

In one aspect of the invention, the PS has a molecular mass of <500 kDa. In another aspect of the invention, the PS has a molecular mass of <70 kDa.

Additionally, the present invention also provides for synergistic, immunogenic conjugates against cancer. In these conjugates, the target X protein is a protein expressed predominantly on the cancer cells, such that the conjugate elicits both potent humoral and potent cellular immunity to this protein. A large number of cancer-associated antigens have been identified, several of which are now being used to make experimental cancer treatment vaccines and are thus suitable for use in the present embodiments. Antigens associated with more than one type of cancer include Carcinoembryonic antigen (CEA); Cancer/testis antigens, such as NY-ESO-1; Mucin-1 (MUC1) such as Sialyl Tn (STn); Gangliosides, such as GM3 and GD2; p53 protein; and HER2/neu protein (also known as ERBB2). Antigens unique to a specific type of cancer include a mutant form of the epidermal growth factor receptor, called EGFRvIII; Melanocyte/melanoma differentiation antigens, such as tyrosinase, MART1, gp100, the lineage related cancer-testis group (MAGE) and tyrosinase-related antigens; Prostate-specific antigen; Leukaemia-associated antigens (LAAs), such as the fusion protein BCR-ABL, Wilms' tumour protein and proteinase 3; and Idiotype (Id) antibodies. See, e.g., Mitchell, 3 Curr. Opin. Investig. Drugs 150-58 (2002); Dao & Scheinberg, 21 Best Pract. Res. Clin. Haematol. 391-404 (2008).

Another approach in generating an immune response against cancer employs antigens from microbes that cause or contribute to the development of cancer. These vaccines have been used against cancers including hepatocellular carcinoma (hepatitis B virus, hepatitis C virus, *Opisthorchis viverrin*), lymphoma and nasopharyngeal carcinoma (Epstei-Barr virus), colorectal cancer, stomach cancer (*Helicobacter pylori*), bladder cancer (*Schistosoma hematobium*), T-cell

leukemia (human T-cell lymphotropic virus), cervical cancer (human papillomavirus), and others. To date, there have been clinical trials for vaccines targeting Bladder Cancer, Brain Tumors, Breast Cancer, Cervical Cancer, Kidney Cancer, Melanoma, Multiple Myeloma, Leukemia, Lung Cancer, Pancreatic Cancer, Prostate Cancer, and Solid Tumors. *See* Pardoll et al.,

- 5 ABELOFF'S CLIN. ONCOL. (4th ed., Churchill Livingstone, Philadelphia 2008); Sioud, 360 Methods Mol. Bio. 277-318 (2007); Pazdur et al., 30(3) J. Infusion Nursing 30(3):173-78 (2007); Parmiani et al., 178 J. Immunol. 1975-79 (2007); Lollini et al., 24 Trends Immunol. 62-66 (2003); Schlom et al., 13 Clin. Cancer Res. 3776-82 (2007); Banchereau et al., 392 Nature 245-52 (1998); Finn, 358 New Engl. J. Med. 2704-15 (2008); Curigliano et
- 10 al., 7 Exp. Rev. Anticancer Ther. 1225-41 (2007). Thus, the present embodiments encompass both preventive/prophylactic cancer vaccines and treatment/therapeutic cancer vaccines.

- Recombinant proteins may be conveniently expressed and purified by a person skilled in the art using commercially available kits, for example PROBOND™ Purification System (Invitrogen Corp., Carlsbad, CA). Alternatively, standard molecular biology protocols may be
- 15 used, as for example described in Sambrook, et al., MOL. CLONING. A LAB. MANUAL (Cold Spring Harbor Press, 1989); CURRENT PROTOCOLS MOL. BIO. (Ausubel et al., eds., John Wiley & Sons, Inc. 1995-1999); and CURRENT PROTOCOLS PROT. SCI. (Coligan et al., eds., John Wiley & Sons, Inc. 1995-1999).

- Specific embodiments of the present invention provide for synergistic, immunogenic
- 20 conjugates which are capable of eliciting an immune response in an animal. More specifically, the compositions elicit both humoral and cellular immunity, and in many instance mucosal immunity. Embodiments of the present invention provide at least partial protection from or partial improvement after infection by, in particular, pneumococcal infection. Pneumococci cause a number of diseases, such as meningitis, pneumonia, bacteraemia, and otitis media.
 - 25 Almost one million children die of pneumococcal diseases worldwide every year. *S. pneumoniae* have been studied extensively, and at least some of the genomes sequenced (*see, e.g.*, U.S. Patent No. 7,141,418). Antibodies to the capsular polysaccharides, which define the known serotypes, confer serotype-specific protection, and have been called the only significant mechanism of acquired immunity (Janeway et al., IMMUNOLOGY (Garland Pub., NY, 2001)).
 - 30 The application of protein-polysaccharide conjugate vaccine, PCV7, has reduced diseases significantly (Black et al., 24(S2) Vaccine 79-80 (2006); Hansen et al., 25(9) Pediatr. Infect. Dis. J. 779-81 (2006)). Yet, recent studies have shown that the lack of other serotypes in PCV7 has resulted in emerging replacement pneumococcal serotypes (Pichichero & Casey, 26(S10) Pediatr. Infect. Dis. J. S12-16 (2007)).

Certain pneumococcal antigens common to all serotypes of the species have been shown to have immunoprotective potential despite the encapsulation, e.g., the surface proteins PspA, PspC, PsaA and the cytotoxin pneumolysin or pneumolysoid mutants (Basset et al., 75 Infect. Immun. 5460-64 (2007); Briles et al., 18 Vaccine 1707-11 (2000)); the use of genomics and mutational libraries has identified several dozen additional species-common proteins (Hava & Camilli, 45 Mol. Microbiol. 1389-1406 (2002); Wizemann et al., 60 Infect. Immun. 1593-98 (2001)). Immunity has been induced by individual antigens in animal models (Alexander et al., 62 Infect. Immun. 5683-88 (1994); Balachandran et al., 70 Infect. Immun. 2526-34 (2002); Chung et al., 33 170 J. Immunol. 1958-63 (2003); Glover et al., 76 Infect. Immun. 2767-76 (2008); Wu et al., 175 J. Infect. Dis. 839-46 (1997)), but no vaccine based on a common antigen has been approved for human use to date.

One disadvantage of any strategy relying on single proteins is that, given the genetic diversity of pneumococci as well as its propensity for genetic transformation, it may be evaded. Further, antibody of a single common specificity may not bind to the capsulated cell in sufficient multiplicity to be effective. For this reason, vaccination with mixtures of species antigens has been proposed; in some studies, synergistic effects of mixtures of three proteins in systemic vaccination were demonstrated (Ogunniyi et al., 68 Infect. Immun. 3028-33 (2000)). Recently, it was shown that a mixture of three species-common proteins administered intranasally (i.n.) with cholera toxin adjuvant confers protection against colonization in an antibody-independent, CD4+ (and likely IL-17A)-dependent manner (Basset et al., 2007).

The present invention provides for a covalent combination of three species antigens: a non-toxic derivative of pneumolysin, PdT (Asp385Asn, Cys428Gly and Trp433Phe), the surface adhesin protein A (PsaA), coupled to cell wall polysaccharide (CWPS) (molecular weight about 26,400; Koenig & Perrings, 1 J. Biophys. Biochem. Cytol 93 (1955)). Surprisingly, the conjugate protected mice against colonization in a synergistic fashion, whereas mice immunized with a mixture of the antigens were not so protected. Additionally, in contrast to wild-type C3H/HeO/J mice, TLR4-deficient C3H/HeJ mice were not protected by the intranasal immunization with conjugate, implying that TLR4 plays a role in protection. Indeed, subcutaneous immunization with the protein fusion conjugate resulted in a 90% reduction in pneumococcal colonization density, and also fully protected mice from lethal lung inhalation challenge by highly virulent serotype 3 strain.

Additionally, mice immunized intranasally with pneumococcal CWPS plus a mucosal adjuvant became resistant to nasopharyngeal colonization, and the immunity is antibody-independent and dependent upon CD4+ T-cells. Protection against colonization in mice is mediated by T_H17 cells, and correlated with IL-17A expression by blood samples stimulated

with pneumococcal antigens *in vitro*. In a mouse aspiration pneumonia model, CWPS also provides protection against invasive disease. In order to enhance immune response to CWPS, conjugation of CWPS to pneumococcal protein carriers was generated with the goal to enhance immune response to both CWPS and pneumococcal proteins. It was shown recently that this

5 IL-17 promotes agar surface killing of pneumococci by polymorphonuclear leukocytes in the absence of antibody and complement, and a similar mechanism might operate at the nasopharyngeal mucosa (Lu et al., 4(9) PLoS Pathog. e1000159 (Sept. 19, 2008)).

Intranasal immunization using enterotoxin or related adjuvants such as CT, although immunologically advantageous, has raised safety concerns due to possible entry into the central

10 nervous system via the olfactory nerve, and also due to evidence following the use of a killed nasal influenza vaccine in Switzerland (Mutsch et al., 350 N. Engl J. Med. 896-903 (2004)). Thus, subcutaneous presentation with alum - the standard adjuvant in human vaccination - was tested. By this mode of immunization, the fusion conjugate was active in antibody induction and priming for IL-17A, and it reduced nasopharyngeal colonization by the two tested

15 serotypes, 19F and 6B. Protection was abrogated by administration of neutralizing antibodies to CD4⁺ T cells, suggesting dependence on IL-17A and non-dependence on the antibody responses. The antibody responses to the conjugate, however, would be highly advantageous in immunization strategy: if pneumococci evaded the T-cell-mediated protection against colonization, the antibodies would represent an additional line of defense.

20 Protection by s.c. administration was tested also in a model of fatal disease due to aspiration of a highly capsulated serotype 3 strain. Here, the fusion conjugate, but not the mixture of its three component antigens, was completely protective, again demonstrating synergy of the fusion conjugate. There was enhancement from coupling to CWPS, seen with the IL-17A priming, which may be a physicochemical effect or due specifically to the potential of

25 zwitterionic polysaccharides to activate T_H17 cells (Briles et al., 153 J. Exp. Med. 694-705 (1981); Chung et al., 170 J. Immunol. 1958-63 (2003); Cobb et al., 117 Cell 677-87 (2004); Kalka-Moll et al., 169 J. Immunol. 6149-53 (2002); Malley et al., 74 Infect. Immun. 2187-95 (2006); Tzianabos et al., 275 J. Biol. Chem. 6733-40 (2000)). The synergy of the conjugate may also depend, in part, upon the TLR4 stimulatory property of the PdT component, which was

30 preserved in the present conjugate. Chemical linkage to PdT gave enhanced responses to PsaA and CWPS individually as well as in the fusion conjugate. This result agrees with the concept that the presence of TLR agonist and antigen on the same particle enhances processing for immunity (Blander & Medzhitov, 440 Nature 808-12 (2006)).

These results are generalizable to other proteins (beyond PsaA and involving pathogens

35 other than pneumococci). Thus, a construct consisting of CWPS conjugated to pneumolysoid

fused with a protein target results in greatly enhanced immunity to the target protein and offers the possibility of providing protection against other pathogens.

Further, regarding the particular embodiments of presented herein, pneumococcal cell wall polysaccharide (CWPS), a ribitol teichoic acid linked to the muramic residues of the cell wall peptidoglycan, and the membrane-bound lipoteichoic acid (LTA) (consisting of the identical teichoic acid with a glycolipid end group), has been identified as a species antigen. CWPS, and compositions including preparations of the polymer or its components, have been tested as a vaccine in animal models (Briles et al., 153(3) *J. Exp. Med.* 694-705 (1981); Szu et al., 39(2) *Infect. Immun.* 993-99 (1983); Wallick et al., 130(6) *J. Immunol.* 2871-75 (1983); Szu et al., 54(2) *Infect. Immun.* 448-55 (1986); Skov et al., 56(3) *Infect. Immun.* 1890-96 (1988)). Serum antibodies to phosphorylcholine protect mice against parenteral challenge with capsulated serotype 3 pneumococci (Briles et al., 153(3) *J. Exp. Med.* 694-705 (1992)).

Additionally, compositions including phosphorylcholine conjugated to a carrier protein and mixed with Freund's adjuvant protected mice against an intravenous challenge with serotype 1 or serotype 3 pneumococci (Wallick et al., 1983). Subsequent studies, however, failed to show protection in parenterally challenged mice by antibodies to phosphorylcholine or to determinants within the polymer "backbone" (Szu et al., 1986; Nielsen et al., 14(4) *Microbial Pathogenesis*, 299-305 (1993)). Studies in humans also failed to show any association between antibodies to phosphorylcholine and protection against invasive or mucosal pneumococcal disease (Musher et al., 161(4) *Infect. Dis.* 736-40 (1990); Koskela et al., 30(6) *J. Clin. Microbiol.* 1485-90 (1992)).

The issue was revisited recently to evaluate whether the initial successes established by Briles et al. could be reproduced using different models and routes of immunization. Intranasal immunization with CWPS confers significant and long-lasting protection against nasopharyngeal pneumococcal colonization and aspiration pneumonia leading to sepsis (Malley et al., 74(4) *Infect. Immun.* 2187-95 (2006)). Treatment of CWPS with periodate, which destroys the PS component, eliminates immunogenicity and protection, suggesting that the PS is a critical component of protection. The dose of CWPS required, however, is quite high: 100µg per dose (given twice i.n.) is required for protection against colonization.

Protection against colonization is antibody-independent, CD4+ T-cell-dependent and IL-17A dependent: antibodies to IL-17A abrogate protection (Malley et al., 2006) and mice lacking IL-17A receptor are not protected by immunization with CWPS. This MHC class II-dependent protection elicited by a PS is consistent with recent data showing that a PS with a zwitterionic charge motif within the repeating unit (such as CWPS) could activate CD4+ T cells in a process that is dependent on MHC class II (Tzianabos et al., 275(10) *J. Biol. Chem.* 6733-40

(2000); Cobb et al., 117(5) Cell 677-87 (2004)). It is believed that these PS (including PS A and PS B of *Bacteroides fragilis*, and the CWPS of pneumococci) are oxidized by nitric oxide and processed via MHC II by B-cells to T-cells through interactions with T-cell receptors.

Pneumolysin, the cholesterol-dependent cytolysin, is 53kDa protein composed of 470 amino acids and is encoded by the pneumolysin (*ply*) gene. Pneumolysin belongs to a family of protein toxins known as the 'thiol-activated cytolysins' and is a common component of almost every *S. pneumoniae* isolate. A non-toxic derivative PdT (Asp385Asn, Cys428Gly, Trp433Phe) activates cells via TLR4 (Malley et al., 100(4) P.N.A.S. USA, 1966-67 (2003); Srivastava et al., 73(10) Infect. Immun. 6479-87 (2005)). It has also been suggested that immunization with an antigen and TLR agonists is significantly more effective when the TLR agonist is part of the antigenic cargo (Blander & Medzhitov, 440(7085) Nature 808-12 (2006)), rather than just added in solution with the antigen. The IL-17A pathway is also known to be dependent on TLR involvement (Kolls & Linden, 21(4) Immunity, 467-76 (2004)).

Pneumococcal surface adhesin protein A (PsaA) is good candidate for vaccine development because studies have shown that PsaA exists in ninety pneumococcal serotypes, including clinically relevant strains (Morrison et al., 38(1) J. Clin. Microbiol. 434-37 (2000)), and immunization with PsaA protected mice from both nasal colonization and lethal infection (Talkington et al., 21(1) Microb. Patho. 17-22 (1996); Oliveira et al., 8(4) Microbes Infect. 1016-24 (2006)). A mixture of three proteins (pneumococcal surface antigen C [PspC], PsaA, and PdT), administered i.n. with adjuvant, confers protection against colonization, also in an antibody-independent, CD4+ (and likely IL-17A)-dependent manner (Basset et al., 75(11) Infect. Immun. 5460-64 (2007)).

A particular nonhemolytic variant of pneumolysin (PdT) (W433F, D385N, and C428G) and PsaA were chosen as the fusion protein carrier because PdT is a TLR4 ligand and PsaA has been shown to provide protection from pneumococcal colonization. Purified PdT and PsaA were conjugated to CWPS by 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) using the method described previously (Lees et al., 14(3) Vaccine, 190-98 (1996)), and then run through a Sepharose S300 column. Fractions from void volume were collected and filter sterilized. Analysis of the conjugate revealed that it contains a CWPS:protein ratio of 5:4 for PdT-CWPS and 2:1 for PsaA-CWPS.

PdT is a TLR4 agonist, and TLR4 signaling is important for protecting mice following intranasal challenge with pneumolysin-positive encapsulated pneumococci (Malley et al., 100 P.N.A.S. USA 1966-71 (2003)). Therefore the fusion protein and PdT conjugates were tested for retention of TLR4 activity: wild-type or TLR-knockout macrophages were incubated *in vitro* with the various antigens for twenty hours, and the production of TNF- α was measured by

ELISA (Figure 2). As expected, neither PsaA nor CWPS induced a TNF- α response, while the lipopolysaccharide (LPS) used as a positive control produced a two-log increase in TNF- α secretion in the TLR4+ cells. PdT, PdT:PsaA fusion protein, and the two PdT-containing CWPS conjugates induced similar TLR4-dependent responses. To exclude that the stimulation was due to LPS contamination of the recombinant proteins, the samples were tested after boiling for 1 hour. Boiling did not affect activity of the control LPS, while TNF- α production was eliminated in all other samples. Thus the TLR4-dependent response was not due to LPS contamination; furthermore, the fusion and coupling of PdT did not diminish its TLR agonist activity.

Intranasal immunizations with two-component conjugates PdT-CWPS and PsaA-CWPS, or uncoupled controls, were compared. Mice were intranasally immunized, twice at one-week interval, with CWPS, PsaA, PdT, mixtures of CWPS and the proteins, or the conjugates PdT-CWPS or PsaA-CWPS, all with amounts of each antigen equalized; 1 μ g of cholera toxin (CT) was used as adjuvant and was always tested alone as one control. The serum IgG antibody responses to PdT, CWPS, or PsaA measured by ELISA in sera taken three weeks after the last immunization are shown in Figure 3: The anti-PdT responses in the PdT-CWPS conjugate immunized mice were also greater than the respective mixture (Figure 3A). Anti-CWPS (Figure 3B) and anti-PsaA (Figure 3D) antibody responses in the PdT-CWPS or PsaA-CWPS conjugate immunized mice were greater than those in mice immunized with mixtures of the two antigens. The mice were challenged intranasally with serotype 6B pneumococcus strain 0603 four weeks after the second immunization. Colony-forming units (CFU) per mouse in nasal washes collected one week after challenge are shown in Figures 3C and 3E. No statistically significant reduction of bacterial colonization, compared to the controls receiving CT alone, was observed in any of the immunization groups.

Regarding the intranasal immunization with PsaA:PdT-CWPS, in order to enhance the protection, a fusion protein of PsaA and PdT with a poly-linker was constructed, as shown in Figure 1A, and was generated and purified as described herein. PsaA:PdT was conjugated to CWPS by CDAP and purified by gel filtration (Figure 1B). The ratio between protein and polysaccharide was determined to be 1/1.1.

Mice were similarly immunized with the fusion conjugate. A mixture of the three antigens or the PsaA:PdT fusion protein alone (all given with the adjuvant CT) was used as control; also included as controls were mice vaccinated with the pneumococcal WCA and CT. IgG antibodies to PdT, CWPS, and PsaA at three weeks after immunization are shown in Figure 4. The conjugate induced higher titers of anti-CWPS than the mixture (Figure 4B); the conjugate and the fusion protein both induced higher titers of anti-PsaA than the mixture

(Figure 4C). Interestingly, however, the conjugate induced less anti-PdT IgG than the mixture or fusion protein (Figure 4A).

Priming for expression of IL-17A *in vitro* is shown in Figure 4D. Priming for IL-17A production was assayed *in vitro* in cells from the three-week blood samples, incubated six days with pneumococcal whole cell antigen (WCA). No IL-17A was detected in cells incubated with DMEM culture medium only. The cells of conjugate-immunized mice produced IL-17A levels substantially higher than those immunized with the antigen mixture or the PsaA:PdT fusion protein and similar to cells from the mice immunized with the WCA. The results of challenge with serotype 6B pneumococci are shown in Figure 4E. There was no significant protection by the mixture or the fusion protein (albeit a suggestion of activity by the latter) as compared to the CT adjuvant alone. In contrast, there was about 40-fold reduction in the geometric mean colonization of the conjugate-immunized mice ($P = 0.009$). This protection was comparable to that produced by the pneumococcal WCA, known to be highly protective in this model (Malley et al., 2003).

Protection by the fusion conjugate PsaA:PdT-CPS was found herein to be TLR4 dependent. To test whether protection by the fusion conjugate is TLR4 dependent, C3H/HeJ (TLR defective) and C3H/HeOuJ (wild type) mice were intranasally immunized then challenged as previously described with strain 0603. The fusion conjugate protected HeOuJ mice from colonization to the same extent as observed in C57/BL6 mice, but there was no protection observed in HeJ mice suggesting that protection by the fusion conjugate is TLR4-dependent (Figure 5A). To investigate the mechanism behind this TLR4-dependent protection, antibodies to the whole cell antigen and IL-17A responses from splenocytes stimulated with WCA or the individual components of the fusion conjugate (PdT, CWPS, and PsaA) were measured in immunized HeJ and HeOuJ mice. No differences in antibody against WCA were noted between immunized HeOuJ and HeJ mice. In contrast, splenocytes from HeJ mice made significantly less IL-17A when stimulated with WCA or PdT (Figure 5B). It was demonstrated previously that protection against NP colonization is mediated by, and correlates with, IL-17A production (Lu et al., 2008). Thus the lack of protection in HeJ mice may be explained by a requirement for TLR4 for priming for IL-17A responses, as has been shown by others (Kolls & Linden, 2004). Immunity 467-76 (2004)).

To examine the fusion conjugate as a systemic immunogen, mice were injected s.c. (two-week intervals, alum adjuvant) with the conjugate or the control mixture of PdT, CWPS, and PsaA. Antibodies were measured two weeks after the secondary immunization. The conjugate generated higher antibody titers than the mixture for all three antigens (6-fold higher for PdT, 140-fold higher for CWPS, and 3-fold higher for PsaA; Figures 6A to 6C). Antibody

responses against WCA also were measured and were likewise greater (40-fold) after the conjugate (Figure 6D). Thus the fusion conjugate strongly elicited antibodies to its component antigens in configurations expressed in the pneumococcal cell. Priming for IL-17A responses and protection against colonization were determined after three s.c. injections. Cells from the s.c.-vaccinated mice produced on average about 100-fold higher IL-17A than the CT control (Figure 7A). The mice were nasally challenged (separately) with serotype 19F or serotype 6B. There was protection against both serotypes by about one log-fold compared to the alum controls ($P < 0.05$, Figure 7B). At the time of challenge with the serotype 6B strain, antibodies to murine CD4⁺ was administered to four mice previously immunized with the fusion conjugate; as a result, protection was abrogated in this group, suggesting that CD4⁺ T cells (and likely IL-17A) are responsible for this protection.

Possible protection against invasive disease was tested using a fatal aspiration pneumonia model with serotype 3 strain WU2 (Malley et al., 74 Infect. Immun. 2187-95 (2006)). Figure 8 shows that only 35% of the alum control mice survived. Survival of mice immunized with the antigen mixture (55%) was not significantly different. In contrast, 100% of mice vaccinated with the conjugate survived ($P < 0.002$ vs. alum controls by Kaplan-Meier).

Protection by the extant 23-valent capsular polysaccharide vaccine depends upon immunological maturity. In general, the vaccine is not effective for subjects under two years old. Broad use of the 7-valent capsular conjugate vaccine (PCV7) has greatly reduced pneumococcal disease, but the limited serotypes in PCV7 limit the vaccine's efficacy for most developing countries, and non-vaccine type serotype replacement has been reported in the U.S.

One embodiment of the present invention provides for a new conjugate vaccine candidate that contains CWPS and a fusion protein of PsaA and PdT, and protects against pneumococcal colonization and diseases in both *in vitro* and *in vivo* models. Additionally, this vaccine should confer protection against all serotypes because all the three components are conserved in pneumococcus.

Moreover, conjugation of CWPS to the fusion protein enhanced antibody responses to PsaA, both from intranasal immunization and subcutaneous immunization. The immunization with conjugate also enhanced T-cell responses. Further, the conjugate vaccine resulted in TLR4 dependent protection against colonization: following subcutaneous immunization, the fusion protein conjugate is more immunogenic and resulted in a 90% reduction in pneumococcal colonization density, and also protected mice from lethal lung inhalation challenge with serotype 3 strain pneumococcus.

Other implications of the present invention include: the fusion of a target protein (PsaA) to the TLR4 agonist PdT and subsequent conjugation of the fusion protein to the conserved

zwitterionic polysaccharide CWPS resulted in significantly higher immune responses to the target protein. These results suggest strongly that the construction of a conjugate consisting of CWPS conjugated to pneumolysin fused with a target protein (i.e., an antigen) should result in greatly enhanced immunity to the target protein. These results are generalizable to other proteins (beyond PsaA, involving pathogens other than pneumococcus). For example, the target proteins StkPR, PcsB, and StkP were found to have enhanced immunogenicity when presented as a PdT fusion conjugated to CWPS. In this sense, the construct/conjugate serves to augment the immune response to the protein, and may act as an effective adjuvant for vaccine development.

The CWPS of the present invention may be generated by any method known in the art (see, e.g., Slade, 90(3) J. Bacteriol. 667-72 (1965)), and the polysaccharide (PS) of non-pneumococcal bacteria may be used as well. For example, the PS of *Bacillus anthracis* is species specific (Choudhury et al., 281(38) J. Biol. Chem. 27932-41 (2006)), and may serve as an antigenic component. Similarly, the PS of the gram-negative bacteria *Brucella abortus* and *B. melitensis* are highly antigenic and well characterized (see, e.g., Rose et al., 2 Protein Sci. 1106-13 (1993)). Additionally, a portion of the CWPS, or a mimetic of the CWPS may be incorporated into a pneumococcal vaccine. Conjugate protein-PS vaccines, including those comprising streptococcal and pneumococcal PS are well-known in the art and provide for methods of conjugation. See e.g., U.S. Patents No. 6,248,570; No. 5,866,135; No. 5,773,007. CWPS mimotopes, such as protein or peptide mimetics of polysaccharide molecules, are also possible alternative antigens or immunogens (see, e.g., Pincus et al., 160. J. Immunol. 293-98 (1998); Shin et al., 168 J. Immunol. 6273-78 (2002)). Additionally, other proteins or nucleic acids may serve as antigens or immunogens in vaccine or vaccine development using any number of techniques known in the art. See, e.g., U.S. Patent No. 6,936,252.

Various pneumolysin mutants are also known in the art and within the scope of the present invention, including combinations of one or more His367Arg, Cys428Gly, Cys428Ser, Trp433Phe (Pdb toxoid), Glu434Gln, Trp435Phe, Asp385Asn, His156Tyr, His367Arg, and Cys428Gly. See, e.g., U.S. Patent No. 6,716,432. Any appropriate pneumolysin mutant may be suitable for the present fusion-conjugate, as long as they retain the immunogenic nature of pneumolysin but have reduced or undetectable hemolytic activity compared to native pneumolysin (Kirkham et al., 74(1) Infect. Immun. 586-93 (2006); U.S. Patent No. 6,764,686). Additionally, a fragment or portion of such a pneumolysin is also suitable for the present invention.

PspA is also well known in the art, as is PspC. An immunogenic region of these proteins has recently been identified, lacking alpha helical structure (WO/2007/089866). As noted above,

the fusion-conjugate composition of the present invention may include any protein of interest, from cultured or recombinant sources, or a fragment of such proteins.

One or more adjuvant agents may be included in such vaccines, as are accepted in the art, such as those described herein and, for example, *E. coli* mutant enterotoxin (U.S. Patent No. 6,019,982), cytokines and complement (Gor et al., 70(10) Infect. Immun. 5589-95 (2002)), or neisserial porins (U.S. Patent No. 6,613,336).

The delivery of pneumococcal vaccines and other vaccines, either by parenteral, mucosal, or other administration, as well as the design, monitoring, and dosing regimens of such vaccines are also well-known in the art.

10 Additionally, the conjugate of the present invention may be useful in vaccine development because it may be used to characterize the response at the cellular or molecular level. Sera or antibodies raised to the conjugate may also be used for passive protection. The antibodies useful for passive immunization may be raised initially against the present fusion-conjugate, but also includes both intact immunoglobulin molecules as well as portions, 15 fragments, peptides and derivatives thereof, such as, for example, Fab, Fab', F(ab')₂, Fv, CDR regions, or any portion or peptide sequence of the antibody that is capable of binding antigen or epitope. An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. The methods of generating such molecules are also well-known in the art.

20 Embodiments of the present invention are described further by these non-limiting examples.

EXAMPLES

Example 1. Construction of an immunogenic conjugate.

Materials. "Pneumococcal cell-wall polysaccharide, purified" (CWPS) was from Statens Seruminstitut, Copenhagen, Denmark. It was prepared from strain CSR SCS2 as described previously (Karlsson et al., 265(3) Eur. J. Biochem. 1091-97 (1999)). Pneumococcal whole-cell antigen (WCA) consists of ethanol-killed cells of a capsule-deficient pneumococcus strain Rx1AL- as previously reported (Malley, et al., 69 Infect. Immun. 4870-73 (2001)). Cholera toxin (CT) was from List Biological Laboratories (Campbell, CA). The monoclonal antibody to the PCCho determinant of CWPS (TEPC-15), 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), triethylamine (TEA), and ethanolamine (EA) were from Sigma (St. Louis, MO). Other chemicals used are of analytical grade.

Protein purification. *S. pneumoniae* nonhemolytic variant of pneumolysin (PdT) with mutations W433F, D385N and C428G, which render the molecule nontoxic but do not interfere with TLR4-mediated inflammatory properties (Berry et al., 63(5) infect. Immun. 1969-74 (1995)), was purified from *E. coli* msbB mutant strain carrying a pQE30 vector that expresses Pdt (Srivastava et al., 2005). PsaA was a gift from Dr. Edwin Ades, CDC, (Atlanta, GA) (Srivastava et al., 19(1) Hybridoma, 23-31 (2000); De et al., 419(2) Arch. Biochem. Biophys. 147-57 (2003)). PsaA and PdT fusion protein PsaA:PdT was generated by linking *S. pneumoniae* truncated PsaA with PdT with a GSGGGGS linker.

Briefly, PsaA was amplified from *S. pneumoniae* genomic DNA by primers 5'-GGGGATCCAGCGGAAAAAAGATACTTCTGGTC-3' (SEQ ID NO:2) and 5'-GCGGATCCACCTCCACCACTACCTTTTGCCAATCCTTCAGCAATC-3' (SEQ ID NO:3). The resulting truncated PsaA lacks N-terminus signal peptide and transmembrane domain and has a GSGGGGS (SEQ ID NO:1) in the C-terminus. This DNA piece was inserted between the 6His-tag and the starting codon of PdT and sequence of the final product was verified in Children's Hospital (Boston, MA) molecular genetics core facility.

E. coli cells were cultured in LB medium with antibiotic at 37°C until the OD₆₀₀ reached 0.6. Cultures were cooled to room temperature and induced with isopropyl-1-thio-β-D-galactopyranoside, added to a final concentration of 1 mM, and incubation continued at room temperature for overnight. Then, cells were collected by centrifugation (6,000 rpm, 4°C, 15 min) and cell pellets were lysed with sonication. Soluble proteins were applied to a Ni²⁺-agarose column and washed with 40 mM imidazole, 20mM Tris-HCl, pH 7.4, 0.5 M NaCl. His-tagged proteins were eluted with 10 mM EDTA. Purified proteins were dialyzed against PBS extensively. Proteins were then treated with ETclean beads (Cellufine, MA) to remove any residual LPS contamination.

Conjugation of CWPS to proteins. Proteins were conjugated to CWPS using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), as described previously (Lees et al., 14(3) Vaccine, 190-98 (1996)). Briefly, 5 mg of CWPS was solubilized in saline at 10 mg/ml; 10 mg of CDAP (100 mg/ml in acetonitrile) was added while vortexing; 30 sec later, 100 µl 0.2 M TEA was added into mixture; 5 mg protein was added into mixture after 2 min. Reaction was carried out overnight at 4°C and terminated with 100 µl 1M EA. The reaction mixture was applied to a Sepharose S300 column, eluted with PBS, and protein-conjugate was separated by collecting void volume fractions. Protein concentration was determined by BCA kit (Pierce) and CWPS content was determined by anthrone method (Roe, 212(1) J. Biol. Chem. 335-43 (1955)).

The composition of the protein-polysaccharide conjugates in the constructs was as follows: PdT-CWPS conjugate protein:CWPS ratio 4:5; PsaA-CWPS conjugate protein:CWPS ratio 1:2; and PsaA:PdT-CWPS conjugate, ratio 1:1.1.

Example 2. *In vitro* analysis of immunogenic conjugate

Assay of IL-17A production in whole blood samples. Fifty (50) µl of heparinized blood was added to 450 µl DMEM (BioWhittaker, Walkersville, MD) containing 10% low-endotoxin defined FBS (Hyclone, Logan, UT) and Ciprofloxacin (10 µg/ml, Cellgro, Manassas, VA). Except for the unstimulated control, the cultures were incubated at 37°C for six days with 10^7 cells of pneumococcal WCA or with purified antigens as specified. Supernatants were collected following centrifugation and stored at -80°C until analyzed by ELISA for IL-17A concentration (R&D Systems, Minneapolis, MN).

TNFα production in cultured macrophage. Wildtype and TLR4 knockout macrophage cell lines were gifts from University of Massachusetts. Cells were seeded at 1×10^5 /ml in 96-well plates and incubated at 37°C overnight. Medium was changed and 36 nM of each stimulus was added into each well and continued to incubate for 20 hr before supernatant was analyzed by ELISA for TNFα production.

Enzyme-linked immunosorbent assay (ELISA). Assays for murine antibodies to PdT and PsaA were done in Immulon 2 HB 96 microwell plates coated with either PdT or PsaA proteins (1 µg/ml). Assays for murine antibodies to CWPS was done in NUNC-immuno 96-microwell plates (Nalge Nunc Int'l, Rochester, NY) coated overnight with CWPS (5 µg/ml). Plates were blocked with 0.05% casin amino acid (PdT) or 5% fetal calf serum (PsaA and CWPS) in phosphate-buffered saline-0.05%Tween (PBS-T). Antibody diluted in PBS-T was added and incubated at room temperature for 2 hr. Plates were washed with PBS-T, and secondary antibody to mouse immunoglobulin G (Sigma) was added and incubated at room temperature for 1 hr. The plates were washed and developed with SureBlue TMB microwell peroxidase

substrate (KPL, Gaithersburg, MD). Antigenicity of CWPS derivatives was assayed by inhibition of the ELISA in which mouse monoclonal antibody to PCho (TEPC-15, Sigma) was premixed with serial dilutions of CWPS or the modified antigens before application to the CWPS coated plates as described previously (Malley et al., 2006).

5 *Neutralization of Pneumolysin.* A neutralization assay was developed to evaluate whether antibodies against pneumolysin may have the capacity to neutralize the hemolytic activity of the molecule. Briefly, 100 μ l of a solution containing pneumolysin at 100 ng/ml in PBS/0.1%BSA with 100 μ M dithiothreitol (DTT, Sigma) was incubated for 30 min at 37°C with serial dilutions of serum from mice immunized s.c. three times with the fusion conjugate or alum
10 alone. Following this incubation, 50 μ l of sheep red blood cells were added and incubated for another 30 min in the same conditions. After centrifugation at 2000g for 5 min, supernatants were harvested and their OD420 measured to quantify hemolysis. These values were read against a standard consisting of 2-fold diluted samples of fully hemolyzed red blood cells; then compared with the dilution of serum at which 50% hemolysis was observed between groups.

15

Example 3. Immunization and challenge of mice.

Mice were obtained from The Jackson Laboratories (Bar Harbor, ME) unless otherwise noted. C57BL/6J (or HeOuJ) were used as wild-type animals and are referred to as "mice". The age at time of first immunization was between four to six weeks. Intranasal injection was done
20 by instilling 10 μ l of saline, adjuvant only, or adjuvant mixed with antigen as specified atraumatically into unanesthetized mice, a procedure that puts no immunogen into the lungs; secondary immunizations were given after one week. The amounts of all antigens applied to mice were all normalized to 5 μ g PsaA per mouse. Subcutaneous immunization was done by mixing the antigen with 200 μ g of alum (Alum Hydroxide, Accurate Chem. & Scientific Co,
25 Westbury, NY) on a volume of 200 μ l and injection into the dorsal surface of the mouse. The amounts of all antigens were normalized to 5 μ g PsaA per mouse.

To determine susceptibility to NP colonization, i.n. challenge with live encapsulated pneumococci was done as described (Malley et al., (2001). Four weeks after the second immunization, mice were i.n. challenged with 10^8 colony-forming units (cfu)/ml serotype 6B
30 strain 0603 containing predominantly transparent phenotype (Weiser et al., 62(6) Infect. Immun. 2582-89 (1994)), or of a strain of serotype 19F in the TIGR4 background (Trzcinski et al., 69 Appl. Environ. Microbiol. 7364-70 (2003)), in 10 μ l PBS as described herein.

To examine whether protection against colonization was CD4+ T cell dependent, a small subset of mice received intraperitoneal injections of 0.35 mg of rat anti-mouse CD4 monoclonal
35 IgG2b (purified from hybridoma GK1.5, American Type Culture Collection [ATCC], Manassas,

VA) one day prior to and on day three of challenge as described previously (Basset et al., 2007; Malley et al., 2005).

To determine NP colonization, an upper respiratory culture was done by instilling sterile saline retrograde through the transected trachea, collecting the first six drops (about 0.1 ml) from the nostrils, and plating neat or diluted samples on blood agar plates containing 2.5 µg gentamicin/ml; thus 1 cfu/100 µl of wash fluid or 10 cfu/ml was detectable. For calculations of geometric means, a sterile sample was assigned half the lower limit of detection, or 5 cfu/ml. As a model for pneumonia and sepsis, the heavily capsulated serotype 3 strain WU2 (Briles et al., 153(3) J. Exp. Med. 694-705 (1981)), was used. The mice were given 10⁶ cfu in 10 µl of phosphate-buffered saline i.n. to establish NP colonization; after two days, to mimic the aspiration of pneumococci, the mice were anesthetized with isoflurane and given an additional dose of 10⁶ cfu of WU2 in 100 µl by i.n. route. In control animals, this challenge produced death within four-to-five days in most mice, and bacteremia in all mice.

Statistical analysis. NP colonization density was compared by the Mann-Whitney U test or by the Kruskal-Wallis test with Dunn's correction for multiple comparisons using PRISM (version 4.0a, GraphPad Software, Inc). Survival analysis by Kaplan Meier was performed using PRISM as well.

Example 4. Fusion to PdT-CWPS confers immunogenic synergy for X

Three conjugates of X: PdT-CWPS were prepared as discussed in the preceding examples. More specifically, in one immunogenic preparation, the PdT was fused to the C-terminal pneumococcal polypeptide StkP (Serine/Threonine Protein Kinase), which has the amino acid sequence:

YLILLASLVLVAASLIWILSRTPATIAIPDVAGQTVAEAKATLKKANFEIGEEKTEASEKV
EEGRIIRTDPGAGTGRKEGTKINLVSSGKQSFQISNYVGRKSSDVIAELKEKKVPDNLK
IEEESNESEAGTVLKQSLPEGTTYDLSKATQIVLTVAKKATTIQLGNYIGRNSTEVISL
KQKKVPENLIKIEEESSESEPGTIMKQSPGAGTTYDVS KPTQIVLTVAKKVT SVAMPSYI
GSSLEFTKNNLIQIVGIKEANIEVVEVTTAPAGSAEGMVVEQSPRAGEKVDLNKTRVKIS
IYKPKTTSATP (SEQ ID NO:4)

In another immunogenic preparation, PdT was fused to the N-terminal pneumococcal PesB protein (derived from *pcsB*, which encodes the protein required for cell separation in group B streptococci), which has the sequence:

ETDDKIAAQDNKISNLTAQQQEAQKQVDQIQEQVSAIQAEQSNLQAENDRLQAESKK
LEGEITELSKNIVSRNQSLEKQARSAQTNGAVTSYINTIVNSKSITEAISRVAAMSEIVSA
NNKMLEQQKADKKAISEKQVANNDIAINTVIANQQKLADDAQALTTKQAEKAAELSL

AAEKATAEGEKASLLEQKAAAEAEARAAAVAEAAAYKEKRASQQQSVLASANTNLTAQ
VQAVSESAAAPVRAKVRP (SEQ ID NO:5)

5 Another immunogenic construct fused PdT with a polypeptide StkPR (Serine/Threonine Protein Kinase repeat unit), which has the amino acid sequence:

VTSVAMPSYIGSSLEFTKNNLIQIVGIKEANIEVVEVTTAPAGSAEGMVVEQSPRAGEKV
DLNKTRVKISIIYKPKTTSATP (SEQ ID NO:6)

10

These recombinant constructs were conjugated to CWPS as described. Immunization groups were given subcutaneous injections of either the conjugate preparation, or equimolar mixtures of the same antigens. Subsequently, elicited IgG was compared for antigen "X." Each of the conjugates elicited more IgG than did the corresponding mixtures. Figure 9.

15

Example 5. Immunogenic constructs including dextran.

Groups of ten C57BL/6 mice each were immunized subcutaneously with one of the following, three times, two weeks apart: (1) alum alone; (2) CWPS-PdT:PsaA (fusion protein of PdT:PsaA conjugated to CWPS); and (3) PsaA:PdT-Dextran (fusion protein of PdT:PsaA
20 conjugated to dextran (35kD)).

Antibody responses to the X protein (represented here by PsaA) and also T-cell responses (both IL-17A and IFN γ) were evaluated and compared with the response to whole pneumococci as well as purified proteins.

Antibody titers to PsaA (the X in the construct): Mice were immunized three times, two
25 weeks apart with vaccine preparations as indicated (alum control, CWPS-PsaA:PdT or Dextran-PsaA:PdT). Blood samples were obtained two weeks after the second and third immunization and serum analyzed for the titer of IgG antibodies directed against PsaA by ELISA. Mice immunized only with alum/control had essentially undetectable antibodies. Mice immunized with the dextran-containing conjugate did not have lower antibody titers ($P>0.2$) than mice
30 immunized with the CWPS-containing conjugate after either two or three doses as shown in Figure 10. Median and interquartile range are shown. N=10 per group.

T cell responses: Same immunization scheme and groups. Two weeks after second and third immunization, whole blood of mice was sampled and stimulated as indicated to measure T-cell cytokine (IL-17A and IFN γ) responses. Mean and SEM are shown, N=10 mice per group.
35 Figure 11A shows IL-17A responses after two or three doses for blood stimulated with whole cell pneumococcal antigen. Figure 11B shows IFN γ responses after three doses in blood stimulated with killed pneumococcus. Figure 11C shows IL-17A responses after three doses in

blood stimulated with PsaA. Figure 11D shows IFN γ responses after three doses for blood stimulated with PsaA.

Colonization studies: Two weeks after the last immunization, the mice were challenged intranasally with a clinical isolate of pneumococcus (strain 0603); colonization status was
5 evaluated ten days later by determining density of bacteria in respiratory washes. Results are shown in Figure 12. Both the CWPS- and Dextran-based conjugates were significantly protective against colonization, suggesting that either sugar may be used. There may be advantages (vs. other forms of pneumococcal infections, such as sepsis/meningitis) to have CWPS in the construct; conversely, making a conjugate with dextran may be easier (as the
10 polysaccharide can be extremely pure).

CLAIMS

1. An immunogenic composition comprising a fusion protein-polysaccharide conjugate consisting of a polysaccharide conjugated to a X:PsT fusion protein where PsT is a nonhemolytic variant of pneumolysin and X is a target protein, such that immunity to X is enhanced.
2. The immunogenic composition of claim 1, wherein the polysaccharide is dextran, Vi polysaccharide of *Salmonella typhi*, or pneumococcal cell wall polysaccharide (CWPS), or another polysaccharide of prokaryotic or eukaryotic origin.
3. The immunogenic composition of claim 1 or 2, wherein X is an antigen derived from *Staphylococci*, *Streptococci*, *Enterococci* species; *Listeria*, *Bacillus*, *Brucella*, *Corynebacteria*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Moraxella*, *Haemophilus typeable*, *Haemophilus nontypeable*, *Pseudomonas*, *Salmonella*, *Shigella*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *E. coli*, *Clostridia*, *Bacteroides*, *Chlamydiaceae*, *Mycoplasma*, *Legionella*, *Treponemes*, *Borrelia*, *Candida* or other yeast or other fungi, *Plasmodium*, *Amoeba*, herpes viruses, cytomegalovirus, Epstein-Barr virus, varicella-zoster virus, influenza, adenoviruses, enteroviruses, or hemorrhagic viruses.
4. The immunogenic composition of claim 3, wherein X is a truncated pneumococcal PsA protein, pneumococcal serine/threonine protein kinase (StkP), pneumococcal serine/threonine protein kinase repeating unit (StkPR), pneumococcal PcsB protein, *Mycobacterium tuberculosis* mtb protein ESAT-6, *M. tuberculosis* cell wall core antigen, *Chlamydia* CT144, CT242 or CT812 polypeptides or fragments of these, *Chlamydia* DNA gyrase subunit B, *Chlamydia* sulfite synthesis/biphosphate phosphatase, *Chlamydia* cell division protein FtsY, *Chlamydia* methionyl-tRNA synthetase, *Chlamydia* DNA helicase (uvrD), *Chlamydia* ATP synthase subunit I (atpI), *Chlamydia* metal-dependent hydrolase, or *Chlamydia* antigens CT144, CT242, CT812, or fragments of these.
5. The immunogenic composition of claim 1 or 2, wherein X is a cancer antigen.
6. The immunogenic composition of claim 1 or 2, wherein the polysaccharide is a cancer antigen.

7. An immunogenic composition comprising a fusion protein of a truncated pneumococcal PsaA protein and a nonhemolytic pneumolysin PdT protein, conjugated to a pneumococcal cell wall polysaccharide (CWPS).

5 8. A method of conferring TLR4-dependent protection against pneumococcal colonization comprising administering the composition of any of claims claims 1 to 4.

9. A composition consisting essentially of a fusion protein (PsaA:PdT) containing truncated PsaA and a nonhemolytic variant of pneumolysin (PdT) conjugated with CWPS.

10

10. An immunogenic composition comprising a fusion protein polysaccharide conjugate (X:PdT-PS) wherein X is an antigen, PdT is a nonhemolytic variant of pneumolysin, PS is a polysaccharide, wherein administration of said composition to a subject elicits enhanced humoral immunity, enhanced T-cell immunity, and enhanced mucosal immunity.

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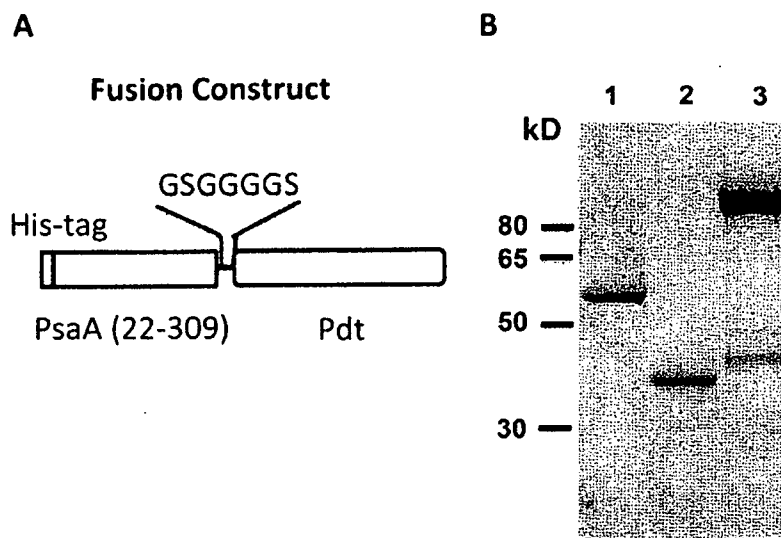


Figure 1

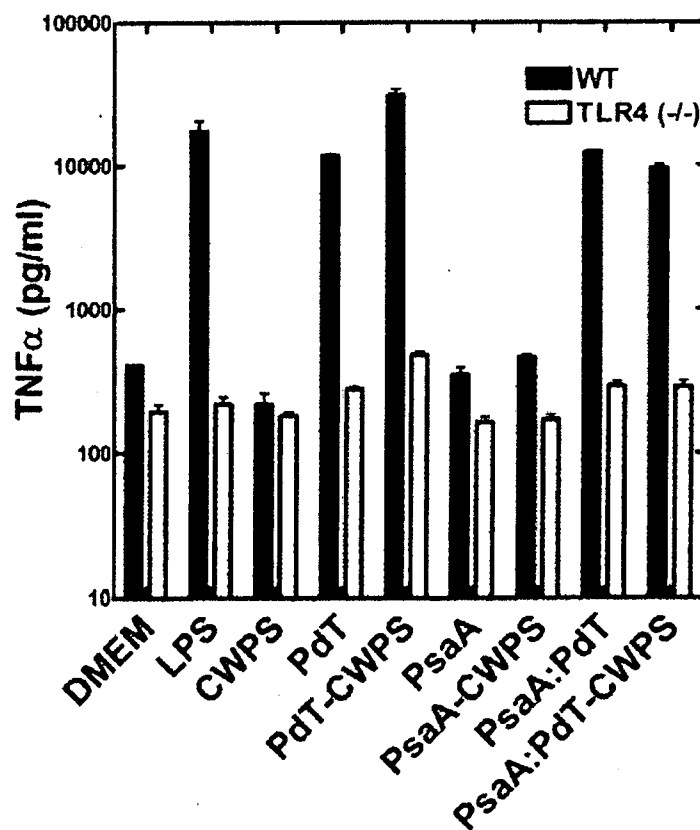
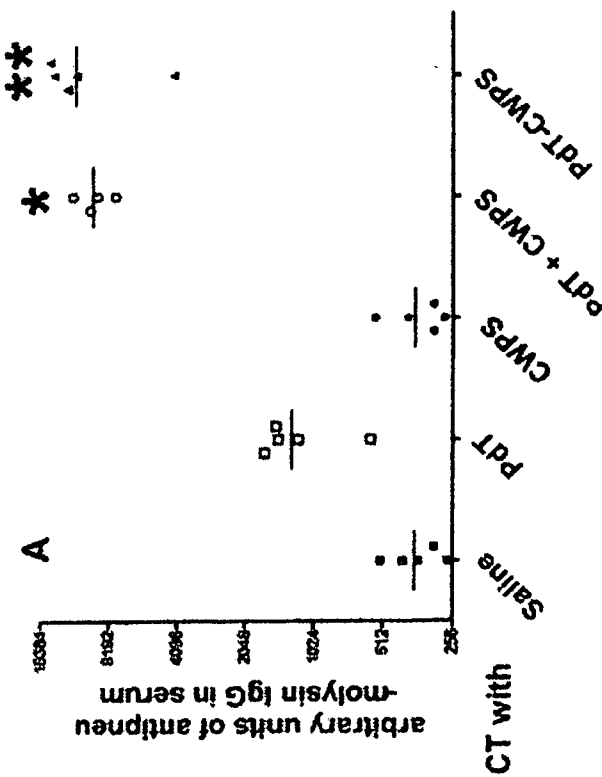
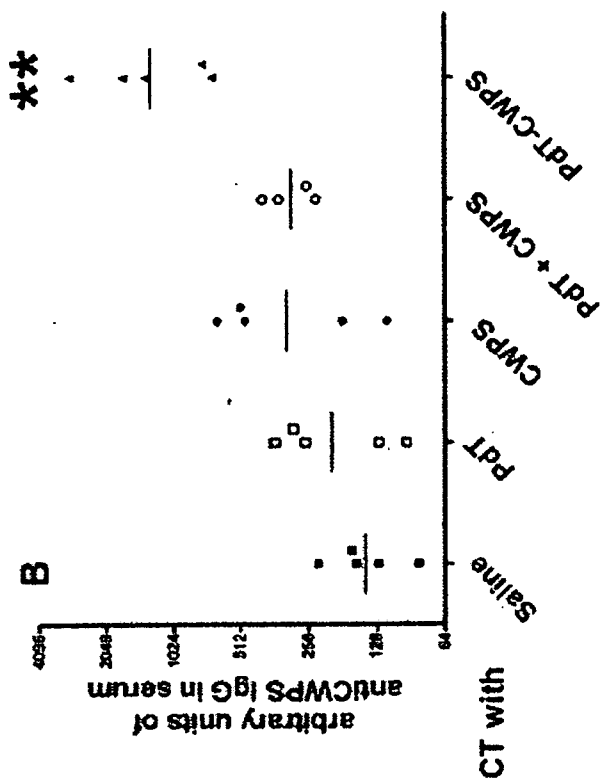


Figure 2

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Figures 3A and 3B

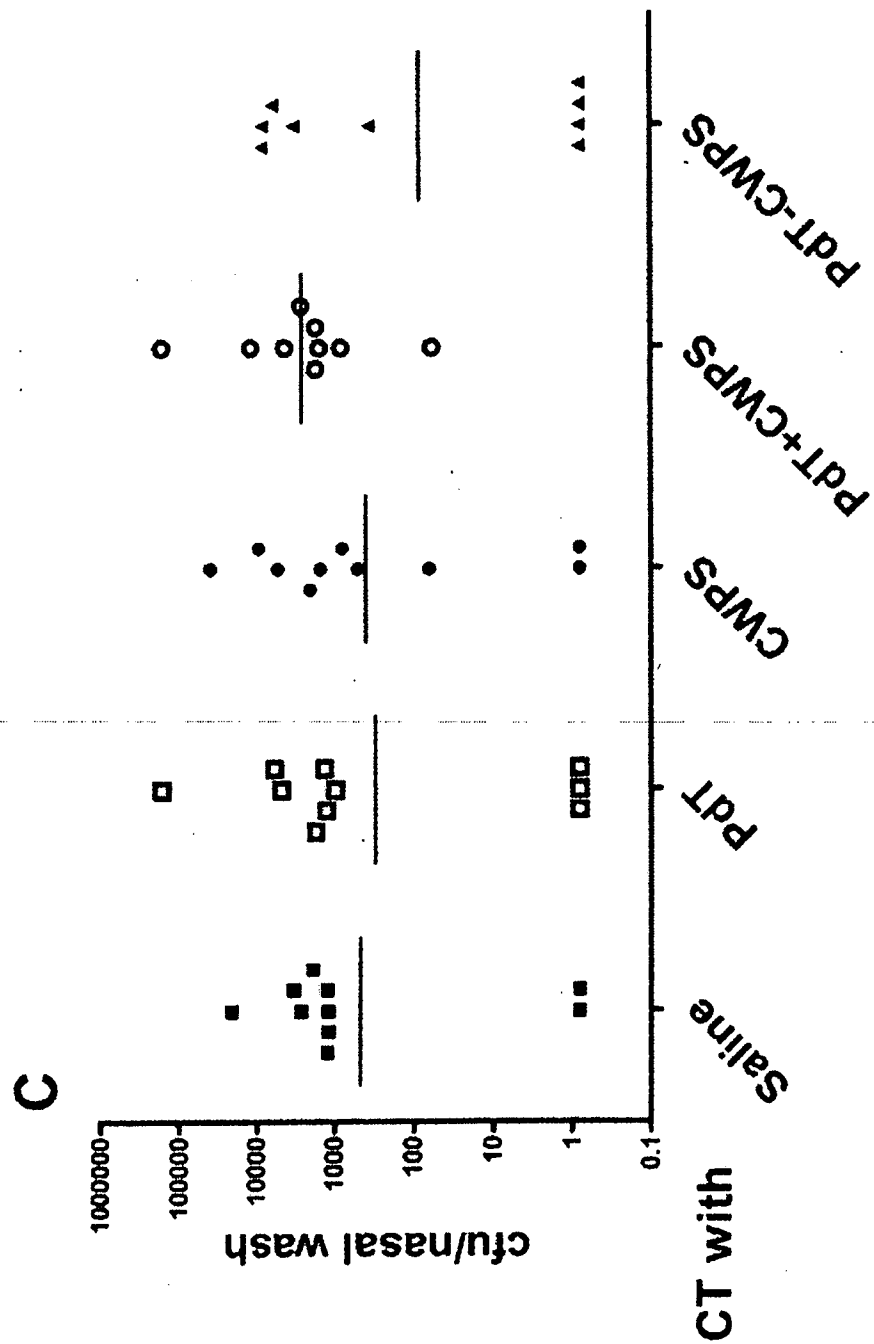
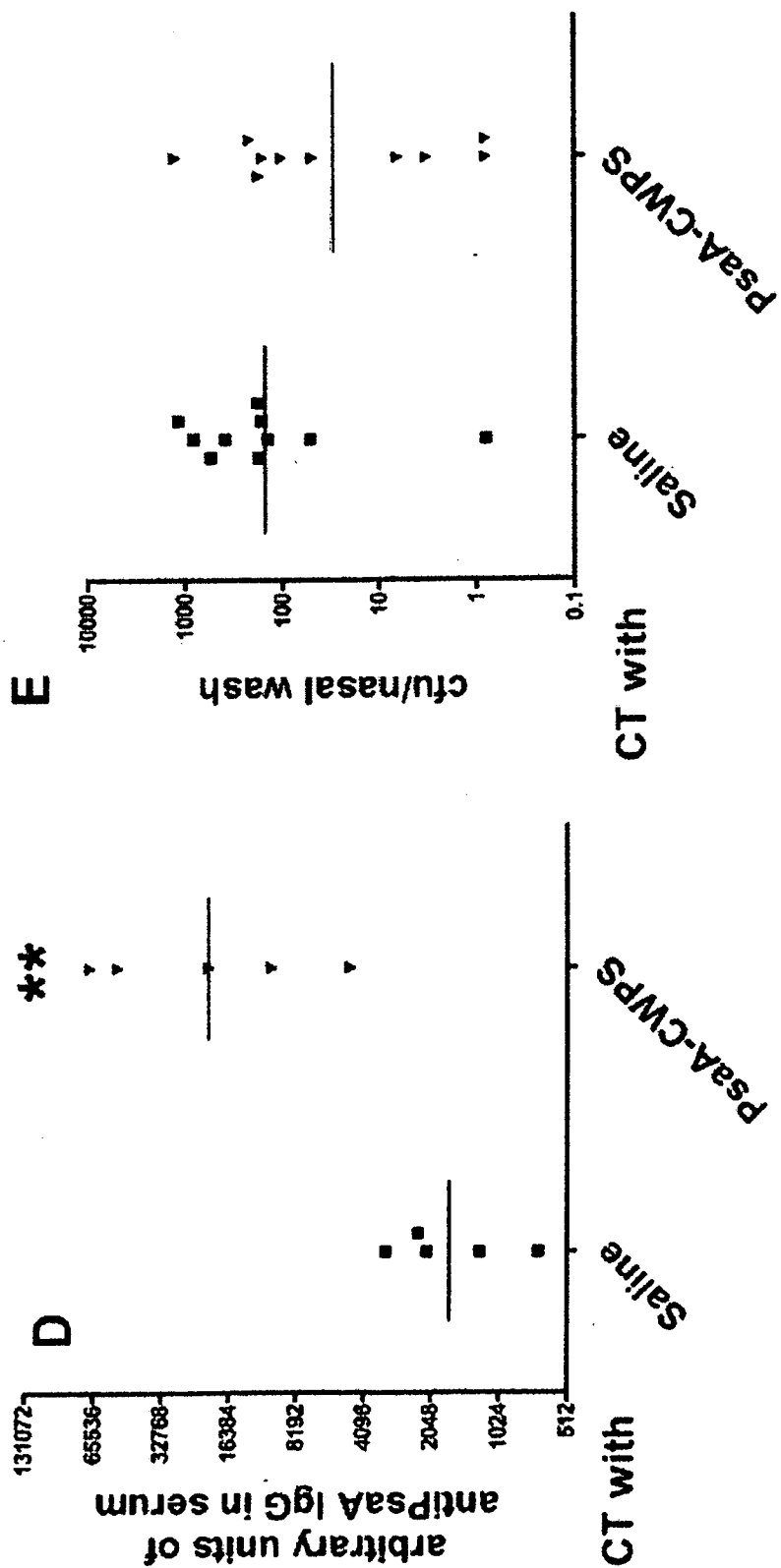


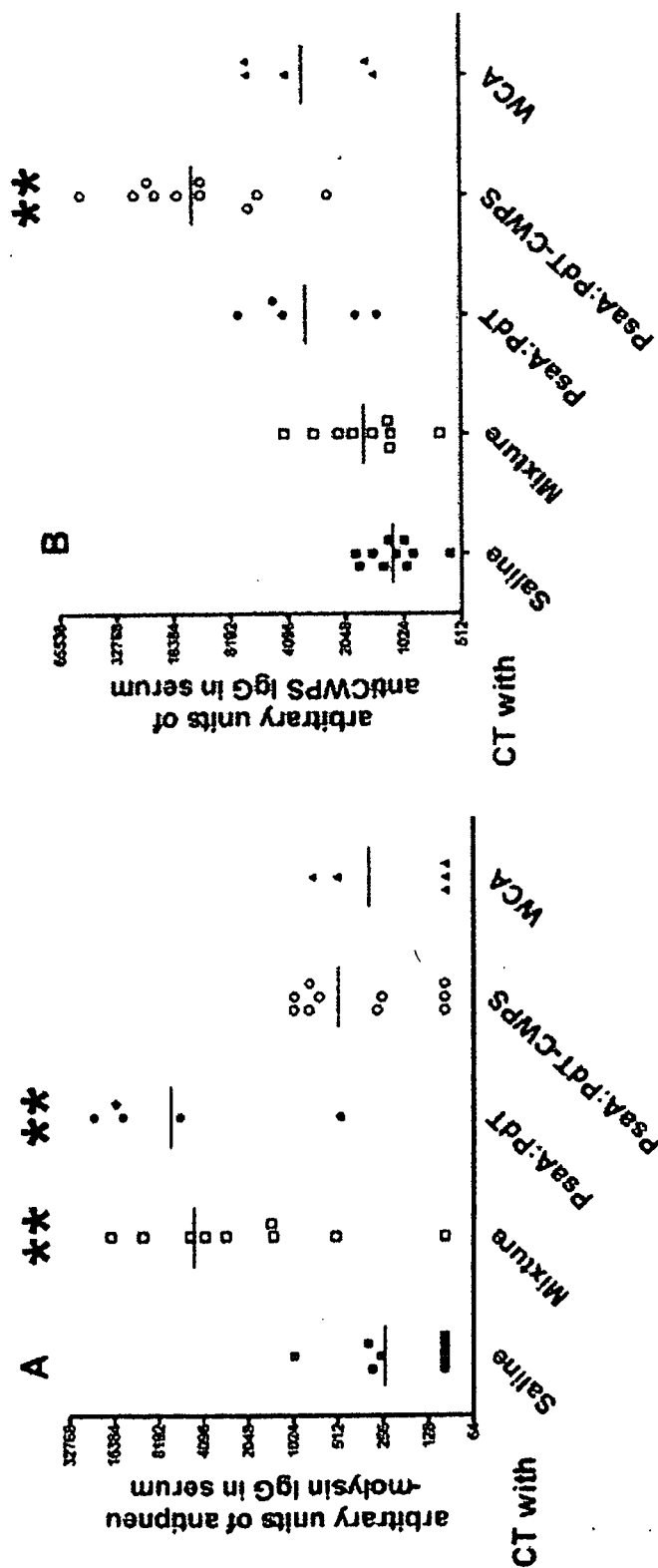
Figure 3C

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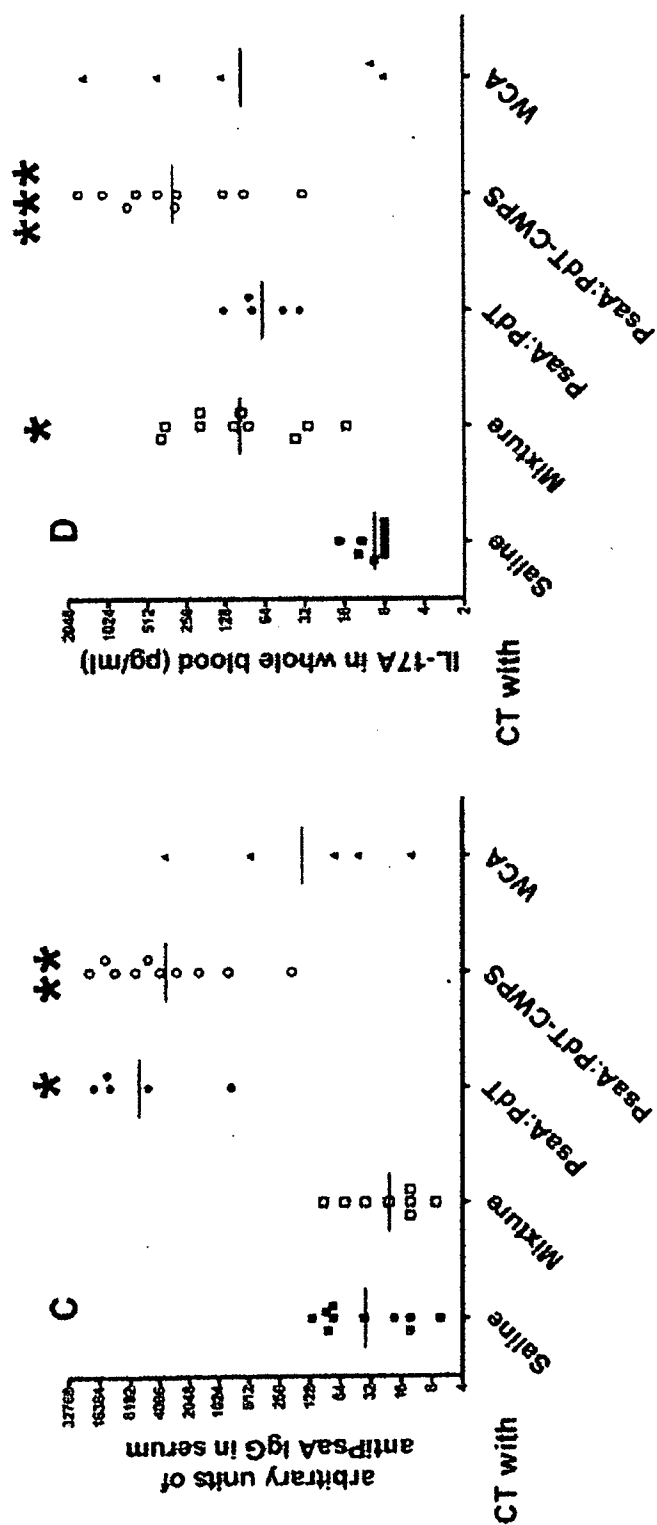
Figures 3D and 3E

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Figures 4A and 4B

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Figures 4C and 4D

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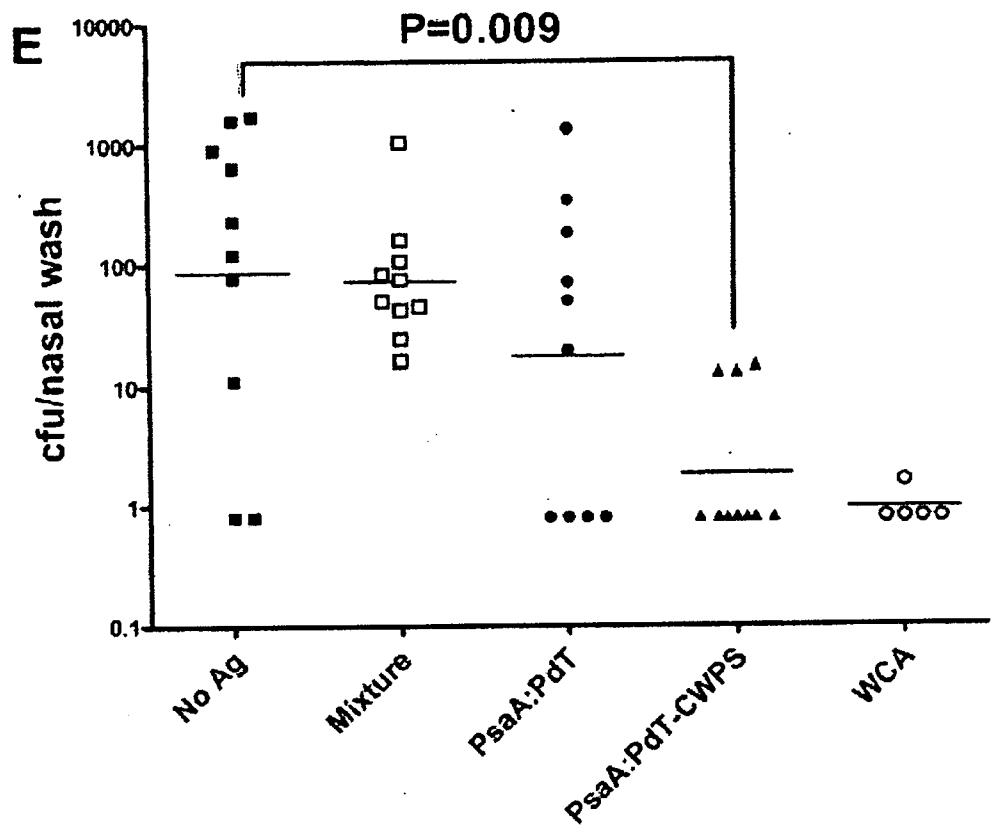
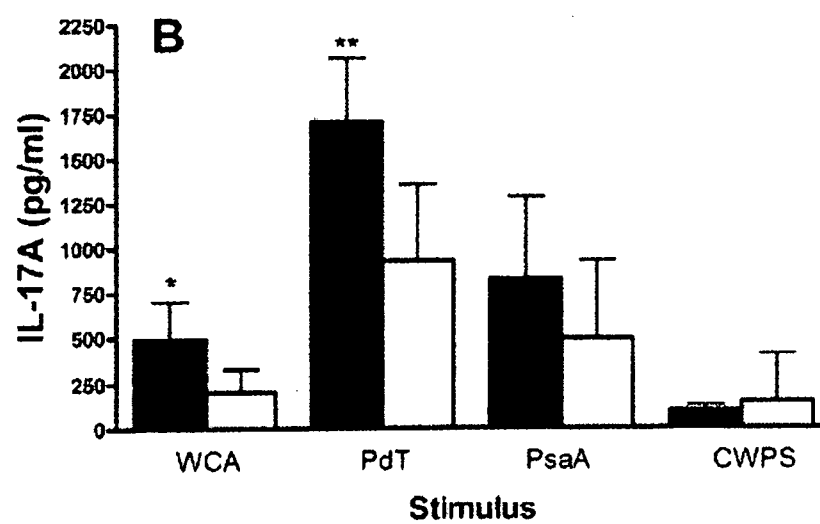
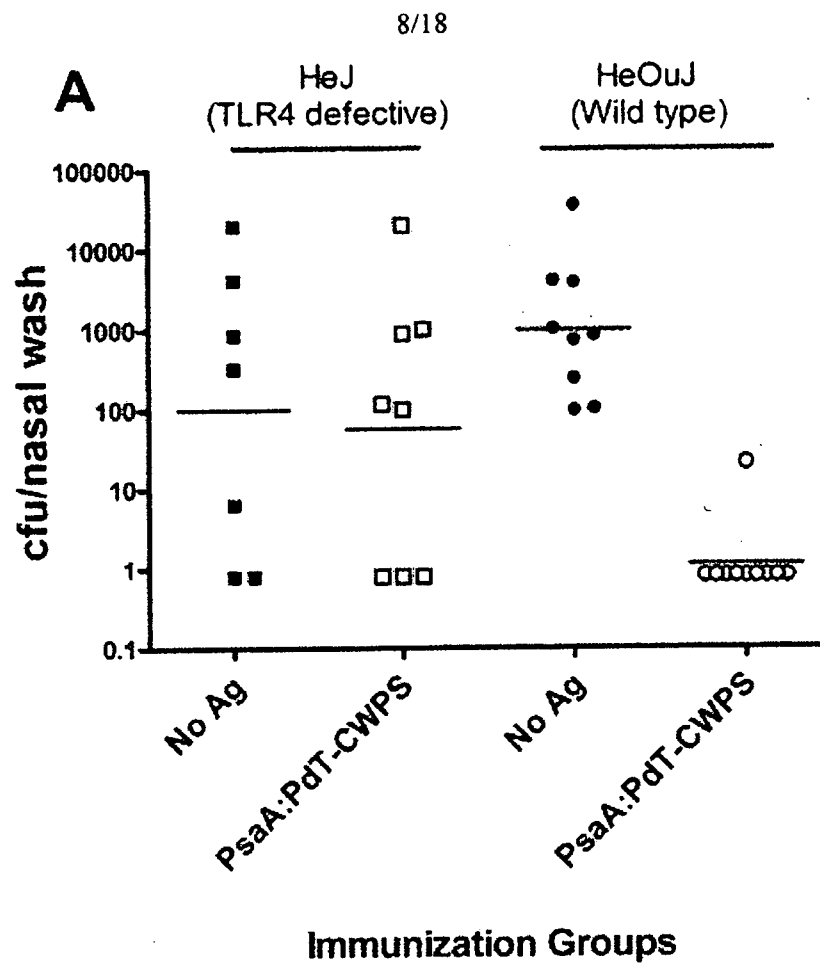
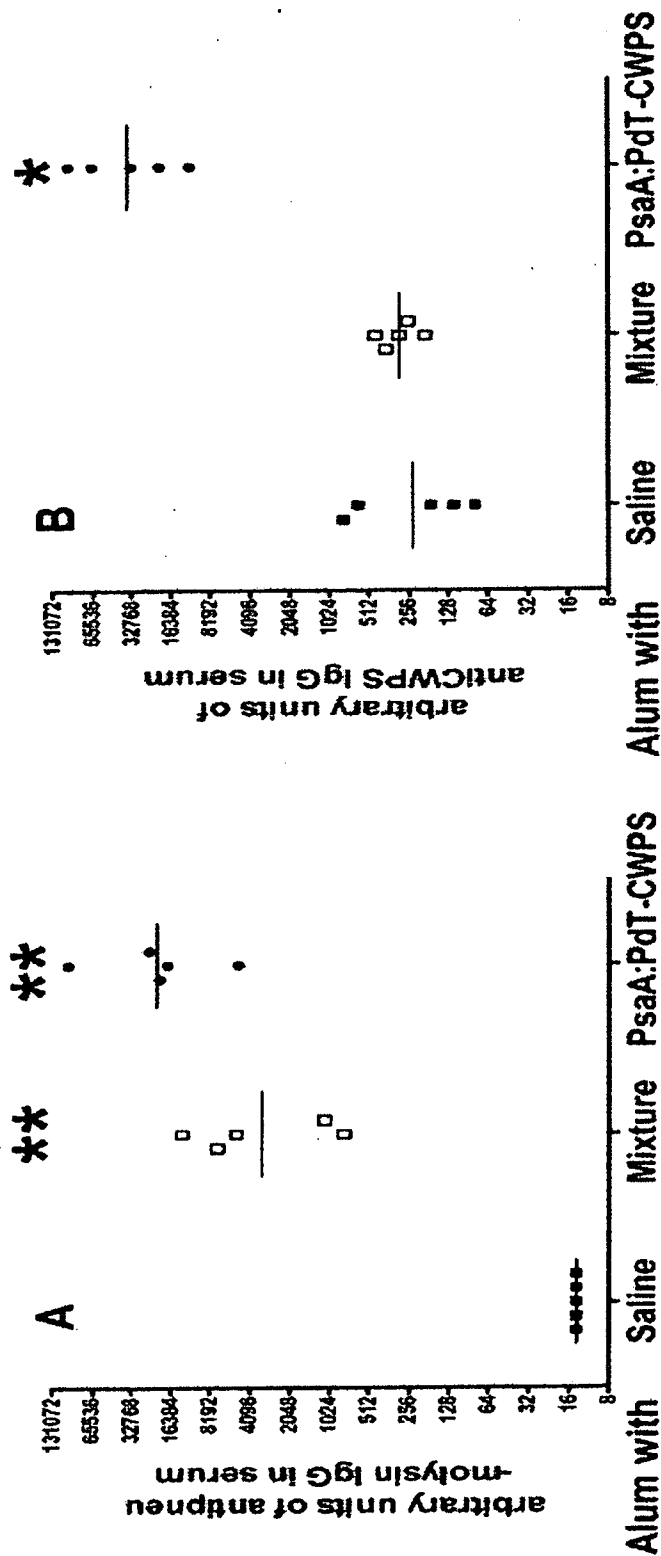


Figure 4E



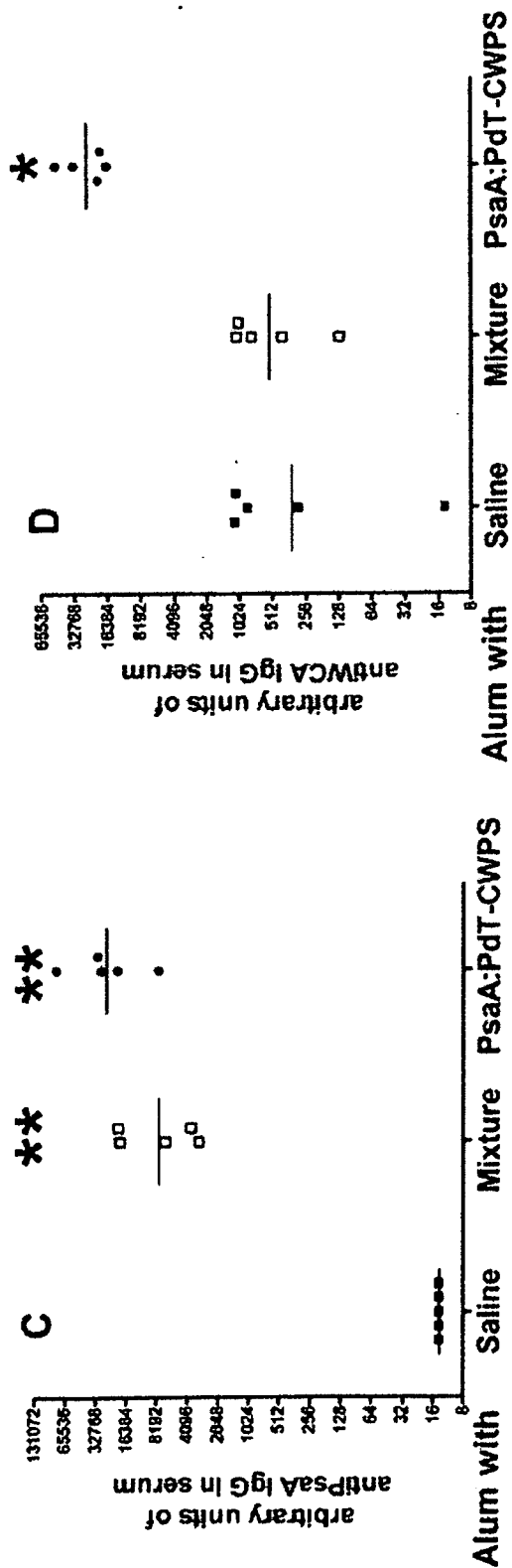
Figures 5A and 5B

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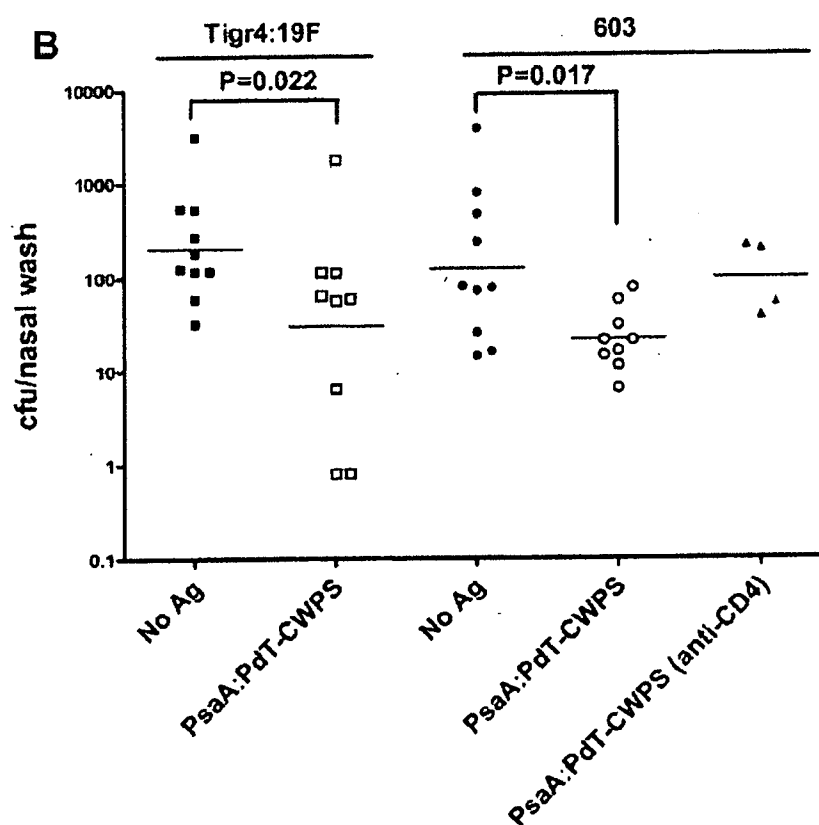
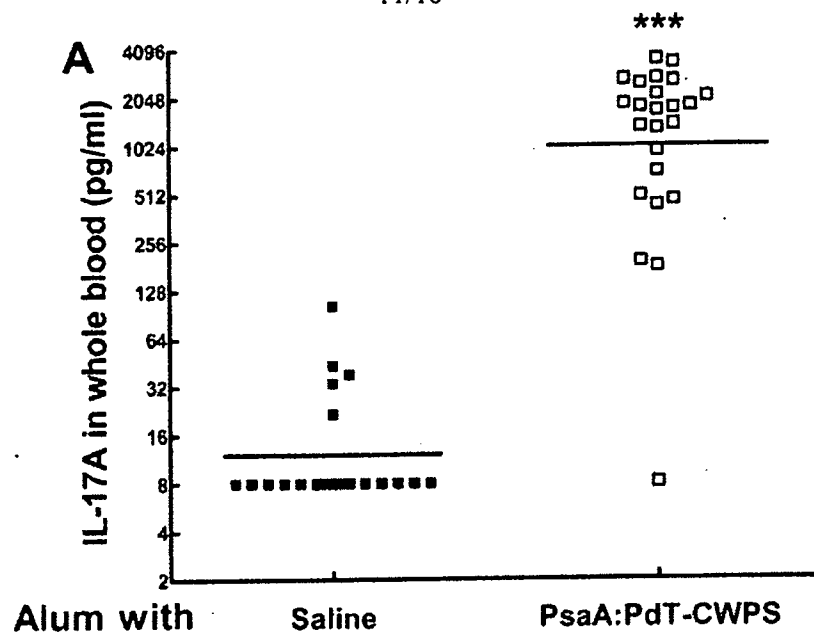
Figures 6A and 6B

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Figures 6C and 6D

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Figures 7A and 7B

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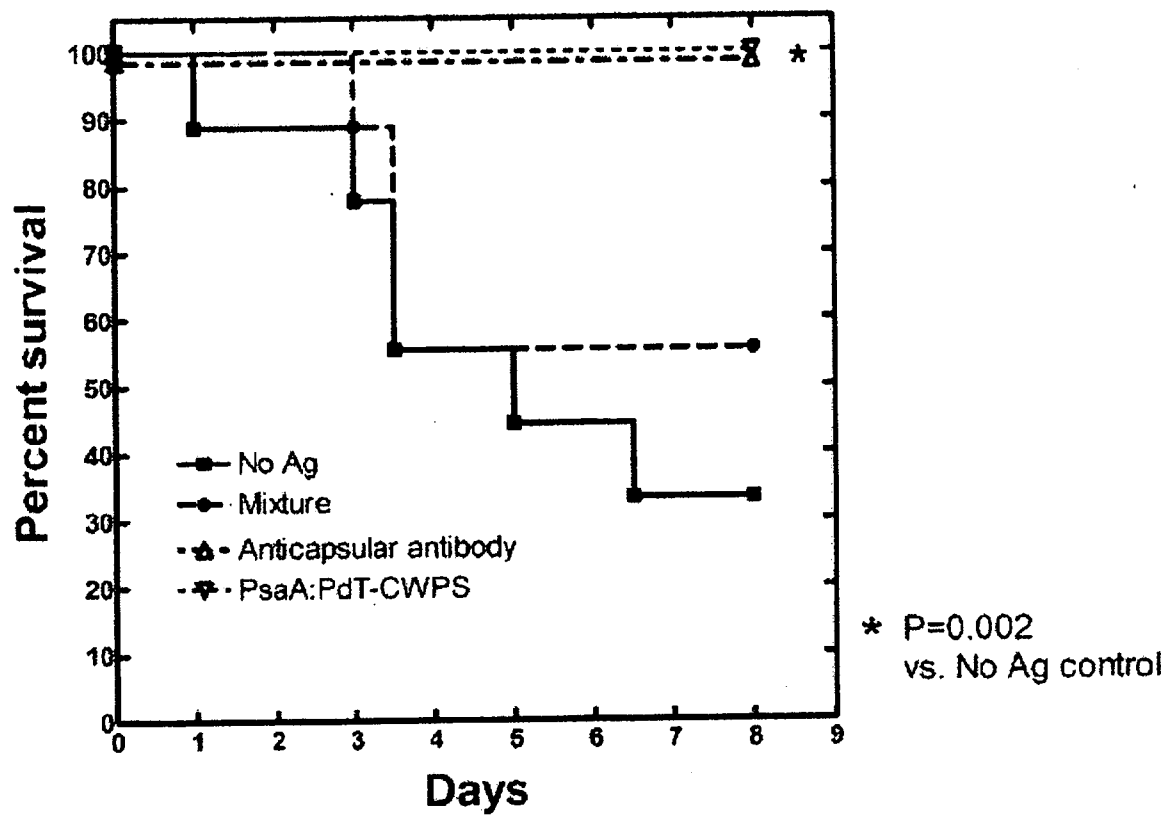


Figure 8

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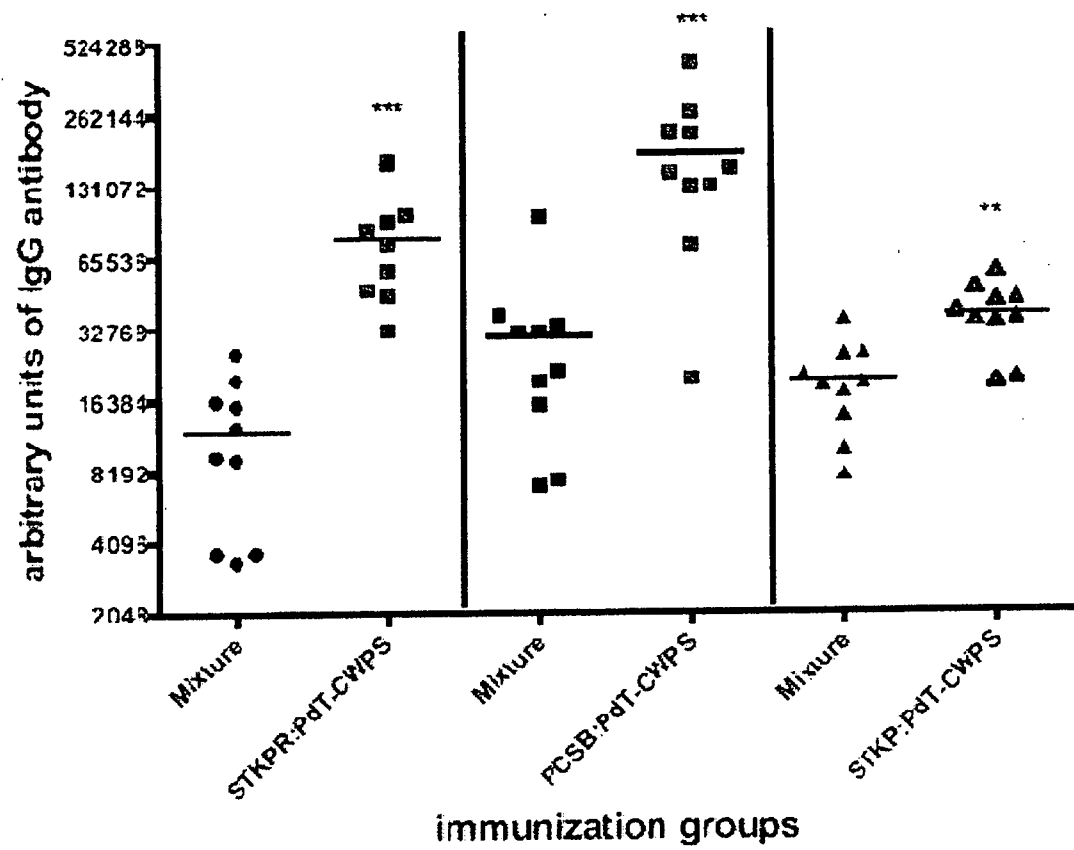


Figure 9

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Antibody titer after 2 doses

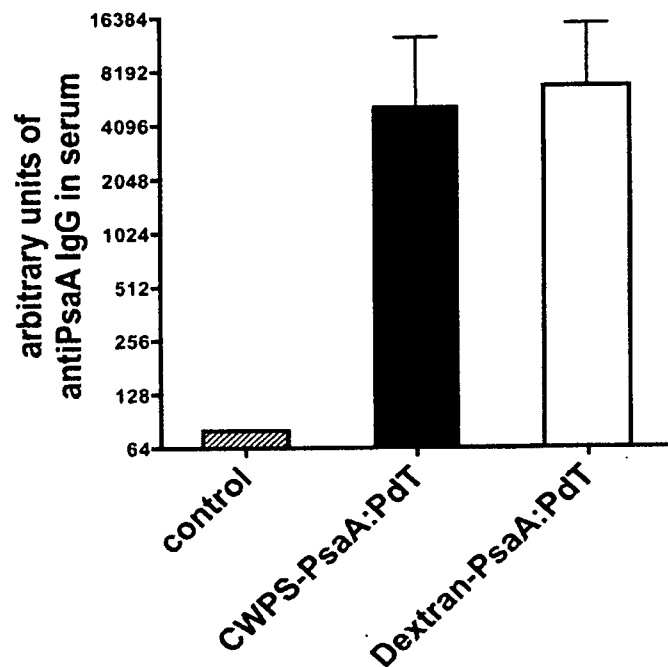


Figure 10A

Antibody titer after 3 doses

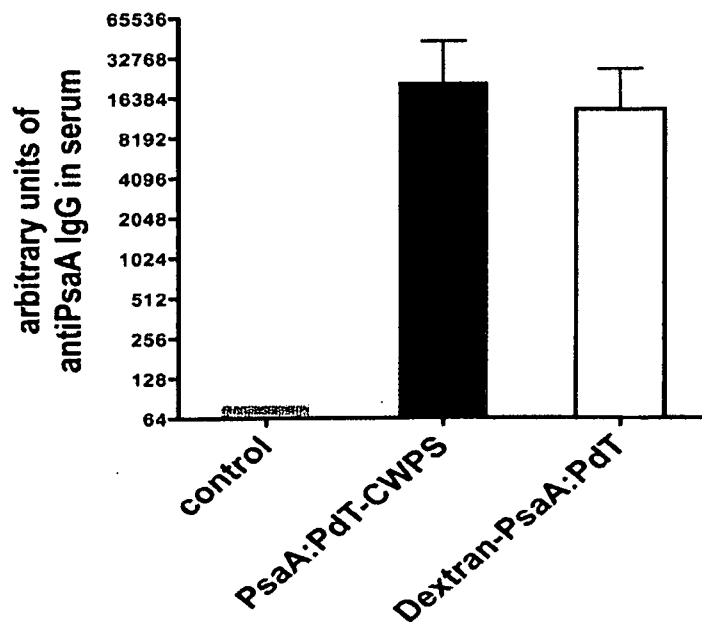


Figure 10B

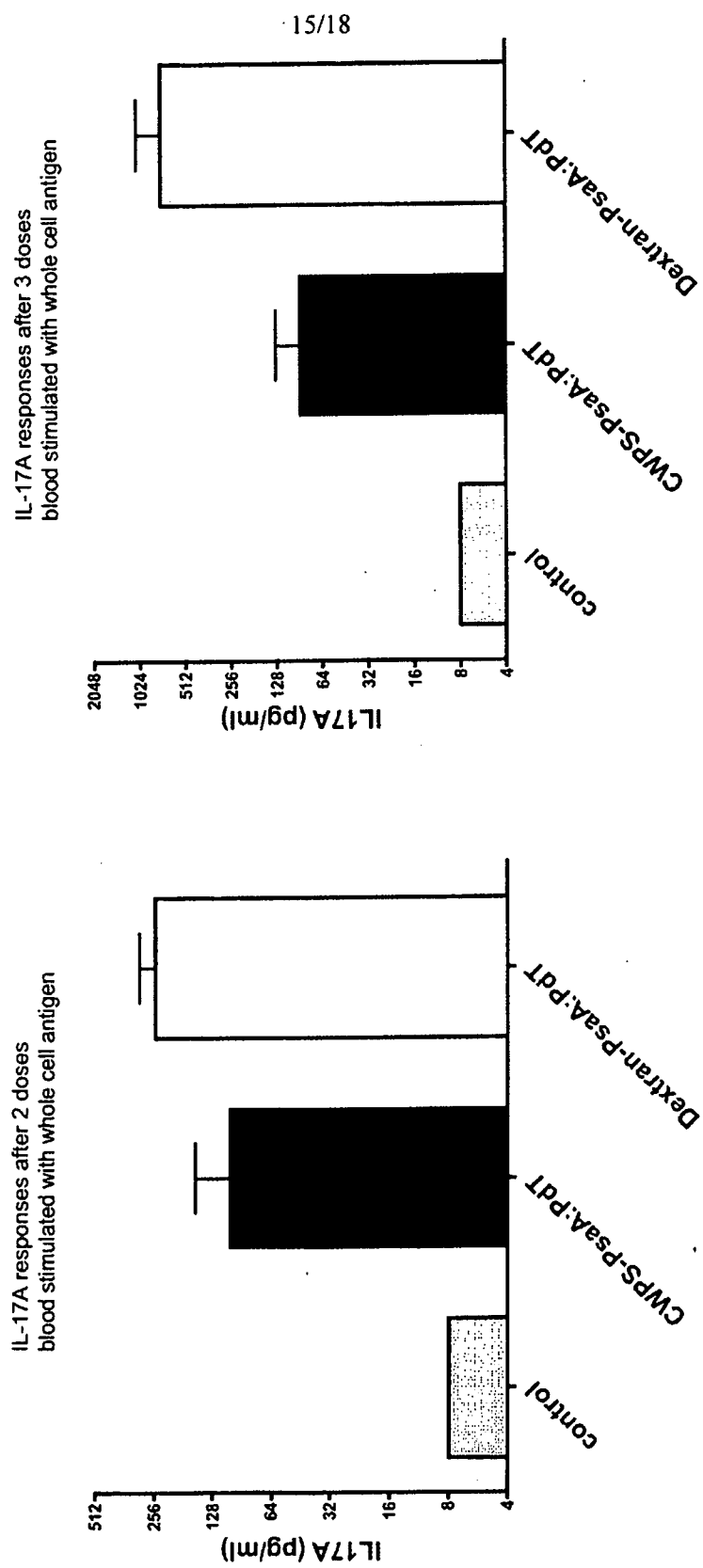


Figure 11A

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IFN-gamma responses after 3 doses
Blood stimulated with whole cell antigen

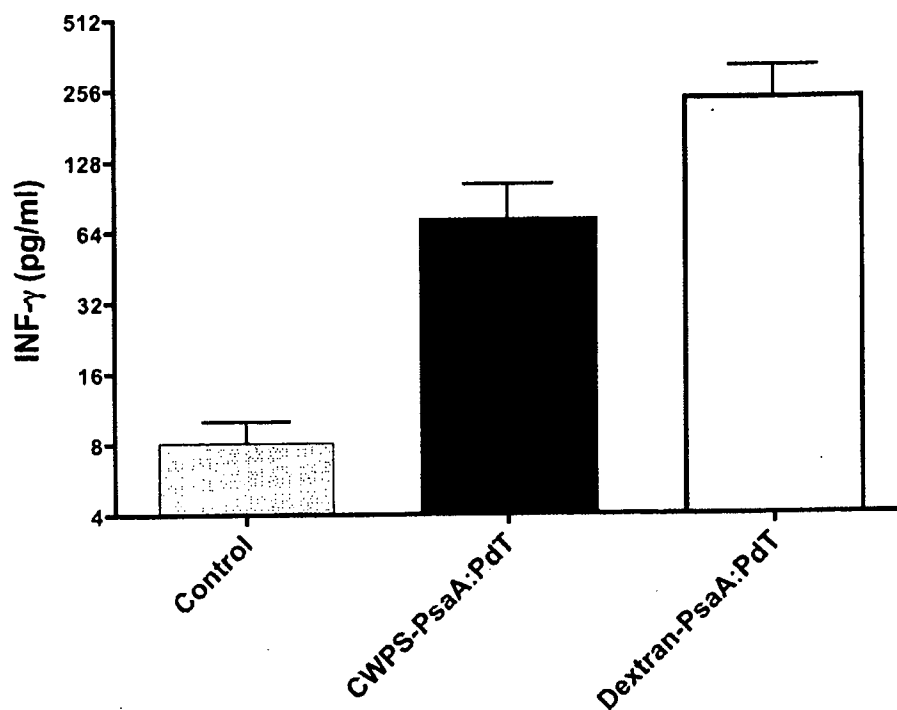


Figure 11B

IL-17A responses after 3 doses
Blood stimulated with PsaA

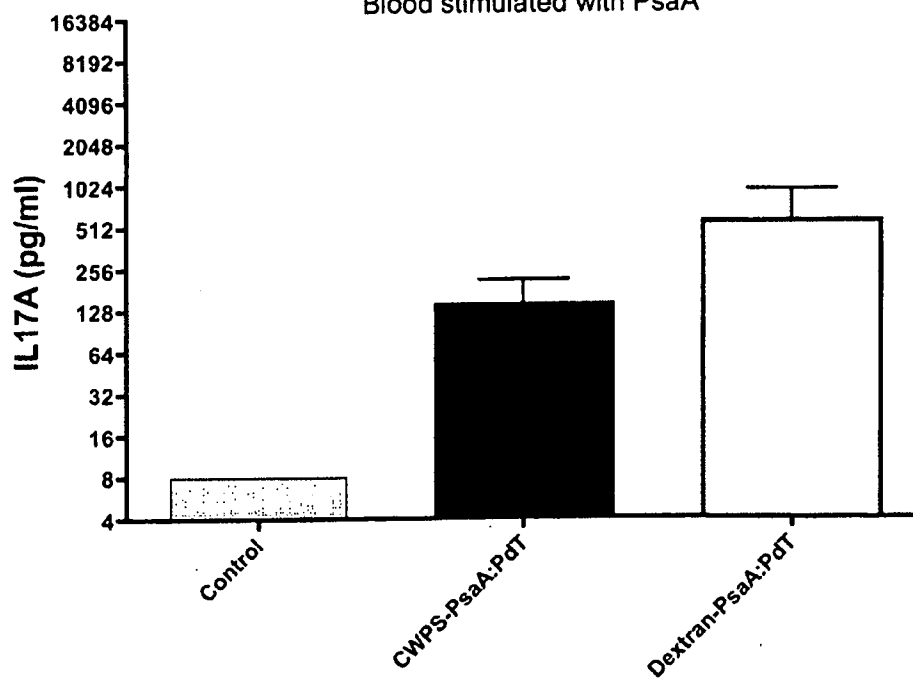


Figure 11C

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IFN-gamma responses after 3 doses
Blood stimulated with PsaA

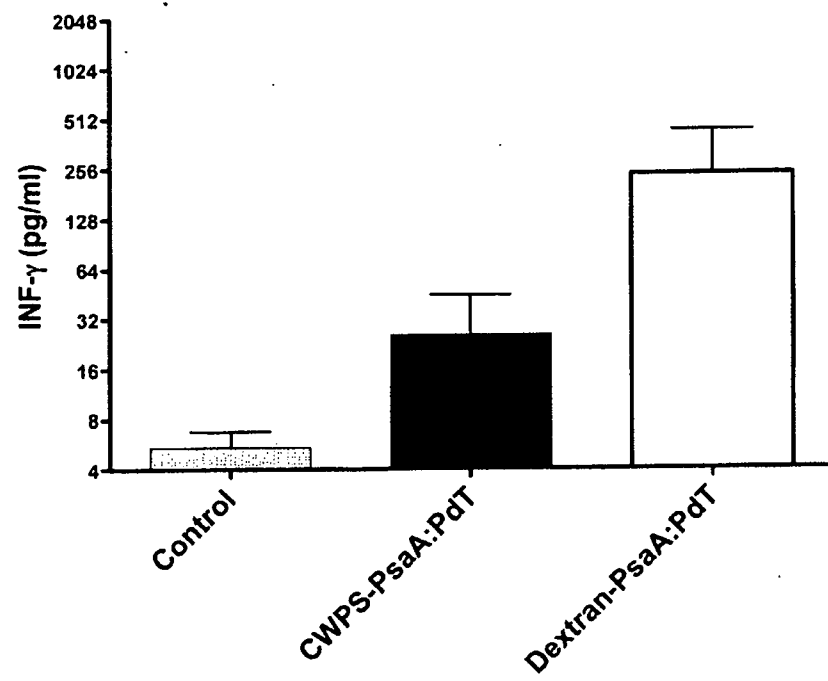


Figure 11D

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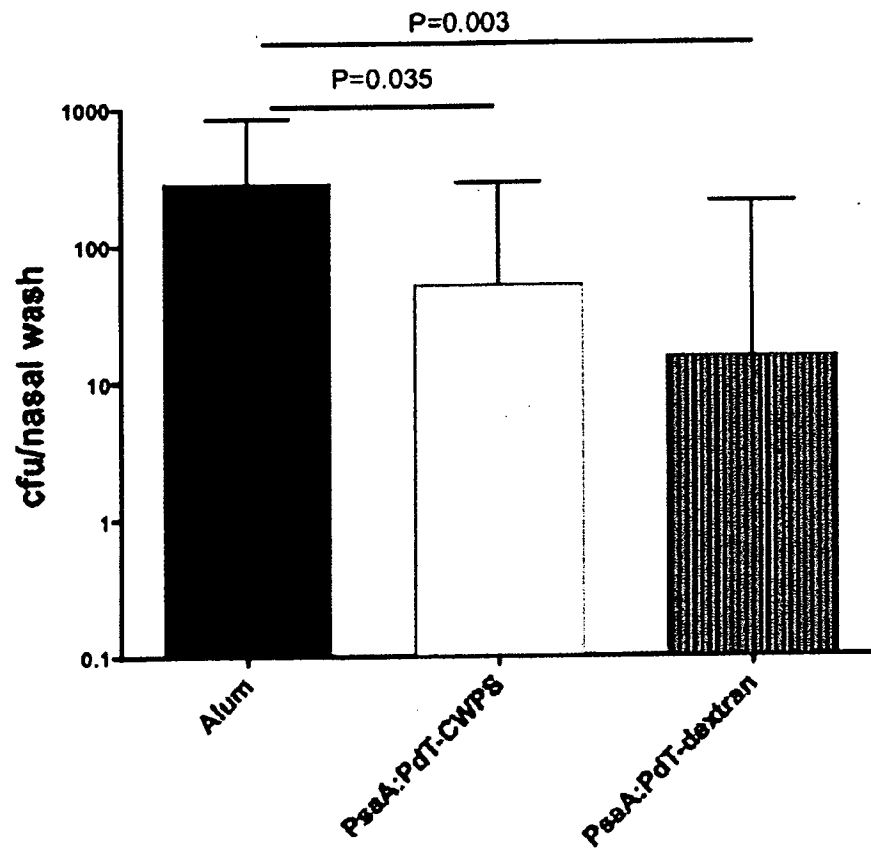


Figure 12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/44956

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/385 (2009.01)

USPC - 424/193.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC: 424/193.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC: 424/193.1, 244 (text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Electronic Data Bases: (EPAB, JPAB, PGPB, USPT); Google Scholar

Search Terms: Streptococcus pneumonia, colonization, cell wall polysaccharide (CWPS), conjugate vaccine, fusion protein, synergism, pneumolysin (non-hemolytic variant), cancer antigen

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2005/0070695 A1 (MINETTI et al) 31 March 2005 (31.03.2005). Especially [0040], [0115], [0116],	1-7, 9, 10
Y	BASSET et al. Antibody-independent, CD4+ T-cell-dependent protection against pneumococcal colonization elicited by intranasal immunization with purified pneumococcal proteins. IN: Infect. Immun.; November 2007; Vol 75, No 11; Pages 5460-5464. Especially pg 5460 left col para 2 and right col para 4, pg 5461 fig 1A, pg 5462 fig 2	1-7, 9, 10
Y	OGUNNIYI et al. Protection against Streptococcus pneumoniae elicited by immunization with pneumolysin and CbpA. IN: Infect. Immun.; October 2001; Vol 69, No 10; Pages 5997-6003. Especially abstract	1-7, 9, 10
Y	EP1834650A1 (SHIKU et al). 19 September 2007 (19.09.2007). Especially para [0010]	5
Y	HO et al. The immunostimulating activities of anti-tumor polysaccharide from K1 capsular (polysaccharide) antigen isolated from Klebsiella pneumoniae. IN: Immunopharmacology; January 2000; Vol 46, No 1; Pages 1-13. Abstract only.	6

☐ Further documents are listed in the continuation of Box C.


* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

22 September 2009 (22.09.2009)

Date of mailing of the international search report

13 OCT 2009

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/44956

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 8
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.