Seton Hall University

eRepository @ Seton Hall

Seton Hall University Dissertations and Theses (ETDs)

Seton Hall University Dissertations and Theses

Summer 8-15-2016

Utilization of Cholera Toxin B as an Adjuvant for the Subunit PspA Vaccine, Provides Effective Protection against Streptococcus pneumoniae Challenge

Daniel R. Pinho
daniel.pinho@student.shu.edu

Utilization of Cholera Toxin B as an Adjuvant for the Subunit PspA Vaccine, Provides Effective Protection against *Streptococcus pneumoniae* Challenge

By

Daniel R. Pinho

Submitted in partial fulfillment of the requirements for the degree of Master of

Sciences in Biology from the Department of Biological Sciences of Seton Hall

University August, 2016

Seton Hall University

Department of Biological Sciences

Approved By:

_____
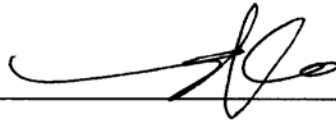
MENTOR

Dr. Constantine Bitsaktsis

_____

COMMITTEE MEMBER

Dr. Brian Nichols

_____

COMMITTEE MEMBER

Dr. Jane Ko

_____

DIRECTOR OF GRADUATE STUDIES

Dr. Angela Klaus

_____

CHAIRPERSON, DEPARTMENT OF BIOLOGICAL SCIENCES

Dr. Heping Zhou

Abstract

Streptococcus pneumoniae is a prevalent human pathogen associated with pneumonia. It is estimated that approximately 1 million people around the world die each year, specifically with young children and the elderly comprising a significant portion. Currently, antibiotics can treat the infection but individuals such as the young or elderly are more susceptible to bodily damage from symptoms, such as pneumonia, meningitis, and sepsis, thus require more preventive measures. As a result, vaccinations are a key solution to providing effective protection against infectious pathogens. Presently, two vaccinations exist in the market: $PPSV_{23}$ and PCV_{13}, which only protect against select 23 or 13 serotypes respectively of *Streptococcus pneumoniae* out of the possible 90. Researchers are searching for alternative antigenic markers conserved throughout the multitude of serotypes to formulate cross-protective vaccinations. One such proponent is known as Pneumococcal Surface Protein A (PspA) which is a surface protein conserved throughout most serotypes and a potential candidate as a subunit vaccine against *Streptococcus pneumoniae*. To increase the immunogenicity of PspA for long term and effective protection, this study utilizes Cholera Toxin B (CTB) as an adjuvant to effectively increase vaccine efficacy. We assessed the efficacy of the subunit vaccine, PspA, administered intranasally (i.n.) in conjunction with CTB as an adjuvant. Our results indicated increased survival in mice immunized with both PspA and CTB accompanied with reduced lung bacterial burden, increased levels of S. pneumoniae-specific IgG subclass antibodies, increased cytokine production, and a reduced acute inflammatory response.

Table of Contents

List of Tables

Table 1

List of Figures

Acknowledgements

1. Introduction

1.1 Streptococcus pneumoniae: An Overview

Pneumonia is a respiratory infection triggered via a multitude of viruses, bacteria, and fungi. Pathogens proliferate within the alveoli sacs of the lungs initiating a strong immune response by the body. Elevated levels of inflammation within alveoli of the lungs may induce large amounts of pus and fluid to form resulting in the reduction of the lung's capacity to successfully absorb oxygen within the body (CDC http://www.cdc.gov, 2015). According to the Centers of Disease Control, Pneumonia is the leading cause of bacterial meningitis for children under the age of five in the United States as many of these children do not possess a fully developed immune system nor bodily endurance to cope with the disease (Bitsaktsis et al., 2011). Elderly individuals and those suffering from immune deficiencies are also at a higher risk of contracting pneumonia (CDC http://www.cdc.gov, 2015). Although there are several viral factors such as Human Parainfluenza virus and Respiratory Syncytical Virus that may result in pneumonia, it is the bacterial pathogen *Streptococcus pneumoniae* that it is the focus of this study.

Streptococcus pneumoniae is a gram positive, facultative anaerobic extracellular bacteria that is a very prevalent human pathogen with high morbidity rates among youths, elderly, and generally immunocompromised individuals (Bitsaktsis et al., 2011). The bacteria is transmitted directly from carrier to carrier through respiratory droplets resulting in several infectious diseases ranging from pneumonia, otitis media, meningitis, or sepsis (CDC http://www.cdc.gov, 2015; Seo, et al., 2001). Upon contracting the disease, symptoms such as coughing, fever, and chest pains quickly begin to onset after one to three days (CDC http://www.cdc.gov, 2015). Although average human adults possess adequate immune defenses to resist infection, medications such as antibiotics are available to treat the infection and prevent further damage to the host. However,

Streptococcus pneumoniae can be far more life threatening to individuals with preexisting infections or immune compromised systems due to age or genetic disorders (CDC http://www.cdc.gov, 2015). As a result, vaccine strategies are ideal routes of preventing pneumococcal infections and providing long term protection against potential future infections.

1.2 Vaccinations and relevance to Streptococcus pneumonia

Vaccinations originated from the work of Edward Jenner with his work on the small pox vaccine and further expanded by other notable researchers such as Louis Pasteur and his work with attenuated rabies viruses. Since then, vaccines have become the highlight of medical research and still remain as a primary means of long term protection against infectious diseases (Owen et al., 2009). Vaccines specifically activate the adaptive immune system to achieve protection by introducing an antigen into the host system. Vaccine strategies may consist of a variable selection of antigenic determinants ranging from an attenuated form of the whole pathogen, an inactivated form of the pathogen, a subunit form vaccine of a specific macromolecule originating from the pathogen, recombinant vectors originating from the pathogen, or DNA vaccines consisting of the plasmid DNA that encodes the antigenic proteins (Owen et al., 2009). Selection of the vaccine strategy is relative to the properties of the pathogen itself ranging from route of infection, virulence, immunogenicity of antigenic determinants, and potential risks to the host. The study primarily focuses on subunit vaccinations since most vaccine strategies against *Streptococcus pneumoniae* comprise of subunit vaccinations.

Streptococcus pneumoniae consist of over 90 serotypes that vary from one strain to next via each serotype's unique capsular makeup (Seyoum et al., 2011). Serotype variation prevents the infected host from acquiring specific immunity against the entire species so that a new serotype can re-infect the same host. As a result, research has been focused on identifying key

surface proteins and polysaccharides that are conserved throughout the majority of serotypes within *Streptococcus pneumonia* to produce effective protection against the varying serotypes (Ferriera et al., 2009). The current market utilizes a polysaccharide based conjugate vaccine with two most popular being 23-valent pneumococcal polysaccharide vaccine ($PPSV_{23}$) which is commonly administered to adults/elderly and the more tolerable Pneumococcal conjugate vaccine (PCV_{13}) that is more frequently administered to youths. $PPSV_{23}$ contains twenty three common pneumococcal polysaccharide antigens and PCV_{13} contains thirteen common pneumococcal polysaccharide antigens which elicit an effective immune response resulting in long-term protection. $PPSV_{23}$ possessing the broadest range of serotype protection is estimated to possess an efficacy of around 60 to 80% in adults sixty five years and older (Kawakami, 2016; CDC http://www.cdc.gov, 2015). However, the cost of isolating all twenty three polysaccharides and producing these vaccines factors into limited distribution within the population. Also, $PPSV_{23}$ is only commonly administered to adults while the more tolerable PCV_{13} is more commonly administered to children which protects against significantly less serotypes of *Streptococcus pneumoniae* (Seyoum et al., 2011). Secondly, the route of administration is intramuscular which is not the common infection route of *Streptococcus pneumoniae* and thus may produce a lower immune protection compared to that of an intranasal administration which has been shown to produce a more effective vaccine (Bitsaktsis; Badabadjanova et al., 2015). Also the cost and ease of intranasal vaccinations versus that of intramuscular presents a better alternative for vaccinating populations in mass (Ferreira, 2009).

1.3 Route of Vaccination and Antigen Immunogenicity against Streptococcus pneumonia

   Streptococcus pneumoniae route of infection most commonly involves the intranasal pathways of the host and ultimately affects the respiratory pathways of the organism and

sometimes resulting in death from three major symptoms. Pneumonia occurs from excess fluid accumulation within the lungs preventing oxygen from entering the bloodstream, sepsis as a result of excessive *Streptococcus pneumoniae* entering the blood stream, or meningitis resulting from infection within the surrounding brain or spinal fluid. Initially, the pathogen is typically spread through mucus droplets created by an infected host and transferred to an uninfected one through touch or airborne. The path of infection plays a vital role in determining the route of immunization and ultimately increasing overall immunogenicity of the vaccine (Dockrell et al., 2012; Seo, J., 2002). In the case of *Streptococcus pneumoniae,* an intranasal route of vaccination has strong immunogenicity thereby eliciting effective and long term protection against said pathogen (Bitsaktsis et al., 2009).

Pneumococcal Surface protein A (PspA) is a protein that exists on the outer surface of *Streptococcus pneumoniae* on the cell wall. PspA is located on the cell wall and conserved throughout most strains of pneumococci (Bitsaktsis et al., 2011; Hollingshead et al., 2006) and thus a primary candidate for providing broad range protection from all serotypes of *Streptococcus pneumoniae* (Gor et al., 2004). Its role within the pathogen is twofold, it plays a vital role in virulence during infection and enables *Streptococcus pneumoniae* to evade phagocytosis by preventing complement deposition onto its surface (Ferreira et al., 2009; Kono et al., 2011). The typical immune response to an extracellular pathogen is a humoral response in conjunction with activation of the complement system upon contact of the pathogen allowing deposition of the complement factor C3b onto the cellular surface of the pathogen. Complement factor C3b is important because it opsonizes a pathogen which ultimately leads to phagocytosis of the bacteria by antigen presenting cells (APC) such as macrophages, which then sequentially release pro-inflammatory cytokines recruiting additional leukocytes to the site of infection

(Owen et al., 2009; Ren et al., 2012). APCs also serve a role presenting the pathogen to T cells, enabling the initiation of an adaptive immune response. Activation of the adaptive portion of the immune system will target the pathogen specifically. In the case of an extracellular bacterium such as *Streptococcus pneumoniae,* the activation of B cells then switch into antibody producing plasma cells or memory cells providing long term protection against that specific pathogen (Kono et al., 2011; Gor et al.,2005).

1.4 Cholera Toxin B adjuvant

Cholera Toxin (CT) is the primary virulence factor for the bacterium *Vibrio cholera* and a key component in the infectious disease of Cholera. CT can be divided into two subunits; Cholera Toxin A (CTA) and Cholera Toxin B (CTB). The first subunit, CTA, provides the toxic and disease function of the two subunits. The second subunit, CTB, provides the anchoring mechanism for CT by attaching the subunits to a cell (Baldauf, 2015). Cholera Toxin B (CTB) has been shown to be an effective mucosal adjuvant that is shown to induce increased antibody production and thus provide enhanced vaccination protection when combined with a mucosal pathogen (Bitsaktsis et al., 2009). CTB lacks inherent dangers associated with Cholera pathogen as it lacks the toxic A subunit which causes cellular damage to the host. As a result, CTB is now regarded as a potentially effective vaccine adjuvant for eliciting effective protection against target pathogens. In the case of *Streptococcus pneumoniae,* there have been previous studies that show the potential increase in immunological protection when utilizing CTB as the adjuvant (Kono et al., 2011).

1.5 Immune System: Humoral Immunity

Humoral immunity is integral for eliciting effective protection against extracellular pathogens. The innate immune system by itself acts as a deterrent to most pathogens from damaging the host body. It utilizes a range of lymphocytes in conjunction with Toll Like Receptors inducing a generalized inflammatory response to most threats (Owen et al., 2009; Seyoum et al., 2011). However, this system possesses limitations and may be unable to successfully neutralize or prevent certain pathogens from successfully entering into a host and potentially inducing system wide damage. As a result, the secondary adaptive immune system possesses the capability of specific immunological targeting against invasive pathogens that escaped the innate immune system's broader protection. Specific antigen presenting cells (APCs) possess the vital role of showing antigens to T cells and providing the necessary secondary signal required to fully activate any naïve B or T cell and thus eliciting specific immunity against the target antigen.

1.6 Significance of Immunological Proteins: Cytokines

There are several key immunological cytokines involved in effective immunological protection. Previous studies have shown correlation between altered levels of immunological cytokines during infection demonstrate immunological functions that are occurring during an immunological response towards infection. Interleukin (IL)-2 is produced via activated T cells which further stimulate proliferation of other T-cells and B-cells within the host. IL-2 also enables activation of Natural Killer (NK) cells in the site of infection. IL-4 is also secreted via mast cells, basophils, and activated T cells, particularly via type 2 T helper cells (TH-2) which are a subset of T cells. IL-4 also leads to the stimulation and proliferation of B cells indicating immunological shift towards humoral immunity. IL-6 is produced and secreted via T and B cells,

macrophages, and numerous other support cells. IL-6's main function is to regulate the B and T cell functions. IL-6 also induces inflammation at the site of infection inducing initial innate immune response and ultimately the shift towards adaptive immune responses (Owen et. al, 2009). Interferon gamma (INF-γ) is primarily produced primarily by TH-1 cells, some CD-8 T cells, and NK cells. INF-γ induces a class in antibody production towards the IgG subclasses. Secretion of INF-γ also activates macrophages which further propagates a shift towards Major histocompatibility complex (MHC) class 2 expression thusly promoting antibody mediated humoral immunity. Tumor necrosis factor (TNF) - α plays an integral role in cellular apoptosis and inflammatory functions by being primarily produced via activated macrophages. An over expression of TNF-α can result in prolonged inflammation ending in damaged host tissue. Specifically, TNF-α ligand binds to receptor TNF-R1 that possesses an intracytoplasmic death domain (DD). The binding between TNF-α and TNF-R1 retains the option of either inducing cellular apoptosis or survival. (Micheau et al.., 2003)

1.7 *Significance of Immunological Proteins: Antibodies*

Immunoglobulins play a vital role in conjunction with the adaptive branch of the immune system to elicit humoral immunity against specific foreign pathogens (Ren et al., 2012). Immunoglobulins or antibodies are divided into two main portions (Janeway et al., 2001). The variable region of the antibody is randomly generated upon B cell maturity within the bone marrow and serves as the attachment point between the immunoglobulin and the specific antigen that a pathogen possesses. This allows antibodies to possess a wide range of many varying antigenic sites that are unique to each B cell no matter the pathogen that enters the host body. The second region, or Fc region or receptor, consists of a constant domain that possesses an

effector function for the host leukocytes. The function of the Fc receptor stimulates leukocytes into phagocytosis of the antibody attached pathogen or can stimulate antibody-dependent cell-mediated cytotoxicity (ADCC) (Owen et al., 2009). Stimulation by antibodies on leukocytes also causes increased secretions of immunological cytokines that further promote immunological functions such as inflammation, leukocyte recruitment, and proliferation. The combination of cytokines in conjunction with leukocytes induces inflammation and initiates an immune response against a pathogen.

The most numerous and prevalent immunoglobulin in sera is the isotype IgG. IgG plays a vital role in humoral immunity for its opsonizing properties allowing phagocytosis of a pathogen or through ADCC with NK cells. Furthermore, it has the ability to activate the complement system which further propagates an immune response against a specific antigen (Vidarsson et al., 2014; Owen et al., 2009). IgG consists of four subclasses; IgG1, IgG2, IgG3, and IgG4 which vary based on their effector functions and slight structural differences, particularly in the hinge region. Presently, the focus of this research examines the presence of IgG1 and IgG2c and their immunological importance against S. pneumoniae. IgG1 is predominant in compliment fixation and associated with TH-2 activation while IgG2c facilitates more of an ADCC role and connected with a TH-1 interaction (Petrushina et al., 2003; Owen et al., 2009; Dahlgren et al., 2015). The presence of IgG1 and IgG2c combined with determined concentrations of type one and type two cytokines is utilized to subsequently determine the response of TH-2 and TH-1.
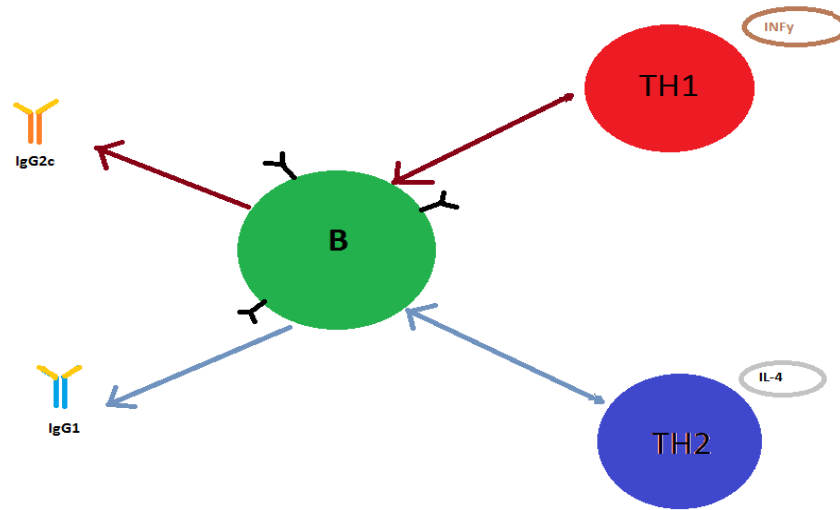
Figure 1 : TH-1 -TH-2 Relationship- Both TH-1 and TH-2 leukocytes possess the ability to elicit humoral immunity by activating naive B-Cells. Secretion of IL-4 stimulates TH-2 cells to activate B cells in producing IgG1 while secretion of INF-γ stimulates TH-1 in the creation of Immunoglobulin IgG2c.

The objective of this investigation was to determine if the subunit PspA vaccine combined with the adjuvant CTB created a potent vaccine that provided effective protection against A66.1 strain of *Streptococcus pneumonia* (serotype 3). This protection was correlated with reduced lung bacterial burden, increased S. pneumoniae-specific antibody production, increased type 1 cytokine secretion during infection, and reduced overall inflammation.

2. Materials and Methods

2.1 Bacterial Strains and Culture Techniques

Todd Hewitt broth was prepared by suspending 37g into 1000 mL of DiH_2O, mixing well until dissolved, and then filtering the media via a vacuum filtration system at 0.22 um. The media was then refrigerated at 4° C upon usage.

Streptococcus pneumoniae strain A66.1 was procured from New York Albany Medical. A66.1 was streaked onto a sheep blood agar plate and incubated at 37° C with 5% CO_2 for 18 hours. Two colonies were removed from the plate and mixed into 3 mL of Todd Hewitt Broth (THB) and incubated at 37° C with 5% CO_2 for 8 hours on a shaker. Removed 200 μL of culture and placed into filtered culturing flask consisting of 50 mL of THB and then incubated overnight for 18 hours at 37° C with 5% CO_2 on a shaker. The Optical Density (OD) of the culture was measured after each incubation phase by sampling 500 μL of culture with 500 μL of PBS and the OD taken via spectrophotometer. The blank utilized consisted of 500 μL THB and 500 μL PBS and referenced against any culture solutions measured. 7.5mL of glycerol was then added to 50 mL culture flask and vortexed creating a 15% glycerol solution. Aliquot 1 mL of culture solution into cryogenic vials and placed into -80° C freezer for storage. When using stocks from storage, remove 1 mL cryogenic vial of A66.1 sample from freezer and thawed in water bed at 37° C for future use.

2.2 Mice Care and Scheduling

WT C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mouse were kept in specialized rodent facilities within McNulty Hall of Seton Hall University. Mice were provided with water and food *ad lib* during the course of each experiment carried out in this study. All mice utilized in experimentation were between the ages of 6-10 weeks old.

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

2.3 Living Viability Study and Immunization Experimentation

The following table provides the vaccination time table utilized.

Immunization	Boost 1	Boost 2	Infection
Day 1	Day 14	Day 28	Day 35

Table 1: Mouse Boost Schedule - This time frame was utilized during each study that involved immunization with WT C57BL/6 mice

The living viability study examined the amount of A66.1 required for lethality of WT C57BL/6 mice. The original stock concentration of A66.1 *Streptococcus pneumonia* is stored in the freezer at -80° C until used and is at the concentration of 1×10^8 CFU. Upon use, a vial containing stock cell lines was thawed in a water bath at 37 °C and then utilized for infections. Doses were created by diluting down the original concentration of 1×10^8 CFU to 1×10^6, 5×10^5, and 1×10^5 CFU and then infecting each mouse intranasally with said concentrations. Mice were inspected daily for observable symptoms and survival for a period of 15 days. If mice were deemed unhealthy and displayed signs of extreme duress, lethal measures were taken to reduce overall suffering.

Immunizations varied from study to study consisting of different doses administered intranasally with PBS, PspA, CTB, or the combination of PspA with CTB. The first experiment consisted of decreasing doses of PspA. The doses administered were as followed: 10 μg, 5 μg, and 1 μg with PBS being the control and followed the boost schedule outline above. After day 35 infection, mice were observed and survivals counted.

2.4 Bacterial Burden

After mice were infected, lung tissue was extracted from the mice and homogenized. A portion of these samples were plated on Sheep Blood Agar and allowed to incubate over the period of 18 hours at 37° C with 5% CO_2. Plates were divided into subcategories which were ten-fold dilutions from the original sample and titrated onto the blood agar plate. Upon incubation, the colonies for each respective titration marked on each plate were counted to determine the bacterial burden within each tissue sample.

2.5 Streptococcus pneumoniae Specific Antibody measurement

Specific antibody concentration titration measurements for Immunoglobulin G antibody (IgG), and Immunoglobulin G antibody subclasses 1 and 2c (IgG1 and IgG2c respectively) specific for A66.1 *Streptococcus pneumoniae* were examined via ELISA technique. Blood samples were extracted submandibularly from immunized mice 2 days prior to infection.

ELISA plates were used for antibody titrations. Assay plates were coated with A66.1. To do this, 1 mL cryogenic vial of A66.1 was added to 50 mL of THB and incubated at 37° C for 18 hours on a shaker. The culture was then centrifuged at 10 x G for a minute forming a pellet consisting of the bacterial cells. The supernatant was removed and the pellet suspended in 200 mL of Carbonate Coating Buffer. Then coating solution was added to each well of the ELISA assay plates and placed into the refrigerator at 4° C overnight until use. The plates were then blotted and coated with blocking buffer (SuperBlock) and placed on a shaker at room temperature for two hours. Plates were then blotted and washed with ELISA washing buffer three times and blotted after each wash. Then blood serum from each respective group (Control, PspA 5 µg, CTB 1 µg, PspA 5 µg + CTB 1 µg) was added to the diluent PBS to the first well of each respective group creating a 1:100 titration each well. After which, each row consisted of a

twofold dilution to the previous row until the final titration being 1:6400. Plates were placed on a shaker for 90 minutes at 37° C. Plates were then washed and blotted three times. IgG, IgG1, or IgG2c detection antibodies with conjugated Horse Radish Peroxidase (HRP) purchased from Thermo Fisher© were added to the plate and placed on a shaker for 90 minutes at 37° C. After which, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added to the plate and shaken for ten minutes until color change. Plates were analyzed at 610nm via a spectrophotometer.

2.6 Cytokine measurement

The Enzyme Linked Immunosorbent Assay (ELISA) was used to count the cytokine concentrations within the lungs and spleen in post infected mice. To do this, the lungs and spleen were removed from the infected mice post mortem and placed into 10 mL falcon tube with 2mL of Phosphate Buffer Saline (PBS). The tissue samples were then homogenized and placed into micro centrifuge tubes to be stored at -20 ° C. On the first day, plates were coated in Capture Antibody solution, which was specific to each cytokine being detected, and then incubated overnight at 2-8° C. On the second day, plates were washed/blotted and then blocked with 10% FBS at room temperature for 1 hour on a shaker. After blocking, plates were washed/blotted. Samples were thawed and kept on ice as the desired amount of each group's sample was aliquoted out. Lung samples were diluted down to 1:10 concentration of the original sample when applied to plate. Plate incubated at room temperature for 2 hours on shaker. Detection antibody for the specific cytokine was then added to the plates and incubated at room temperature for 1 hour. Next, Avidin-HRP was added to the wells and incubated for 30 minutes. Then, TMB substrate was added in and incubated in the dark for between 20-30 minutes until color change. The final step was the addition of stop solution. Plates were read with a spectrophotometer at 450 nm and 570 nm. The 570 nm absorbance values were subtracted from

18

the 450nm absorbance values. All plates were washed 4 times in between steps until after the

addition TMB substrate step.

2.6 Statistical Analysis

The Log-Rank (Mantel-Cox) test was used for survival curves using the GraphPad Prizm 4 software.

3. Results

3.1 Determining Living Viability of WT C57BL/6

Three groups of five WTC57BL/6 mice were infected intranasally at various titrated doses of A66.1 *Streptococcus Pneumoniae*. The three concentrations were $1x10^6$ CFU, $5x10^5$ CFU, and $1x10^5$ CFU. The purpose of this experiment was to determine the lowest dose required to terminate 100% of mice in a group of five. Mice were monitored for 15 days. All mice died at 1.0×10^6 CFU, 80% of mice died at 5.0×10^5 CFU, and all mice survived at 1.0×10^5 CFU. This experiment concluded that the lethal dose was around 5.0×10^5 CFU and thus all further infections would use the same dose.
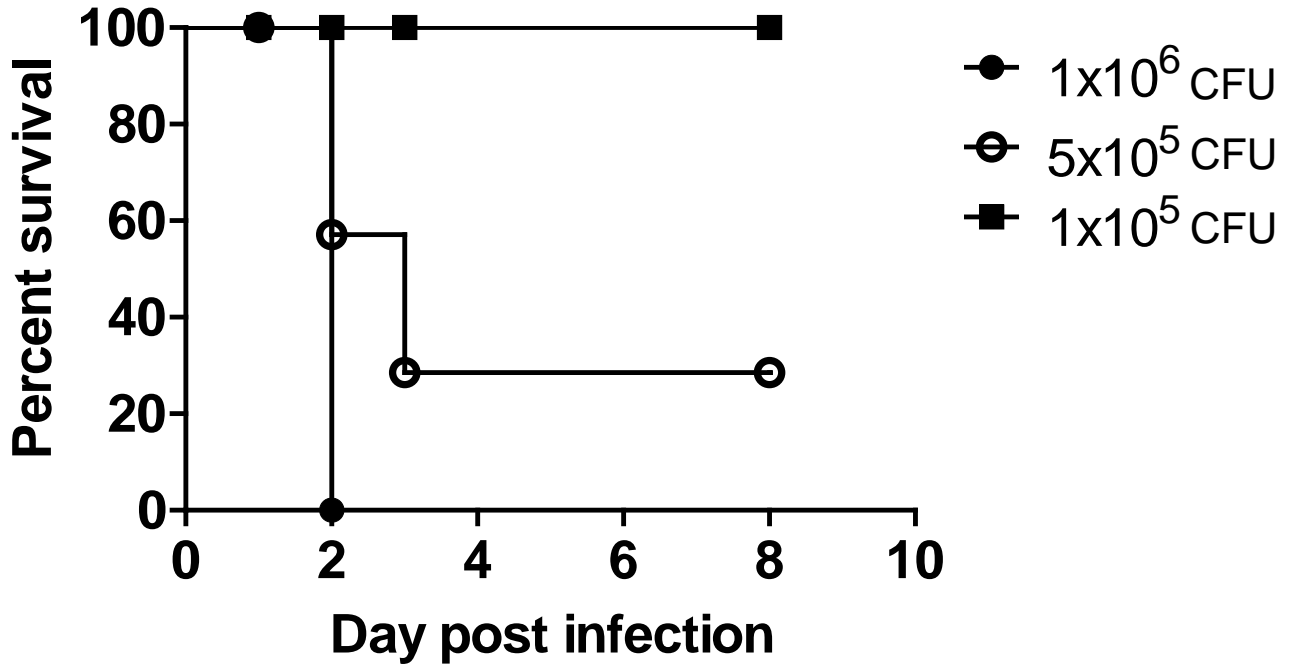
Figure 2: Survival Study- After eight days of monitoring with no change in status: All five mice died at the concentration of 1×10^6 CFU, four of the mice died at 5×10^5 CFU leaving one survivor, and none of the mice died at 1×10^5 CFU. No change in survival rate was observed after 15 days.

3.2 PspA and CTB titrations

3.2.1 PspA Titration

From determining the lethal dose of A66.1 to C57BL/6 WT mice, next step of the study was to determine the dosage of PspA and CTB required to effectively immunize and protect C57BL/6 WT mice from a lethal dose of A66.1 at 5.0×10^5 CFU. The initial objective was to titrate the PspA and establish the dose required to effectively protect C57BL/6 mice. 1 µg, 5 µg, and 10 µg against a control were used. PBS served as the control with 0% of the mice surviving. At 1 µg, 20% of the mice survived. At 5 µg, 40% of the mice survived. At 10 µg we saw the highest percentage with 100% of the mice surviving.

3.2.2 PspA + CTB titrations

Subsequently, we sought to determine if the use of the adjuvant CTB would increase the effective protection of a midpoint survival dose of PspA which was determined to be approximately 5 µg of PspA. To do this a dose of 1 µg of CTB was utilized. Four groups were assigned with the following doses: Control (PBS), PspA (5µg), CTB (1µg), and PspA (5µg) + CTB (1µg). Of the four groups, PspA + CTB had the highest survival rate at 100%. At 5 µg PspA, 40% of the mice survived. Both PBS and 1 µg CTB had 0% survival.
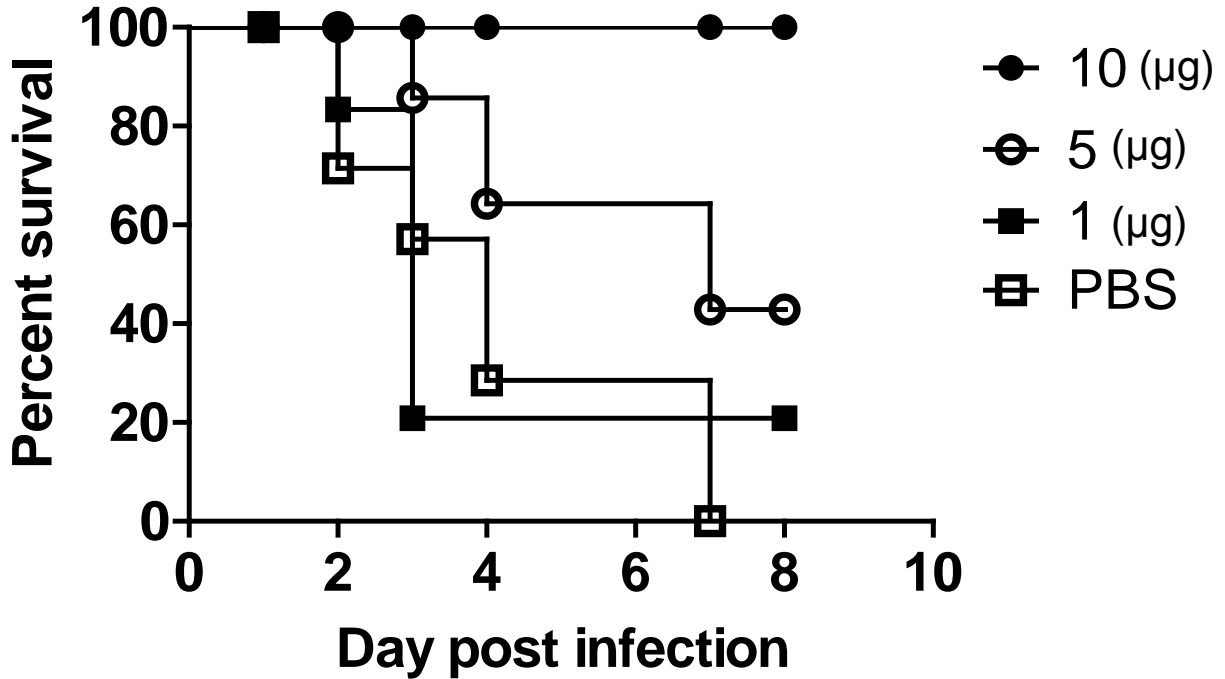
PspA immunization titration (2 boosts)

Figure 3: PspA Immunization Titration- Four groups of five WTC57BL/6 mice were immunized with varying doses of Pmeumococcal Surface Protein A (PspA) and boosted with the same initial dose on week 2 and 4 of the study. The groups were then infected with A66.1 Streptococcus Pneumoniae at $5x10^5$ CFU. All five mice immunized with 10 μg PspA survived after eight days. Of the five mice immunized with 5 μg PspA, two survived. Of the five mice immunized with 1 μg PspA, one survived. Of the control group immunized with PBS, zero survived.
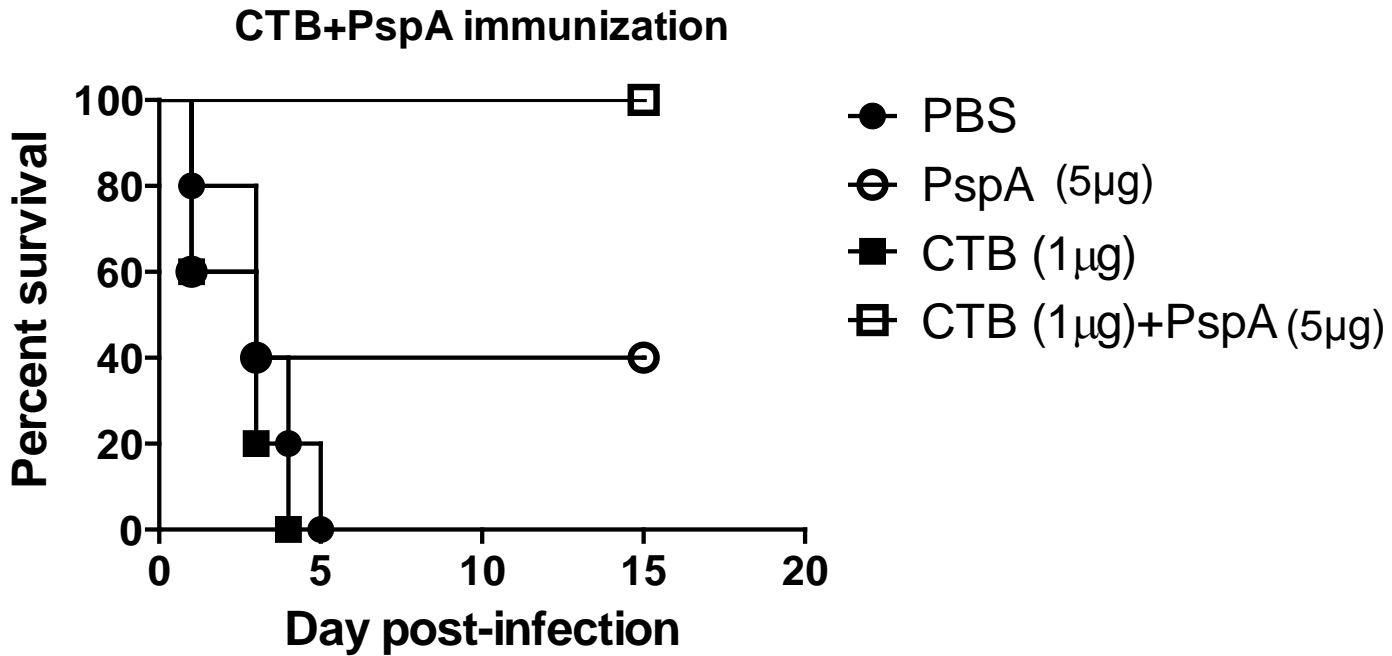
Figure 4: CTB + PspA Immunization - Four groups of five WTC57BL/6 mice were immunized with the following doses: Control group with 20 μL PBS, group dosed with only 5 μg PspA each, group dosed with 1 μg CTB only, group dosed with 1μg CTB and 5 μg PspA. Each group was boosted with the same initial dose on week 2 and 4 of the study. The groups were then infected with A66.1 Streptococcus Pneumoniae at $5x10^5$ CFU. All five control mice died after 15 days of observation. All five mice immunized with 1 μg CTB died. Two mice immunized with 5 μg PspA survived and three died. All five mice immunized with 5 μg PspA and 1 μg CTB survived.

3.3 Bacterial Burden in Post infected mice

Samples were titrated on Blood Agar plates and allowed to incubate over the period of 18 hours. Mice immunized with PBS, CTB(1μg), and PspA(5μg) exhibited colony growth up to 1.0×10^{6} compared to PspA(5μg) + CTB (1μg) which exhibited no colony growth on any titration.
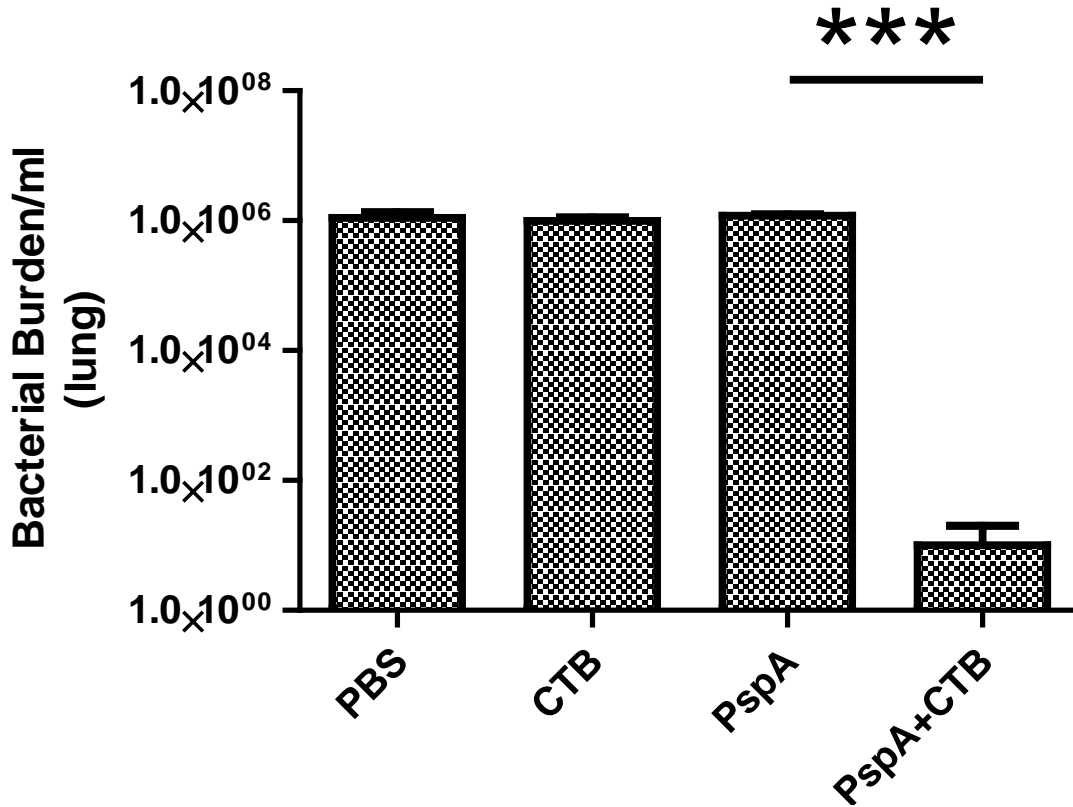
Figure 5: Bacterial Burden - Four groups of five WTC57BL/6 mice were immunized with the following doses: Control group with 20 uL PBS, group dosed with only 5 μg PspA each, group dosed with 1 μg CTB only, group dosed with 1μg CTB and 5 μg PspA and boosted twice. After two days of infection, the lungs were extracted from the mice, homogenized, and then plated at decreasing titrations. The lungs of mice immunized with PBS, CTB(1μg), and PspA(5μg) exhibited colony growth up to 1.0×10^6 where as PspA(5μg) + CTB (1μg) exhibited small growth.

3.4 Antibody titres in pre-infection C57BL/6 Mice

       After immunizations with PspA and CTB, our aim was to determine the antibody levels

of IgG targeting A66.1 *Streptococcus pneumoniae* within WT C57BL/6 mice. Four groups of

mice followed the same protocols highlighted in section 3.2.2 of the PspA and CTB study. Prior

to infection, blood samples were taken from the mice and Antibody titrations created in

conjunction with the ELISA technique. Results found that of the four groups (PBS, PspA, CTB,

and PspA + CTB) the vaccine/adjuvant combination of PspA + CTB resulted in the greatest

concentrations of IgG, IgG1, and IgG2c. Sample group PspA demonstrated the second highest
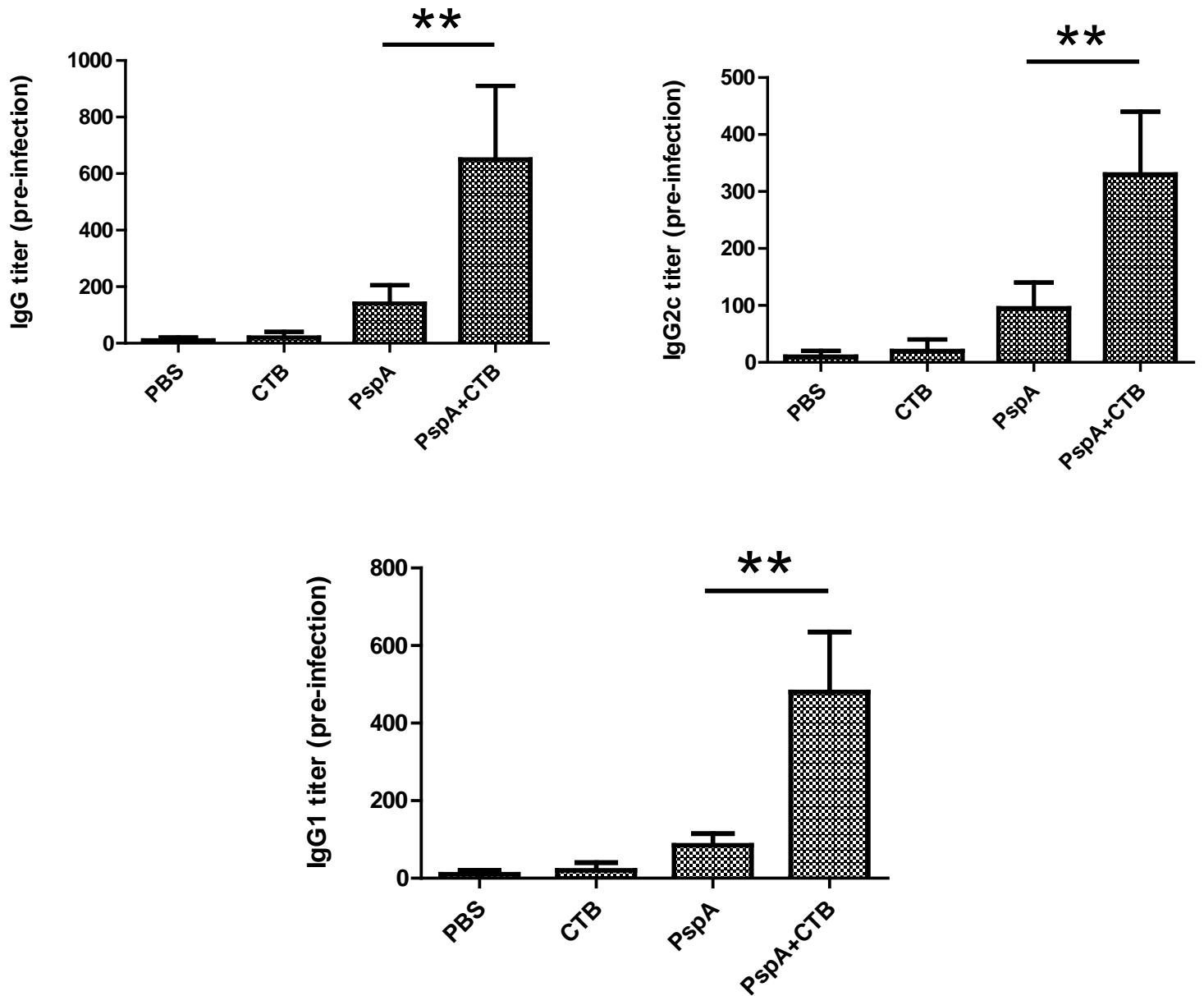
levels

Figure 6: IgG, IgG1, IgG2c Titration - Four groups of five WTC57BL/6 mice were immunized with the following doses: Control group with 20 uL PBS, group dosed with only 5 μg PspA each, group dosed with 1 μg CTB only, group dosed with 1μg CTB and 5 μg PspA and boosted twice. Two days before infection, blood was extracted submandibularly from three of the five mice of each group and pooled. Three separate ELISAs was performed on each of the groups to determine the antibody concentrations of IgG, IgG1, and IgG2c specific for A66.1. In all cases, PspA (5μg) + CTB (1μg) exhibited the highest concentration and PspA (5μg) exhibiting some levels of antibody. PBS and CTB exhibited no significant production of antibody.

3.5 Cytokine Concentrations in post-infection C57BL/6 Mice

      For the last portion of this study, we wanted to establish the cytokine concentrations within immunized mice after infection. Four groups of mice followed the same protocols highlighted in section 3.2.2 of the PspA and CTB study. Post infection, lung tissue samples were taken from the mice and homogenized. ELISA technique was used to determine the cytokine concentrations of IL-2, IL-4, IL-6, TNF-α, and INF-γ in all four groups of mice. Results found that of the four groups (PBS, PspA, CTB, and PspA + CTB) the vaccine/adjuvant combination of PspA + CTB resulted in the greatest concentrations of IL-2 and INF-γ and the lowest concentration of TNF-α and IL-6. IL-4 concentrations were relatively even throughout all four groups.
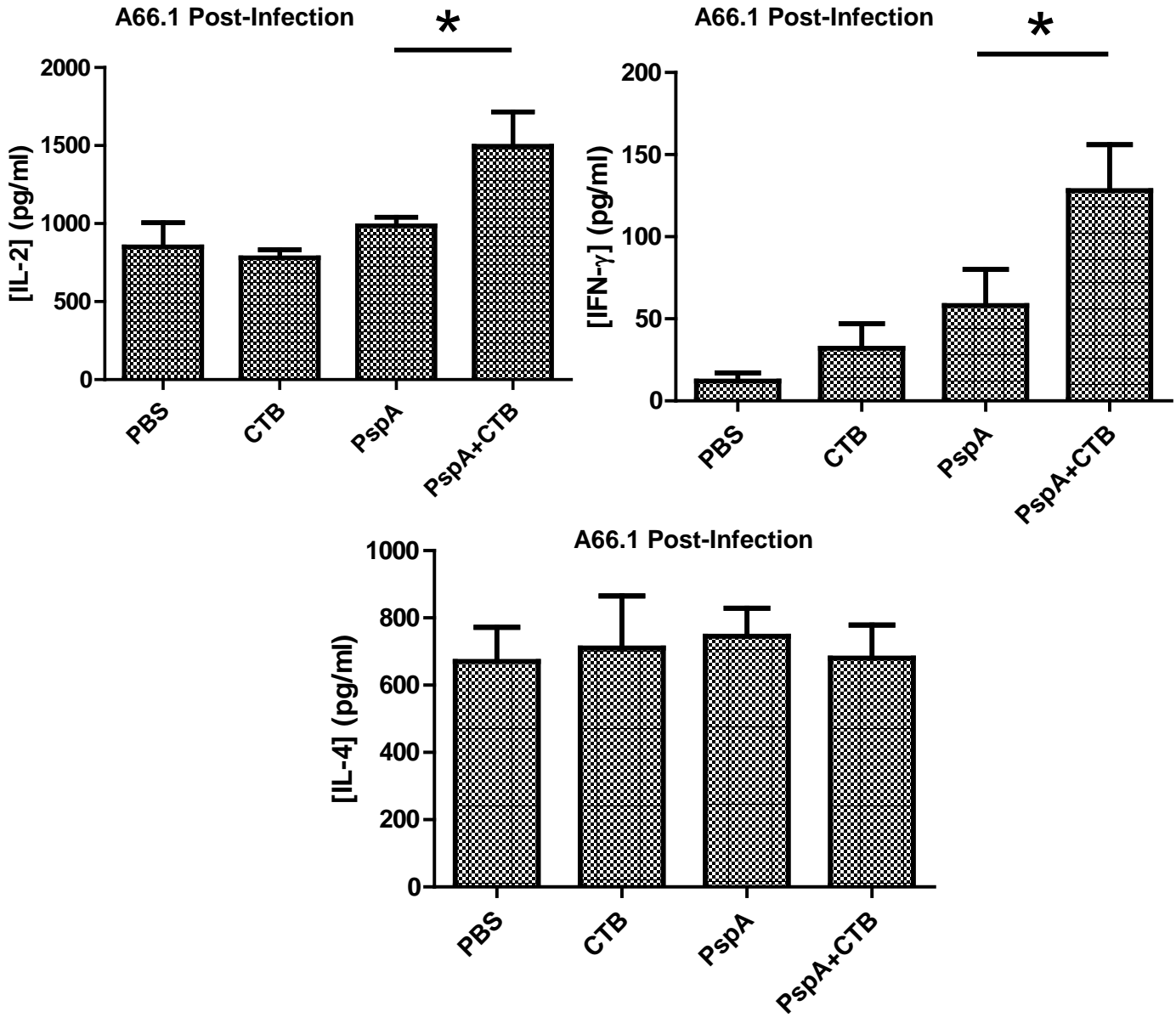
Figure 7: Cytokine INF-γ, IL-2, and IL-4 Concentrations Post-Infection:
Four groups of five WTC57BL/6 mice were immunized with the following
doses: Control group with 20 uL PBS, group dosed with only 5 μg PspA each,
group dosed with 1 μg CTB only, group dosed with 1μg CTB and 5 μg PspA
and boosted twice. Blood samples were then taken submandibulararly two days
post infection. Group PspA+CTB had the highest concentrations of IL-2 and
INF-y at approximately 1500 pg/mL and 130 pg/mL respectively. PspA had the
second highest with approximately 1000 pg/mL and 70 pg/mL. All groups had
no significant difference in IL-4 concentrations.

A66.1 Post-Infection

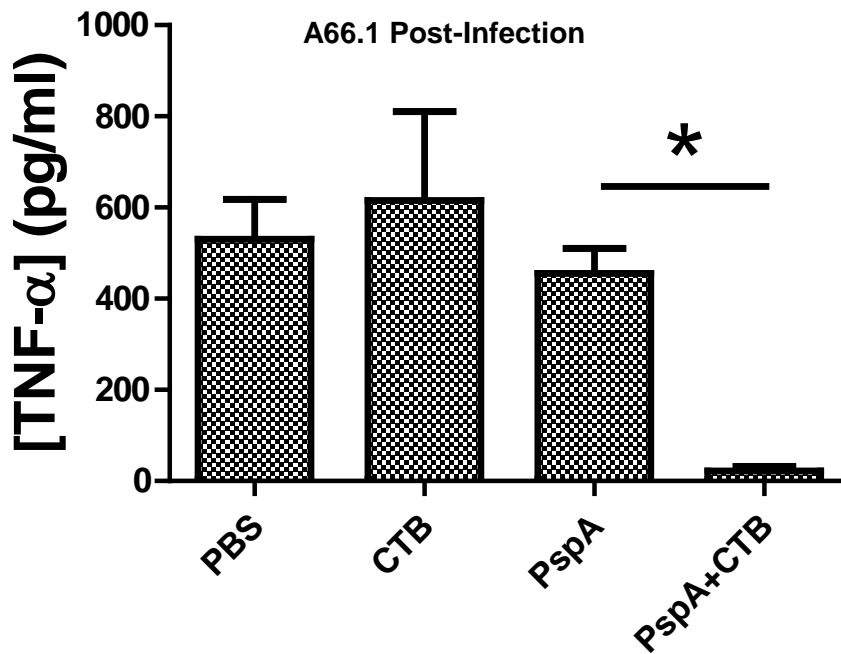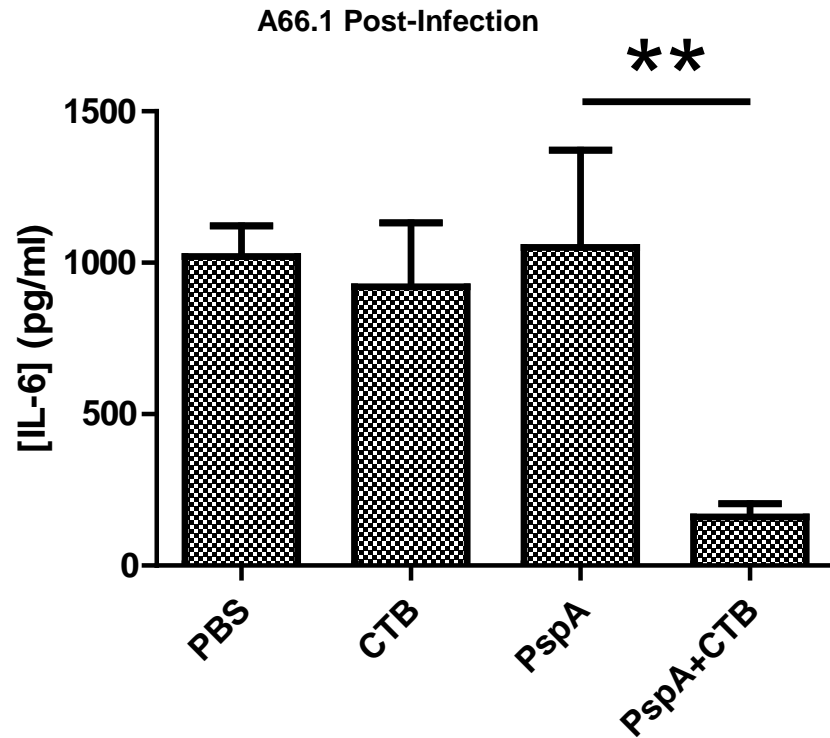**Figure 8: Cytokine TNF-α and IL:-6 Concentrations Post-Infection**: Four groups of five WTC57BL/6 mice were immunized with the following doses: Control group with 20 uL PBS, group dosed with only 5 μg PspA each, group dosed with 1 μg CTB only, group dosed with 1μg CTB and 5 μg PspA and boosted twice. Sample group PspA +CTB had significantly lower concentrations of IL-6 and TNF-α compared to the remaining groups.

4. Discussion

Analysis of our findings concluded that the subunit vaccine PspA with CTB was able to successfully protect mice infected with lethal doses of A66.1 *Streptococcus pneumoniae.* Initially, the lethal dosage of A66.1 for WTC57BL/6 had to be determined for the additional survival studies. After establishing the protection conferred by the CTB as an adjuvant we next sought to determine the immunological mechanisms involved in the protection involved. Both cytokines and antibodies are key factors in humoral immunity which plays a key role in protection against extracellular pathogens. By assessing these correlates of protection, we concluded that mice immunized with PspA and CTB had the highest titres of immunoglobulin subclass IgG specific for the A66.1 *Streptococcus pneumoniae* strain. Additionally, cytokine concentrations suggested that immunized mice possessed higher concentrations of type-1 cytokines (IL-2, INF-γ) compared to control groups as well as lower pro-inflammatory cytokines (TNF-α, IL-6). Essentially, mice immunized with PspA and CTB demonstrated a stronger antibody and cytokine responses compared to mice immunized with PspA alone which offered protection against lethal doses of A66.1 *Streptococcus pneumoniae,* thus highlighting the effectiveness of CTB as a vaccine adjuvant.

The first portion of experiments aims to determine the dosage of A66.1 *Streptococcus pneumoniae* required for lethality in WT C57BL/6 mice. At a dosage of 1 x 10^6 CFU A66.1, all of the C57BL/6 mice died after two days while all of the mice survived with the dosage of 1 x 10^5 CFU A66.1 indicating that the LV50 or Living viability of half the mice would be between these two values. At the dosage of 5 x 10^5 CFU A66.1, four of the five mice had died allowing us to consider it as the lethal dose for WT C57BL/6 mice. This dose was then utilized throughout

the study in an effort to assess the effectiveness of PspA as a subunit vaccine and the effect CTB has as a mucosal vaccine adjuvant.

The survival study includes the subunit vaccine PspA in order to determine if it alone is viable as an effective vaccine strategy against *Streptococcus pneumoniae* and at what dosage is there a significant increase in mouse survival against the control. The PspA titration survival study (Figure 3) indicates that PspA provided protection for the WT C57BL/6 mice against lethal doses of A66.1 with 10 µg of PspA resulting in highest survival. With only PspA mouse groups surviving, this coincides with the premise that PspA exists on the surface of A66.1 and that administering it was able to elicit an immune response within mice and form memory against A66.1 (Bitsaktsis et al., 2011). It also evident that increased doses of PspA proportionally increased immunogenicity resulting in higher levels of protection in mice at the highest doses versus mice with lower doses of PspA. These results coincides with previous studies (Bitsaktsis et al., 2011; Gor et al., 2005; Ferreira et al., 2009) on the ability of PspA as a vaccination in the protection against A66.1 *Streptococcus pneumoniae*.

The last stage of the survival studies was the combination of PspA with the adjuvant CTB. To reaffirm, adjuvants increase the immunogenicity of a vaccine eliciting a stronger immune response towards the vaccine, ultimately increasing protection against a target pathogen. This survival study (Figure 4) indicates that PspA (5µg) was able to protect 40% of WT C57BL/6 mice against a lethal dose of $5x10^5$ CFU A66.1 which correlates to findings seen previously (Figure 3). However, the addition of only 1µg of CTB was able to significantly increase the survival to 100% for all five mice. CTB was able to successfully increase the immunogenicity of the PspA vaccine and synergistically improve the efficacy of the vaccine to full protection of the entire sample population. Corresponding to findings found in previous

literature (Baldauf, 2015; Bitsaktsis et al., 2009; Kono, 2011), CTB was able to increase immunogenicity of 5 μg PspA to the effects seen with 10 μg of PspA seemingly through the activation of TH-1 cells. As anticipated, CTB alone did not drive any protection against S. pneumoniae challenge, indicating the non-specific mode of action of adjuvants.

To assess the antibody titres generated in mice following immunization, blood samples were taken pre-infection as outlined in section 2.4 and the titres were measured by ELISA. *Streptococcus pneumoniae*-specific IgG and its subclasses IgG1 and IgG2c were detected and compared among the different experimental groups. These findings (Figure 6) conclude that mice vaccinated with PspA+CTB displayed the highest titre of IgG, IgG1, and IgG2c versus the other groups. The mouse group immunized with PspA alone was able to elicit antibody production of the IgG isotype but its titres were significantly less than the concentrations found in vaccine/adjuvant sample group. Data from the titres emphasize the importance of immunogenicity that each dose has within the host and the ability of the vaccine to from memory cells against *Streptococcus pneumoniae.* Ultimately, these results coincide with the survival studies which concluded that PspA is able to elicit an immune response and facilitate immunoglobulin production against A66.1 *Streptococcus pneumoniae*. Secondly, when PspA is administered with the adjuvant CTB, the antibody production of IgG is over two fold that compared to the levels found in the mouse group immunized with PspA alone. These findings reinforce the hypothesis that CTB is able to increase the immunogenicity of PspA against A66.1 and thusly generate better protection via a TH-1 activation which coincides with the survival study outlined in Figure 4. It is evident that increased antibody production specific towards A66.1 induced by PspA and enhanced via CTB results in increased humoral immunity and allowed for all mice to survive lethal doses.

Progressively, it was determined if A66.1 was growing at a decelerated rate within PspA+CTB immunized mice compared to the other mouse groups. The bacterial burden (Figure 5) determined that the control, CTB, and PspA groups both expressed elevated levels of A66.1 growth within the lungs after day two of post infection. Meanwhile, the PspA+CTB sample group possessed very little to no growth within the lungs compared to the other sample groups. Since PspA+CTB possessed the greatest immunogenicity compared to the other mouse groups, the formation of memory was evident in the overall reduction in bacterial colonization within the lungs. This indicates that immunological protection is evident and strongest within immunized PspA+CTB mice which successfully prevented A66.1 growth within the lungs. This correlates with the heightened presence of total IgG, IgG1, and IgG2c specific for A66.1 within the PspA+CTB mouse groups.

The last portion of this study analyzes the cytokine concentrations within immunized mouse samples and their role in the inflammatory response. On day two post infection, lung and spleen tissue samples were extracted from the four mouse groups immunized with PBS, PspA, CTB, and PspA+CTB. With Cytokines IL-2 and INF-γ, mouse group PspA+CTB (Figure 7) possessed the largest concentrations amongst four groups while the PspA mouse group having slightly higher levels over CTB and PBS. Both IL-2 and INF-γ play an integral role in immunity by stimulating immunoglobulin production and inducing B cell proliferation through their interaction with TH-1 and TH-2 cells thusly initiating IgG class switching for IgG2c and IgG1 respectively (Owen et al.,2009). As discussed in Figure 1, IgG2c is predominantly generated via TH-1 activation which is generated via INF- γ. With cytokines TNF-α and IL-6, mouse group PspA+CTB exhibited the lowest concentrations of pro-inflammatory cytokines. This coincides with the known increase in immune response time via the presence of memory cells in order to

elicit a faster immune response in order to clear out the target pathogen. As a result, pro-inflammatory cytokines are expected to be at lower concentrations compared to the other mouse groups due to the increased immune response generated within PspA-CTB mice.

By connecting results of the viability studies in conjunction with bacterial burden, antibody titers, and cytokine concentrations, it can be concluded that the C57BL/6 mice immunized with PspA and adjuvant CTB possessed the strongest protection against A66.1 *Streptococcus pneumoniae*. Mouse group PspA+CTB exhibited the greatest increase in type-1 and type-2 related cytokine secretion levels of INF-γ and IL-2/IL-4 respectively along with the greatest titers of IgG isotype and its subclasses IgG2c and IgG1. PspA+CTB also possessed the lowest concentrations of TNF-α and IL-6 compared to other mouse groups indicating lowered pro-inflammatory cytokine levels which correlates to the lowered A66.1 growth within the lungs determined via the bacterial burden study. The cytokine IL-4 was equally distributed between all four groups which was anticipated as all sample groups would exhibit a shift towards type-2 cytokine production against an extracellular A66.1 *Streptococcus pneumoniae*. PspA+CTB exhibits with the highest concentration in type-1 cytokine INF-γ compared to all other mouse groups which correlates to the largest titre of IgG isotype subclass IgG2c. This correlates to findings with previous studies which have also shown that CTB is associated with elevated levels of TH-1 proliferation and recruitment during immunizations (Bitsaktsis et al., 2009)

Future research will examine cross species protection against varying serotypes of *Streptococcus pneumoniae*. For example, Serotype 3 strain ATCC 6303 *Streptococcus pneumoniae* is another commonly utilized pneumococcal pneumoniae utilized in murine models and tested against the PspA-CTB vaccine in the future. Cytokines IL-5 and IL-10 are also considered for analysis for examining the shift between the innate immunity towards the humoral

adaptive immunity and for their roles in generating TH-2 and TH-1 responses. Lastly, the

immunoglobulin isotype IgA, which is predominately associated with mucosal sited immunity,

would be examined for its potential increased titres within C57BL/6 mice immunized with PspA

and adjuvant CTB.

5. References

1. Bitsaktsis, C., Iglesias, B. V., Li, Y., Colino, J., Snapper, C. M., Hollingshead, S. K., . . . Gosselin, E. J. (2011). Mucosal Immunization with an Unadjuvanted Vaccine That Targets Streptococcus pneumoniae PspA to Human Fc Receptor Type I Protects against Pneumococcal Infection through Complement- and Lactoferrin-Mediated Bactericidal Activity. *Infection and Immunity, 80*(3), 1166-1180. doi:10.1128/iai.05511-11

2. Hollingshead SK, et al.. (2006). Pneumococcal surface protein A (PspA) familydistributionamongclinicalisolatesfromadultsover50yearsofage collected in seven countries. J. Med. Microbiol. 55:215–221.

3. Gor, D. O., Ding, X., Briles, D. E., Jacobs, M. R., & Greenspan, N. S. (2005). Relationship between Surface Accessibility for PpmA, PsaA, and PspA and Antibody-Mediated Immunity to Systemic Infection by Streptococcus pneumoniae. *Infection and Immunity, 73*(3), 1304-1312. doi:10.1128/iai.73.3.1304-1312.2005

4. Kono, M., Hotomi, M., Hollingshead, S. K., Briles, D. E., & Yamanaka, N. (2011). Maternal Immunization with Pneumococcal Surface Protein A Protects against Pneumococcal Infections among Derived Offspring. *PLoS ONE, 6*(10). doi:10.1371/journal.pone.0027102

5. Ferreira, D. M., Darrieux, M., Silva, D. A., Leite, L. C., Ferreira, J. M., Ho, P. L., . . . Oliveira, M. L. (2009). Characterization of Protective Mucosal and Systemic Immune Responses Elicited by Pneumococcal Surface Protein PspA and PspC Nasal Vaccines against a Respiratory Pneumococcal Challenge in Mice. *Clinical and Vaccine Immunology, 16*(5), 636-645. doi:10.1128/cvi.00395-08

6. Bitsaktsis, C., Rawool, D. B., Li, Y., Kurkure, N. V., Iglesias, B., & Gosselin, E. J. (2009). Differential Requirements for Protection against Mucosal Challenge with Francisella tularensis in the Presence versus Absence of Cholera Toxin B and Inactivated F. tularensis. *The Journal of Immunology, 182*(8), 4899-4909. doi:10.4049/jimmunol.0803242

7. Weber, S. E., Tian, H., & Pirofski, L. (2010). CD8 Cells Enhance Resistance to Pulmonary Serotype 3 Streptococcus pneumoniae Infection in Mice. *The Journal of Immunology, 186*(1), 432-442. doi:10.4049/jimmunol.1001963

8. Dockrell, D. H., Whyte, M. K., & Mitchell, T. J. (2012). Pneumococcal Pneumonia Mechanisms of Infection and Resolution. *CHEST, 142*(2), 482-491. doi:10.1378/chest.12-0210

9. Seo, J. (2002). Cross-Protective Immunity of Mice Induced by Oral Immunization with Pneumococcal Surface Adhesin A Encapsulated in Microspheres. *Infection and Immunity, 70*(3), 1143-1149. doi:10.1128/iai.70.3.1143-1149.2002

10. Seyoum, B., Yano, M., & Pirofski, L. (2011). The innate immune response to Streptococcus pneumoniae in the lung depends on serotype and host response. *Vaccine, 29*(45), 8002-8011. doi:10.1016/j.vaccine.2011.08.064

11. Baldauf, K., Royal, J., Hamorsky, K., & Matoba, N. (2015). Cholera Toxin B: One Subunit with Many Pharmaceutical Applications. *Toxins, 7*(3), 974-996. doi:10.3390/toxins7030974

12. Ren, B., Li, J., Genschmer, K., Hollingshead, S. K., & Briles, D. E. (2012). The Absence of PspA or Presence of Antibody to PspA Facilitates the Complement-Dependent Phagocytosis of Pneumococci In Vitro. *Clinical and Vaccine Immunology, 19*(10), 1574-1582. doi:10.1128/cvi.00393-12

13. Micheau, M. and Tschopp, J. (2003). "Induction of TNF Receptor 1 – mediated apoptosis via two sequential signaling complexes". *Cell* 114: 181-190

14. Owen, J., & Punt, J., & Stranford, S. (2009). Kuby immunology (Seventh ed.). New York, NY: W.H. Freeman & Company.

15. Kawakami, K., Kishino, H., Kanazu, S., Toshimizu, N., Takahashi, K., Sterling, T., . . . Musey, L. (2016). Revaccination with 23-valent pneumococcal polysaccharide vaccine in the Japanese elderly is well tolerated and elicits immune responses. *Vaccine, 34*(33), 3875-3881. doi:10.1016/j.vaccine.2016.05.052

16. Cho, H., Park, I. H., Burton, R. L., & Kim, K. (2016). Impact of IgM Antibodies on Cross-Protection against Pneumococcal Serogroups 6 and 19 after Immunization with 7-Valent Pneumococcal Conjugate Vaccine in Children. *Journal of Korean Medical Science J Korean Med Sci, 31*(6), 950. doi:10.3346/jkms.2016.31.6.950

17. Janeway, CA Jr. Travers, P. Walport M, et al.. (2001) Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science. The structure of a typical antibody molecule. Available from: http://www.ncbi.nlm.nih.gov/books/NBK27144/

18. Allegrucci, M., Hu, F. Z., Shen, K., Hayes, J., Ehrlich, G. D., Post, J. C., & Sauer, K. (2006). Phenotypic Characterization of *Streptococcus pneumoniae* Biofilm Development. *Journal of Bacteriology*, *188*(7), 2325–2335. http://doi.org/10.1128/JB.188.7.2325-2335.2006

19. Pneumococcal Diseases. (2015, June 10). *Centers for Disease Control and Prevention*. Retrieved March 2, 2016, from http://www.cdc.gov/pneumococcal/clinicians/clinical-features.html

20. Bast, D. J., Yue, M., Chen, X., Bell, D., Dresser, L., Saskin, R., de Azavedo, J. C. S. (2004). Novel Murine Model of Pneumococcal Pneumonia: Use of Temperature as a

Measure of Disease Severity To Compare the Efficacies of Moxifloxacin and Levofloxacin. *Antimicrobial Agents and Chemotherapy*, *48*(9), 3343–3348. http://doi.org/10.1128/AAC.48.9.3343-3348.2004

21. Bitsaktsis, C., Babadjanova, Z., & Gosselin, E. J. (2014). In Vivo Mechanisms Involved in Enhanced Protection Utilizing an Fc Receptor-Targeted Mucosal Vaccine Platform in a Bacterial Vaccine and Challenge Model. *Infection and Immunity Infect. Immun., 83*(1), 77-89. doi:10.1128/iai.02289-14

22. Vidarsson, G., Dekkers, G., & Rispens, T. (2014). IgG Subclasses and Allotypes: From Structure to Effector Functions. *Frontiers in Immunology*, *5*, 520. http://doi.org/10.3389/fimmu.2014.00520

23. Petrushina, I., Tran, M., Sadzikava, N., Ghochikyan, A., Vasilevko, V., Agadjanyan, M. G., & Cribbs, D. H. (2003). Importance of IgG2c isotype in the immune response to β-amyloid in amyloid precursor protein/transgenic mice. *Neuroscience Letters*, *338*(1), 5–8. doi:10.1016/s0304-3940(02)01357-5

24. Dahlgren, M. W., Gustafsson-Hedberg, T., Livingston, M., Cucak, H., Alsen, S., Yrlid, U., & Johansson-Lindbom, B. (2015). T follicular helper, but not th1, cell differentiation in the absence of conventional Dendritic cells. *The Journal of Immunology*, *194*(11), 5187–5199. doi:10.4049/jimmunol.1401938