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Development of a protein antigen based pneumococcal vaccine utilizing a polyanhydride nanoparticle delivery platform

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Development of a protein antigen based pneumococcal vaccine utilizing a polyanhydride nanoparticle delivery platform

by

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CHAPTER 1. INTRODUCTION

Streptococcus pneumoniae is the primary cause of bacterial pneumonia and
contributes to millions of deaths worldwide annually. The current capsule polysaccharide
based vaccines do provide some protection, but the 7 to 23 valent formulations cannot protect
against the over 90 serotypes currently known. Pneumococcal surface protein A (PspA) is a
promising candidate for a protein-based vaccine against *S. pneumoniae*, showing protection
in immunized mice and providing the advantage of universal protection from all serotypes of
the bacterium. This project shows the results of immunizing CBA/N mice intranasally and
subcutaneously with PspA encapsulated into a novel polyanhydride nanoparticle delivery
platform in attempt to create a protective protein-based pneumococcal vaccine against
bacterial challenge.

Literature Review

Vaccination History

Immunization, the practice of developing immunity to a pathogen by a controlled
exposure to the specified microbe or a component it produces, has been one of the great
success stories of science and medicine. This claim is best exemplified by the story of
controlling the dreaded disease of smallpox. Attempts to induce resistance to the smallpox
virus *Variola major* have been documented in 16th century China, 18th century Europe, and
colonial America (46, 82). These early attempts would use pathogen-containing pus from
infected individuals to inoculate healthy people, an approach that possessed a risk of
developing severe side-effects or the actual disease from the virulent virus. Incidences of
death from this process of 'variolation' claimed to be 1 % or less, which was a stark

improvement from the up to 30 % death rate of those who contracted smallpox naturally (8).

In the 1790's, Edward Jenner of England conducted the same procedure with the milder

cowpox virus *Vaccinia,* another member of the orthopox virus family, with much reduced

side-effects and was still able to confer immunity to the more deadly smallpox virus (18).

Smallpox 'vaccination' based on this live *Vaccinia* virus method by Jenner continued

through the 20th century, eventually leading to the declaration by the World Health

Organization (WHO) in 1980 that naturally contracted smallpox had been eradicated

worldwide (9).

In the late 1800's, the field of microbiology had taken root and techniques to reduce

pathogen virulence were being developed by multiple scientists such as Pasteur, Toussaint,

Salmon, and Smith (19) to name a few. With the knowledge gained by studying the

cultivation of disease causing microbes, whole cell vaccines were generated by utilizing in

vitro tissue growth or non-host animal passages of the microbes to attenuate them, meaning

that their virulence was reduced (50). This allowed people to be exposed to a weakened form

of the actual causative agent for a disease and to develop specific immunity to the pathogen,

rather than relying on cross reactivity as seen with the *Variola* and *Vaccinia* viruses.

Vaccines for yellow fever (12), measles, mumps, rubella, varicella, tuberculosis, and polio

were created from attenuated infectious agents (86).

At the turn of the 20th century, scientists utilized similar bench top processes to tackle

the issue of creating vaccines against toxin-producing microbes. Substances such as formalin

and aluminum salt precipitations in combination with prolonged heating created the first

toxoid (inactivated microbial toxin) vaccines to diphtheria and tetanus in the 1920's. These

inactivated toxins combined with mammalian antiserum, usually from horses or sheep,

created the first toxoid vaccines (15, 69). Such diseases as typhoid, cholera, pertussis, and typhus began to be countered by comparable bench top techniques, which would create killed whole cell vaccines to protect against these disease-causing pathogens (86).

With utilizing attenuated live microbes in vaccine formulations, there runs a risk of reversion to the fully virulent pathogen, and killed, whole cell vaccines can produce highly noticeable and undesirable side-effects after administration. In attempt to counter these circumstances, molecular techniques were employed to identify and produce immunogenic components of pathogens to make minimal antigen (i.e., subunit) vaccines. In more recent decades, technology has advanced to allow for better recombinant antigen production and purification from microbial pathogens. Since the days of purification from whole cell or virus cultures, recombinant DNA technology has become a common tool in the laboratory for producing and isolating whole or subunit proteins (45). The inherent nature of such procedures also contributes to a better knowledge and characterization of the pathogenic mechanisms of the infectious agent and the necessary components needed for a protective vaccine formulation.

Adjuvants

While producing and utilizing non-viable vaccines (such as toxoids) against microbial pathogens, it was observed that these formulations, while producing fewer side-effects, also created less protective immunity in people (69). Fortunately, some of compounds used as co-precipitates to prepare the early diphtheria and tetanus toxoids proved to enhance the immunogenicity of these microbial components. The use of alums (aluminum salts) became common and were the first adjuvant (an immune enhancing vaccine additive) approved for

human use in the United States (63). In the first half of the 20th century, oil-in-water

adjuvants also became popular. These formulations, similar to alums, help to create a depot

effect and the gradual exposure of an antigen to the host, which lengthens the time of an

antigen's presence to the immune system, resembling a replicating microbe. But with the

improved immune responses induced by the oil-in-water adjuvants, there arose some

troublesome side-effects. The popular oil-in-water Freund's adjuvants were extremely

effective, but could induce a degree of autoimmunity in some individuals after vaccination

(45, 63). There were also concerns that some of the slow degrading oils or surfactants used

could become carcinogenic. The alum salts, while milder in their inflammatory effects, can

produce granulomas at injection sites and their adjuvanticity was not reproducible from batch

to batch of vaccine (63). Increasing safety concerns like these helped to promote the

expansion of adjuvant research; however, it is also becoming more difficult for new

adjuvants/immune stimulators to get approved.

Similar to vaccines, the development of new adjuvants started to focus on microbial

components, chemical molecules, or microbial subunits in order to enhance vaccine

immunogenicity. Bacterial cell wall components such as waxes from mycobacteria, or lipids

from *Salmonella* were isolated and tested. Some of these compounds would have greater

specificity for stimualting specific components of the immune system allowing for immune

response targeting. These targeting systems frequently fall into one of two categories,

delivery systems or immune stimulators (45).

Immune stimulating components that are being studied are frequently molecules that

are recognized by immune cells. Constituents such as monophosphoryl lipid A (MPLA), a

modified component from surface membranes of the Gram negative bacterium *Salmonella*

minnesota, the protein flagellin from bacterial locomotive appendages, and unmethylated bacterial CpG-rich DNA all have specific receptors on most immune cells (45, 74). Each receptor to these immune stimulators induces activation of the immune cells often with characteristic outcomes, allowing for immune reactions against intracellular or extracellular pathogens to be initiated (54).

Adjuvants that enhance the delivery of vaccine antigens tend to be in the micrometer to nanometer scale, mimicking the size of bacterial and viral pathogens and allowing them to disseminate within body tissues and lymphatics (14, 74). Liposomes and virosomes are simple spheres of a lipid composition and thus are able to hold membrane bound surface antigens. The immune stimulating complexes (ISCOMS) are a more complex mixture of lipids, cholesterol, saponins, and antigens which can provide a more robust immune stimulation because of their heterogeneity (45).

Recently, MPLA was approved for use as an adjuvant in the new human papilloma virus vaccine, and, thus, became the second approved vaccine adjuvant for use in the United States (2). In 2009, the vaccine Cervarix, which is composed of virosomes loaded with L1 antigen from two human papilloma virus strains and adjuvanted with a combination of aluminum hydroxide and MPLA, was approved for use in teenagers in a three dose regimen (27). The combined use of delivery (alum) and immunostimulating (MPLA) adjuvants together in a vaccine such as Cervarix can enhance the effectiveness of a subunit vaccine resulting in protective immunity (26).

Variation in Vaccine Effectiveness: Stimulation of the Immune Response

Why are these vaccines successful at producing protection against pathogens? Within

the body is a system of various molecules, hematopoetic (i.e., blood derived) cells, and

tissues that react to infectious agents in order to eliminate them, and these innate and

adaptive components together create the immune system (52).

When a pathogen first crosses the primary physical and chemical barriers of the body,

such as mucosal epithelia, skin, and secreted antimicrobial molecules and enzymes, they are

encountered in the bodily tissues by phagocytic cells. Typically macrophages and dendritic

cells (DCs) move through tissues sampling for foreign matter, which they recognize by

pattern recognition receptors (PRRs). Many microbes have repeating patterns of protein, lipid,

and/or carbohydrates on their surfaces which are well conserved and thus good targets for

recognition and are referred to as pathogen-associated molecular patterns (PAMPs) (54).

Intracellular pathogens can also be recognized by some internal classes of PRRs that reside in

the cytoplasm and intracellular membrane compartments, thus allowing the phagocytic cells

to alert other immune components of the invading microbe. PRRs can initiate the

internalization of the invading microbes by phagocytosis and their subsequent destruction in

the lysosome. These processes also induce the production of cytokines that are released from

activated phagocytes to initiate local inflammation and alert other immune cells to the region

of infection (52).

The four hallmarks of inflammation are 1) swelling from vasodilatation and edema,

2) heat, 3) redness from the influx of blood, and 4) slight pain from the pressure of the extra

fluid. It is this rush of blood that brings additional lymphocytes and neutrophils to assist in

the continued clearance of foreign microbes (35). If any microbial cells escape into the blood

stream, a cascade system of serum proteins, called the complement system, is also bacteriocidal. There are three variations of the complement cascade which are triggered by different starting components. These triggers are antibody-bound to pathogens, mannose-binding lectin which binds bacterial surface carbohydrates, or activation of the C3 complement protein by alternative means resulting in the binding to exposed hydroxyl residues on the bacterial surface (56). When the complement cascade is completed, no matter the initiation pathway, an approximately 10 nanometer pore is formed in the cell membrane by the membrane attack complex that results in the disruption of the delicate ion and solute balance within the bacterial cell (35).

Dendritic cells, after ingesting a microbe at the site of infection, mature and migrate to regional lymph nodes via the lymphatic vessels. The ingestion and breakdown of the invading pathogen generates many small peptide fragments within the phagolysosome which the cell then displays on surface proteins called major histocompatability complexes (MHC) (54). In the lymph node follicles, DCs interact with local naïve B and T lymphocytes, searching for the T cell that will recognize the antigenic peptide-loaded into the MHC molecule on the surface of the DC. When a lymphocyte's (T cell receptor, TCR) and DC's (MHC I or II) surface receptors bind, a secondary surface receptor binding event occurs to stablized the interaction so lymphocyte activation can begin (52). A release of cytokines from the DC triggers the lymphocyte to differentiate into a specific effector cell. The lymphocyte then actively proliferates, differentiating into short-lived effector cells, and long-lived memory cells. Once a primary infection is cleared, it is the memory lymphocytes which will be able to respond quickly to any subsequent infections by the same pathogen (55).

The B cell is the lymphocyte that differentiate into plasma cells that then secrete antibodies, which are critical for the defense against extracellular bacteria, viruses, and toxins. There are two types of binding site on the Y-shaped antibodies, the two arms (i.e., Fab) are for binding the antigenic epitopes (molecular conformations along the antigen) and the carboxy terminal end (i.e., Fc) for binding with the Fc receptors on phagocytic cells or to complement protein (Figure 1) (52). The interaction of the antigen binding site (the Fab) to the foreign cell or toxin marks it for destruction and prevents it from interacting with and possibly destroying host cells, processes called opsonization and neutralization, respectively. It is this component of the antibody that vaccines try to specify for their antigen, so that upon re-infection with the same microbe the antibody will immediately recognize the critical virulence components of the invader and help eliminate it before the infection becomes established and leads to host tissue damage (54).

Antigen specific plasma cells were once thought to possess short life spans to only produce antibody in times of need (e.g., during an infection). When there is no antigen to stimulate antibody production in the body, the cells would die and memory B cells would remain as sentinals until the next infection prompts them to proliferate. More recently, it has been discovered that plasma cells can become long-lived and quietly produce transient amounts of antibody from their niches in the bone marrow or spleen for years or even decades (13, 75). Experiments designed to track the persistence of plasma cells in mice depleted of memory B cells (13), showed that plasma cells can be long-lived and continue to produce antigen-specific antibody (33).

B cell activation to create antibodies can happen independently of interaction with T effector cells. If molecular structures on a microbe's surface are highly repetitive and

promote good binding and cross-linking of the B cell receptor (membrane bound antibodies),

then B cell activation can occur independent of T cell help (75). The antibodies produced

following T cell-independent stimulation of B cells tend to be less diverse and less avid than

those that were produced following a T cell-dependent interaction. Antibodies produced
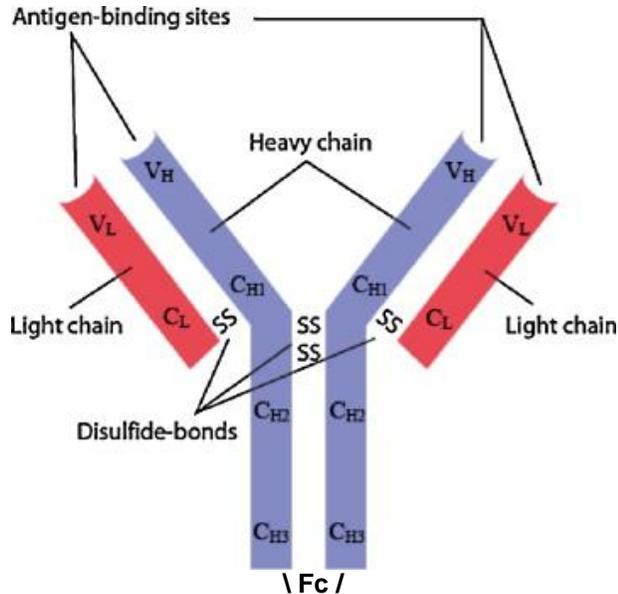
Figure 1. Basic antibody structure. Antibodies are composed of two identical heavy chains (in blue) and two identical light chains (in red), and are held together by covalent disulfide bonds (denoted SS). The variable region of one heavy and one light chain together create an antibody antigen binding fragment (F_{ab}), or antigen binding site. The ends of the heavy chains constant regions (C_{H3}) together create the antibody receptor binding site. Figure from Moser and Leo, 2010.

in this manner also tend to be short-lived, which can become troublesome with frequent re-

infections that can cause disease. The target for creating the most avid (more strongly

binding) antibody memory to antigens therefore requires T cell interactions (37, 86).

Pneumonia

Pneumonia is a respiratory disease caused by many different microbes, including

bacteria, viruses, and fungi. Such pathogens are transmitted via respiratory droplets and

cause inflammation in deep lung tissues when they bypass the innate defenses of the

nasopharyngeal region (4). All ages of humans worldwide are susceptible, especially

children under 5 years of age, the elderly, and any who have chronic conditions that may

impair the immune system. Every year, hundreds of thousands of children die from

pneumonia and it remains one of the leading causes of childhood death around the globe (1,

58), prompting the development of the Global Coalition Against Child Pneumonia in 2009

(10). Pneumonia can occur as a secondary infection when the immune system has been taxed

by another illness such as influenza (32, 48, 58). Community environments contribute to

disease development as well through crowded quarters, increasing person to person

transmission, and air pollution from smoke and chemicals which depress ciliary action in the

trachea (5, 48).

Streptococcus pneumoniae

The bacterium *Streptococcus pneumoniae* is the primary cause of bacterial

pneumonia. It is a Gram positive, polysaccharide capsule-producing extracellular microbe

which naturally inhabits the human nasopharynx and can asymptomatically colonize a vast

majority of a population (1). There are over 90 serotypes, or capsule polysaccharide variants,

that have been identified and all capsular types contribute in varying degrees to immune

avoidance by the pathogen. This polysaccharide coat prevents the phagocytosis of *S.*

pneumoniae via direct recognition by innate phagocytic cells, such as macrophages, that

normally rely on the use of PRRs or bacteria-bound C3b complement protein (11). Only

when the bacterium is opsonized by IgG antibodies can it be readily phagocytosed and

cleared from the site of infection (54). Yu *et al.* (85) have found that as little as 1.0 μg/mL of

secreted polysaccharide is enough to thwart antibody-mediated opsonization of *S. pneumoniae*, thus, reducing the clearance of *S. pneumoniae* by phagocytic cells.

In this regard, a capsule-based pneumococcal vaccine has been available for many decades to curtail the incidence of pneumonia caused by *S. pneumoniae*. At the turn of the 21st century, a newer version of this vaccine was released, and has helped to boost immunity to some of the most at risk groups for contracting *S. pneumoniae*-mediated respiratory disease.

Current Pneumococcal Vaccines

PPSV23, PCV7, and PCV13

The 23-valent pneumococcal polysaccharide vaccine (PPSV23) was the first multi-serotype vaccine developed against *S. pneumoniae*. The PPSV23 was approved for use in the United States in 1983 and contains 23 pneumococcal polysaccharide antigens in isotonic saline solution, with a 0.25 % phenolic preservative. Currently, this vaccine is recommended for children over two years of age or those who have medical conditions that may pre-dispose them to pneumococcal infection (59).

The pneumococcal conjugate vaccines (PCV) have capsular polysaccharide covalently linked to nontoxic, cross-reactive material (CRM) carrier protein from *C. diphtheriae*. These are suspended in succinate buffer with aluminum phosphate adjuvant. The PVC7 vaccine contains polysaccharides from seven *S. pneumoniae* serotypes that cause the vast majority of reported pneumonia cases in the United States. In 2000, the FDA approved use of this vaccine for children under two years of age employing a multiple-dose schedule. In 2010, the PVC13 pneumococcal vaccine was approved for use in the United

States. The main difference between the PCV7 and PCV13 is the inclusion of six additional serotype polysaccharides in the 13-valent conjugated vaccine (59).

Outcomes of Vaccine Use

Over the past decade, worldwide use of the PCV7 vaccine as part of routine vaccination schedules has produced commendable results in reducing the incidence of early childhood pneumonia. While bacterial carriage is not eliminated, vaccination has decreased the incidence of serious invasive pneumococcal diseases (IPD) (59). IPD can cause such complications as otitis media, meningitis, and bacteremia; a condition that can be especially serious for children younger than five years (59). A noticeable drop in prevalence of vaccine-included serotypes has been seen in bacterial isolates grown from pneumonia cases as well (70, 85).

Part of this success is due to the composition of the PCV vaccines in comparison to the PPSV23. The inclusion of a protein carrier attached to the capsule polysaccharide (a T cell-independent antigen, which is also a weak immunogen) enhances the immune system's response to the polysaccharide by promoting T cell help, as evidenced by promoting an adaptive response. The aluminum phosphate adjuvant in the PCV formulation also provides an immediate innate immunity stimulator for recruiting leukocytes (54). In addition, the wide-spread use of PPSV23 as a general booster after 18 to 23 months of age has shown signs of protection in PCV vaccinated children (70).

Obstacles

The capsule-polysaccharide based vaccines, while effective, still require multiple doses for the best results. When the recommended vaccination schedule is followed a child can receive three to four immunizations in the first 18 months of life for pneumococcus alone.

Russell *et al.* (70) found that a minimum of two vaccinations before the age of 12 months, plus a PPSV23 booster at 18 months, was required for noticeable protection from *S. pneumoniae* to occur. While the PCV vaccines can be given at the same time as other infant immunizations, patient compliance in maintaining clinic visits can become difficult, even more so in developing countries (64). This multiple immunization schedule also requires a lot of vaccine product to be purchased, and this can quickly become expensive.

The other limitations associated with the use of the polysaccharide vaccines are that they only protect against the specific bacterial serotypes to which the included capsule types match. All three of the discussed vaccine formulations contain capsule polysaccharides from the *S. pneumoniae* strains that have been epidemiologically identified as the most prevalent in causing disease. But, as these strains are reduced or eliminated from the nasopharyngeal region, other serotypes not included in the vaccine formulations become established (70, 85). Unless these emerging capsule types are included in the current polysaccharide vaccines, no serotype-specific immune memory can be developed. Countries and regions of the world also vary in which *S. pneumoniae* serotypes are most prevalent in their reported cases of pneumonia and IPD (48, 64). In these areas of the world, even if a full 3 dose PCV vaccination schedule is administered, the reduction in disease-causing strains may be 30-40 % less than in American clinical use (42).

To address some of these obstacles, many researchers are now looking at the possibility of using protein antigens expressed on the *S. pneumoniae* cell wall as potential vaccine candidates. With recombinant protein technology far more advanced than polysaccharide production and synthesis, there is the potential for greatly reducing the cost of production for a new pneumococcal vaccine. The use of a recombinant protein antigen also

raises hopes of being able to induce protective immunological memory in all age groups including children.

Pneumococcal Surface Protein A

In order for an antibody-mediated immune response to prevent infection, it must recognize antigens that are secreted or expressed on the extracellular surface of a microbe. Thus, proteins presented on the *S. pneumoniae* cell wall have been the primary target of new vaccine antigen searches. Despite the presence of a polysaccharide capsule, some virulence-associated proteins are sufficiently exposed on the surface of *S. pneumoniae* such that antibodies can bind and effect opsonization or complement fixation (29). With the advent of modern molecular biology techniques, these proteins can be readily cloned and the resultant recombinant proteins used for research purposes including vaccine development.

One extensively studied protein is pneumococcal surface protein A (PspA), which has many variants. Despite there being three family types of this protein expressed in *S. pneumoniae* covering 6 different clades, immunization of mice with recombinant PspA leads to demonstrable antibody-mediated protection across multiple capsular serotypes (38), and sometimes can be cross-reactive between protein clades (30, 51). All PspA proteins share a similar structure (as shown in Figure 2) which consists of a N-terminal alpha-helical coiled region, a proline rich intermediate region, and a C-terminal choline binding domain which attaches to liptotechoic acids within the bacterial cell wall (29). Recent research has uncovered that certain portions of this protein are accessible to antibody above the secreted capsule (30). The function of PspA is to bind apolactoferrin, an iron binding and bacteriocidal serum protein and prevent it from reaching the bacterial cell surface (72); and to

bind immune-activating complement protein to prevent deposition and opsonization on the cell surface (60).

In order for a protein such as PspA to be part of an effective subunit vaccine the delivery of stable, structurally in tact protein antigen within the body is essential in the vaccine design. Current work with polyanhydride nanoparticles for antigen delivery is showing great promise for recombinant protein vaccine application, and demonstrating protection in initial *in vivo* experimentation.
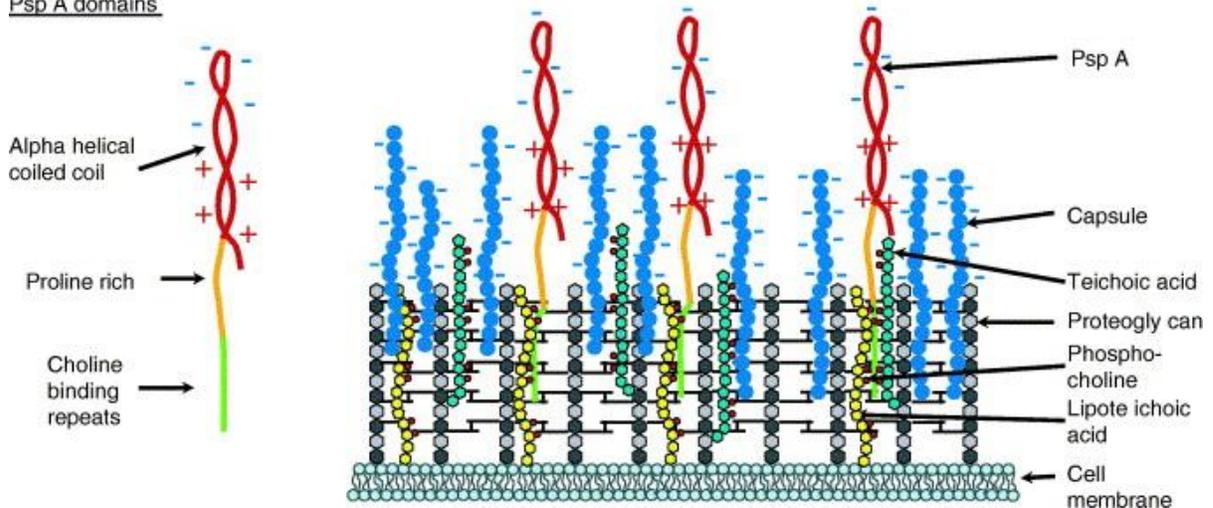
Figure 2. Representation of pneumococcal surface protein A (PspA) secondary structure. This cartoon represents the overall structure of PspA and its attachment to the *Streptococcus pneumoniae* cellular surface. The protein is bound to lipotechoic acids protruding from the cell wall by non-covalently binding to choline moieties with its C-terminal end. The coiled N-terminal portion protrudes out from the capsule polysaccharides where it is available for binding to apolactoferrin or antibodies. Figure originally from Daniels, et al., 2006.

Polyanhydrides

For over a decade, biodegradable polyanhydride polymers have been approved for human use with the drug-releasing product Gliadel, an implantable wafer loaded with an anti-cancer therapeutic drug (3). These polymers offer the great advantages of controlled release of their load due to surface erosion degradation, and the resultant carboxylic acid by-products

are biocompatible and induce no adverse side-effects *in vivo* (76). These load-releasing properties can be fine tuned further by the creation of copolymers with varying erosion kinetics by adjusting the molar ratio of the two polyanhydride monomers used to formulate the polymers.

The commonly used sebacic acid (SA) erodes quickly and the homopolymer can deliver the entirety of the encapsulated product in little more than a week within the body. An aromatic polyanhydride called 1,6-bis(p-carboxyphenoxy)hexane (CPH) is more hydrophobic than SA, and the resulting homopolymer has a slower degradation rate (months to years) which would facilitate the persistence of the encapsualted payload after being implanted or injected (76). Previous studies have demonstrated that copolymer formulations of these two compounds encapsulating an immunogenic protein can induce robust antibody responses in mice against known immune-stimulating antigens, such as tetanus toxoid, administered in a single vaccination dose (44). These results were similar to more traditional multiple dose vaccination regimen employing soluble antigen, and promoted a memory cell response upon in vitro stimulation of isolated T cells from vaccinated mice as well (79). A third, more amphiphilic polyanhydride chemistry called 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), which is based on CPH with triethylene glycol moieties added into the monomer backbone (78), can also be utilized to tailor the erosion characteristics of a copolymer as well as facilitate the stability of the encapsulated protein.

The incorporation of CPTEG into a copolymer results in a polymer particle with the erosion characteristics between bulk and surface erosion, which can allow for a better "burst" of antigen release when administered. This initial burst that releases some of the encapsulated immunogen enhances the initiation of the immune response against the

delivered antigen. The remaining protein payload within the polyanhydride particles is released gradually, prolonging antigen persistence and is believed to facilitate the affinity maturation of antibody and inducte long-lived memory lymphocytes for the specific antigen (44). Affinity maturation is a key component for the development of an efficacious antibody-based protective immune response.

A

B

C
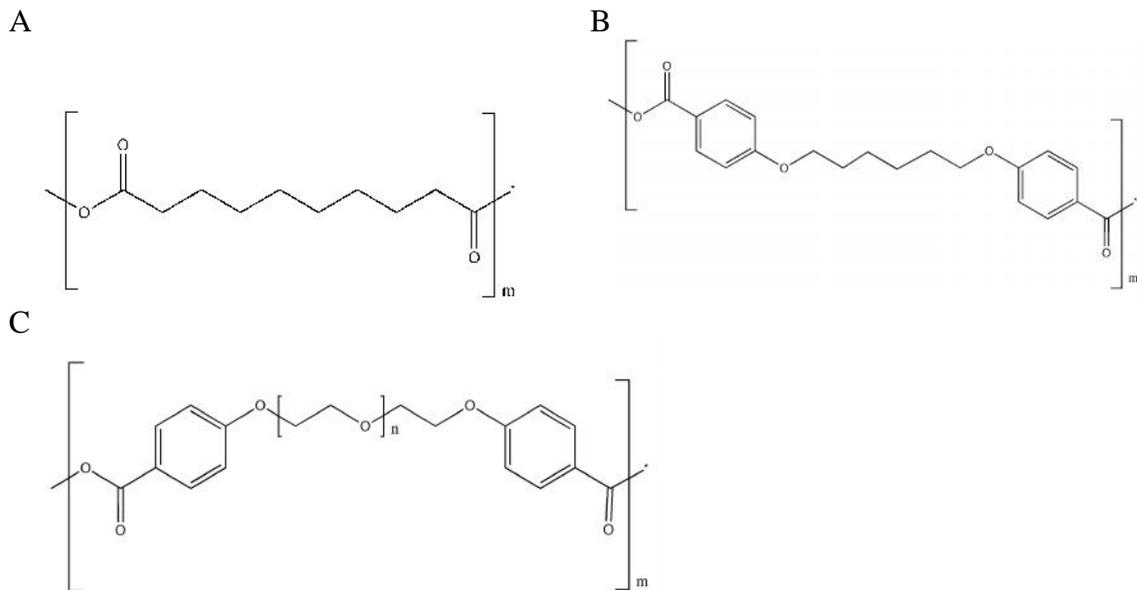
Figure 3. Molecular structure of polyanhydride polymers. Molecular stuctures of A) sebacic acid, B) 1,6-bis(p-carboxyphenoxy)hexane, and C) 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane.

Polyanhydride copolymer formulations can also enhance interactions with some phagocytic cell PRRs due to their hydrophobicity, thus, enhancing the uptake of the nano-to-micro scale particles and subsequent presentation of processed immunogens to the adaptive immune system (68, 81). The adjustment of the monomer ratios in the polymer formations as well as particle size can be used to tailor the kinetics of the resultant immune response induced against a particular encapsulated antigen. Repeating patterns on the surface of the polyanhydride particles, such as oxygen containing repeats, can also encourage phagocytic

update by mimicking PAMP-like patterns and activating antigen presenting cells (79). Work experimenting with surface modification of the polyanhydride particles with monosaccharides or disaccharides common to bacterial surfaces has shown that these modified particles are more readily internalized by DCs cells, encouraging antigen presentation and subsequent activation of T lymphocytes (22, 24).

The process by which polyanhydride nanoparticles are produced, which includes a cold anti-solvent method, provides a production process that is less degradative for protein antigens, better preserving their secondary structure and immunological epitopes (23, 66). With near 100 % encapsulation efficiency, this method of polymer fabrication also greatly reduces the amount of protein needed to produce or formulate a vaccine preparation (77). The nanoscale size of the produced particles facilitates the dispersion of the antigenic load through the lymphatic vasculature to draining lymph nodes from the site of injection, reducing the dependence for DC uptake and migration to secondary lymphoid tissue in the regional lymph nodes (14).

Recently a 50:50 CPTEG:CPH polyanhydride nanoparticle formulation administered in a single intranasal vaccination showed protection against the bacteria *Yersinia pestis*, the cause of pneumonic plague (80). The combination of soluble antigen in buffer, to prompt a primary immune response, combined with nanoparticle-encapsulated protein, for prolonged release and exposure, was 100 % protective in laboratory mice against a bacterial challenge up to 23 weeks after a single immunization. The persistent antibody observed in the mouse serum, as well as its high avidity (compared to the MPLA adjuvanted control) demonstrates the potential of the polyanhydride nanoparticle platform to create viable single dose

vaccinations (80). With this published procedure as a guide, our attempt to create a single

dose protein-based pneumococcal vaccine was tested.

Experimental Purpose

The purpose of the experiments described in the following sections was to determine

if recombinant PspA encapsulated into polyanhydride nanoparticles would induce protective

immunity against a lethal *S. pneumoniae* challenge in mice. The regularly utilized

recombinant protein PspA/Rx1 (AA1…302) (7), consists of the N-terminal alpha-helical

region and a portion of the proline rich mid-section of the native bacterial protein, was the

antigen used. Because a previous single dose protein-loaded nanoparticle regimen had been

used to successfully protect C57BL/6 mice against a lethal challenge with *Yersinia pestis*

(80), we hypothesize that a single immunization with PspA encapsulated into this vaccine

delivery platform will induce protective immunity against a lethal challenge of *S.*

pneumoniae.

CHAPTER 2. MATERIALS AND METHODS

Materials

Monomer Synthesis

The materials used for monomer synthesis include sodium hydroxide, hydrobenzoic acid, dibromohexane, 1-methyl-2-pyrrolidinone, and triethylene glycol, which were purchased from Sigma Aldrich (St. Louis, MO). Acetone, sulfuric acid, potassium carbonate, dimethyl formamide, toluene, acetonitrile, N,N-dimethylacetamide, and acetic acid were purchased from Fisher Scientific (Fairlawn, NJ). 4-p-fluorobenzonitrile used in the synthesis of CPTEG monomer was purchased from Apollo Scientific (Chesire, UK). Sebacic acid monomer was purchased from Sigma Aldrich (St. Louis, MO).

Acetylation, Polymerization, and Nanoparticle Fabrication

The following chemicals were used for acetylation and polymerization. Acetic anhydride, ethyl ether, petroleum ether, chloroform, methylene chloride, and hexane were purchased from Fisher Scientific (Fairlawn, NJ). Deuterated chloroform and dimethyl sulfoxide were used in ^{1}H NMR analysis of the polymers and monomers, respectively, and were purchased from Cambridge Isotope Laboratories (Andover, MA). Pentane and methylene chloride used in nanoparticle fabrication were purchased from Fisher Scientific (Fairlawn, NJ).

Recombinant protein and Bacterial Stocks

All *Streptococcus pneumoniae* strains used in these studies as well as recombinant

pneumococcal surface protein A (PspA) (UAB055, PspA/Rx1 AA1…303) were generously provided by Dr. David Briles of the University of Alabama in Birmingham. *Streptococcus pneumoniae* was cultured on trypticase soy agar petri plates supplemented with 0.5 % yeast extract and 5 % sheep blood (blood agar) or in brain heart infusion (BHI) broth medium supplemented with 0.5% yeast extract at 37 °C overnight.

Bacteriological medium and Taxo P-discs containing optochin, for *S. pneumoniae* verification, were purchased from BD Biosciences (Franklin Lake, NJ) and fresh whole sheep blood (with acid citrate dextrose to prevent clotting) was obtained from the USDA National Animal Disease Center facility (Ames, IA). Gentamicin sulfate salt used in blood agar plates, to select for Gram + bacteria like *S. pneumoniae*, for colony forming unit (CFU) determination was purchased from Sigma-Aldrich (St. Louis, MO).

Endotoxin Removal and Lyophilization of Antigen

Endotoxin removal was performed with Miltenyi endotoxin removing magnetic beads (Auburn, CA). The *Limulus* amoebocyte lysate assay from Lonza (Switzerland) was used to evaluate the endotoxin levels in protein stocks. Dialysis cassettes of 10 kDa cutoff were purchased from Thermo Scientific (Rockford, IL), and the lyophilizer used was from VirTis (Gardiner, NY).

Immunizations

The components used to create vaccines were phosphate buffered saline (PBS, 0.05 M and pH 7.4) from Mediatech, Inc. (Manassas, VA) and monophosphoryl lipid A (MPLA) was purchased from Sigma-Aldrich (St. Louis, MO).

Antibody Evaluations

Enzyme Linked Immunosorbent Assay

Assay components include microtiter 96 well high-binding plates were from Corning Costar (Tewksbury, MA), granulated gelatin from BD Biosciences (Franklin Lake, NJ), Tween 20 from Fisher Scientific (Fairlawn, NJ), and goat serum from Sigma-Aldrich (St. Louis, MO). Alkaline phosphatase-conjugated goat anti-mouse detection antibodies for IgG (H+L), IgG1, and IgG2a were purchased from Jackson ImmunoResearch (West Grove, PA). The phosphatase substrate was obtained from Fisher Scientific (Pittsburgh, PA) and was solublized in sodium carbonate (50 mM) magnesium chloride (2 mM) buffer (pH 9.3).

Avidity Assay

MagPlex-C microspheres of xMap technology reagents were purchased from Luminex (Austen, TX). The flat bottom 96 well plates, BioPlex 200 machine, BioPlex Pro II plate washer, and BioPlex Manager 6.0 software were purchased from BioRad Laboratories (Hercules, CA). Detection reagents, anti-mouse polyclonal IgG biotin-conjugated antibody and streptavidin-phycoerythrin (PE) (0.2 mg/mL), were purchased from eBioscience (San Diego, CA).

Animals

Five to eight week old female CBA/CaHN-*Btk^{xid}*/J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and were housed in specific pathogen-free conditions where bedding, cages, and feed were sterilized before use. All procedures performed were approved by the Iowa State University Institutional Animal Care and Use Committee.

Methods

Monomer Synthesis

The CPH and CPTEG monomers were fabricated as previously described (28, 78). The synthesis of 1,3-bis(p-carboxyphenoxy)propane from 4-hydrobenzoinc acid was adapted using 1,6 dibromohexane. First, sodium hydroxide and hydrobenzoic acid were dissolved in water and heated to 100 °C under refluxing conditions. Dibromohexane was then added dropwise and the solution was allowed to react for at least 5 hours, or until all of the solution had formed a white precipitate. The product was then dissolved in water, and concentrated sulfuric acid added until a pH of 2 was reached. The precipitate was filtered and redissolved in acetone to remove impurities. The recrystallization step was repeated until all impurities were removed from the product, as determined by ^{1}H NMR (Varian VXR300).

The CPTEG monomer was synthesized from triethylene glycol, dimethyl formamide, and potassium carbonate. First, a dinitrile was formed by reacting triethylene glycol and potassium carbonate in dimethyl formamide and toluene. This solution was reacted at 170 °C under refluxing conditions until all of the toluene was distilled. This was followed by the addition of 4-*p*-fluorobenzonitrile and the resultant solution was heated at 160 °C for 12 hours. Next a diacid was formed. Most of the solvent was removed using a rotary evaporator followed by the addition of water, acetic acid and sulfuric acid. The solution was refluxed under nitrogen at 170 °C for 6 hours. The resultant solution was precipitated in deionized water and the product collected. The product was redissolved in acetonitrile and filtered. Recrystallization was performed until purity was confirmed by ^{1}H NMR.

Prepolymer Synthesis (Acetylation)

Pre-polymers of CPH and SA were synthesized from monomers as previously described (73). In order to perform the acetylation, the SA and CPH monomers were heated to 100 °C under reflux conditions and nitrogen purge. The solution was allowed to reflux for 30 minutes before the solvent was completely removed through rotary evaporation. The resultant mixture was then dissolved in chloroform and precipitated with equal parts petroleum and ethyl ether.

Polymer Synthesis

Polymers and co-polymers of CPH, CPTEG, and SA were synthesized through melt condensation as previously described (43, 78). Polymer purity and molecular weight was determined using ^{1}H NMR and gel permeation chromatography (Waters GPC, Milford, MA) was used to confirm molecular weight.

Endotoxin Removal and Lyophilization of Antigen

Endotoxin was removed from the PspA protein stocks using endotoxin removal magnetic beads per the manufacturer's instructions with 82-85 % protein recovery, and endotoxin levels were assessed using a chromogenic *Limulus* amoebocyte lysate assay according to the manufacturer's instructions. By using this procedure, the endotoxin content was reduced from 80 EU/mg protein to 1.9 EU/mg protein. A portion of this PspA preparation was dialyzed against sterile deionized water using a dialysis cassette in sterile equipment at 4 ^{o}C for 18-20 hours. This protein was then frozen at -80 ^{o}C in a sterile vial for

1 hour, and lyophilized at -50 ^{o}C overnight. The lyophilized PspA was stored frozen on

dessicant until used in protein-loaded nanoparticle fabrication.

Nanoparticle Fabrication

The PspA-loaded polyanhydride nanoparticles were formulated using a solvent-anti

solvent precipitation method as previously described (49, 67, 81). PspA (sufficient for 1 %

loading) and polyanhydride polymer were dissolved in methylene chloride at a concentration

of 20 mg/mL. The solution was then sonicated at an output of 40 Hz (VibraCell) to ensure a

homogenized mixture. The resulting solution was then rapidly added to pentane at a ratio of

1:250 at room temperature for the CPH:SA formulation or at -40 °C for the CPTEG:CPH

polymers due to the lower glass transition temperature for these polymer formulations.

Nanoparticles were then collected from the solution using vacuum filtration. Nanoparticles

were characterized using scanning electron microscopy (FEI Quanta SEM, Hillsboro,

Oregon) and size distribution was determined from resultant images using ImageJ software.

Prior to use, the nanoparticles were stored in a dessicator at -80 ^{o}C.

Immunizations

Subcutaneous

Eleven to 14-week-old CBA/N mice were immunized subcutaneously at the nape of

the neck on experimental day zero with the treatments described in Table 1; there were six

mice per group. Protein suspended in PBS with or without MPLA was administered first,

followed by the 1 % PspA loaded nanoparticle formulations. Prior to administration, the

nanoparticles were suspended in PBS and sonicated at 40 Hz for 10-15 seconds. The MPLA

x 3 treatment group received three immunizations in total, administered on days 0, 21, and 42.

Blood samples for antibody analyses were collected via the saphenous vein at -2, 14, 28, 49, and 59 days post-immunization.

Table 1. Immunization dose compositions. The majority of the CBA/N mice were immunized once on day zero. *MPLA 3X dose group received immunizations at day 0, 21, and 42. In the IN immunization experiment n = 8, and for the SC immunization experiment n = 6. IN = intranasal administration, SC = subcutaneous administration at the nape of the neck, NP = not performed, PBS = phosphate buffered saline pH 7.4, PspA = pneumococcal surface protein A, MPLA = monophosphoryl lipid A, CPTEG:CPH = 50:50 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane : 1,6-bis(p-carboxyphenoxy)hexane, CPH:SA = 20:80 1,6-bis(p-carboxyphenoxy)hexane : sebacic acid.

Treatment Group	Component				
	PBS (µL) IN / SC	PspA (µg/dose)	MPLA (µg/dose)	CPTEG:CPH (µg/dose)	CPH:SA (µg/dose)
Control	50 / 100	---	---	---	---
PspA Only	50 / NP	25			
MPLA X 3*	50 / 100	8.33	10	---	---
MPLA X 1	50 / 100	25	10	---	---
CPTEG:CPH	50 / 200	25	---	500	---
CPTEG:CPH + MPLA	NP / 200	25	10	500	---
CPH:SA	NP / 200	25	---	---	500
CPH:SA + MPLA	NP / 200	25	10	---	500

Intransal

Eight-week-old CBA/N mice were immunized intranasally (IN) on experimental day zero with either the saline, MPLA x 1, MPLA x 3, 25 µg PspA in PBS, or CPTEG:CPH treatments described in Table 1, with 8 mice per group. Prior to IN immunization, mice were anesthetized by administering an intraperitoneal injection (80 µL) of a 20 mg/ml ketamine / 1 mg/ml xylazine cocktail; once immobilized, the mice were held vertically and a 25 µL volume of the designated vaccine formulation was administered to each nare, resulting in a 50 µL total vaccination volume. Immediately prior to administration, the nanoparticles were

added to the soluble protein solution and sonicated at 40 Hz for 10-15 seconds to assure

suspension. The MPLA x 3 treatment group received three immunizations administered on

days 0, 21, and 42. Blood samples for antibody analyses were collected via the saphenous

vein at -4, 14, 28, and 49 days post-immunization. Because of the magnitude of the antibody

responses measured in the serum samples collected at day 49, a subcutaneous booster

immunization of 5 µg PspA (100 µL PBS total volume) was administered (day 56) to mice in

the protein only treatment group and the CPTEG:CPH nanoparticle treatment group.

Bacterial Challenge

Streptococcus pneumoniae serotype A66.1 was grown overnight, and about 15-25

colonies were taken from the blood agar plate to inoculate 10 mL of BHI broth medium as

described above. The cultures were incubated until an optical density of 0.45-0.50 at 600 nm

was reached. A fraction of bacterial culture was diluted in PBS to create the inoculum. One

hundred microliters of the prepared inoculum was taken and serially diluted and plated for

actual CFU counts (full method description in Appendix). CBA/N mice were intravenously

challenged at 8-9 weeks post-immunization with 1.01×10^7 (subcutaneously immunized, OD

$= 0.533$) or 3.75×10^5 (intranasally immunized, OD $= 0.493$) CFU via the tail vein in a 200

µL volume. Prior to the intravenous injection, the mice were placed under a heat lamp for 3-5

minutes to dilate the tail veins. The mice were observed three times per day for clinical signs

of infection including ruffled fur, hunched backs, lethergy, and ability to respond to stimuli

(full description in the Appendix, Table 2). Body temperature was monitored once daily with

a Fluke-566 infrared thermometer (Fluke Corporation, Everett, WA). Mice found moribund

were euthanized by CO_2 asphyxiation and necropsied. After euthanasia, blood was collected

via cardiac puncture, and the right lung and spleen were collected to measure bacterial

burden (data not shown).

Antibody Evaluations

Enzyme Linked Immunosorbent Assay

Microtiter 96 well plates were coated with PspA (0.5 μg/mL in 100 μL PBS per well)

and allowed to bind overnight at 4 ^{o}C. Plates were washed three times with PBS and then

blocked with 2 % granulated gelatin prepared in PBS supplemented with 0.05 % Tween 20

(PBS-T) for 2 hours at room temperature. Plates were placed at 37 ^{o}C for 10 minutes to

liquefy the gelatin, then washed three times with PBS-T. Individual mouse serum samples

were diluted 1:100 or 1:200 in the first well of a 96 well microtiter plate in duplicate, and

then serially diluted two- or three-fold in PBS-T supplemented with 1 % goat serum for titer

assessment. For the assessment of IgG isotypes, serum samples were diluted 1:500 and

tested in duplicate wells. Serum antibodies were allowed to bind overnight to the microtiter

plates coated with PspA at 4 ^{o}C. Plates were washed three times with PBS-T and 100 μL of

alkaline phosphatase-conjugated goat anti-mouse IgG (H+L), IgG1, or IgG2a antibody (1

μg/mL in PBS-T supplemented with 1% goat serum) was added to the wells and incubated

for 2 hours at room temperature. Plates were washed four times with PBS-T and 100 μL of

phosphatase substrate (1 mg/mL) in 50 mM sodium carbonate and 2 mM magnesium

chloride buffer (pH 9.3) was added and the colorimetric changes were measured at 405 nm

after 15 minutes (for isotypes) or 30 minutes (for titer) of incubation.

Avidity Assay

MagPlex-C microspheres (magbeads) were activated and coupled to PspA (20 µg/mL in PBS) as described by the manufacturer. Following the conjugation step, the PspA-magbeads were washed with PBS-T and stored in PBS with 1 % bovine serum albumin and 0.05 % sodium azide and covered to block overnight at 4 ^{o}C. Serum samples were diluted in PBS-T and 50 µL was added to each well of a 96 well flat bottom microtiter plate along with 50 µL of vigorously vortexed magbeads (120,000/mL) conjugated with PspA suspended in blocking buffer. Plates were immediately covered and placed on a plate shaker for 1 hour at room temperature. Plates were washed three times using a BioPlex Pro II plate washer with the blocking buffer, and 100 µL of either PBS-T or with 6 M urea in PBS were added to the appropriate wells. Plates were covered and put on a shaker for 15 minutes, and then washed three more times. Biotin-conjugated goat anti-mouse IgG was diluted 1:200 and 50 µL was added to each well, and plates were covered and shaken at room temperature for 1 hour. The plates were washed three times, and 50 µL of streptavidin (PE) diluted 1:20 was added to each well and the plates were incubated for 30 minutes. Plates were washed three times, 125 µL of blocking buffer added to the wells, and the mean fluorescent intensity was measured using a BioPlex 200. To calcualte the relative avidity of a serum sample having a demonstrable antibody response to the target antigen, the mean fluorescent intensity (MFI) obtained for the sample well treated with urea was divided by the MFI measured for the corresponding serum sample wells incubated only with PBS-T. This value was multiplied by 100 to obtain a relative percent binding for each serum sample.

Statistics

Statistics were run with SAS JMP program using the Student's t-test, $p \leq 0.05$.

CHAPTER 3. RESULTS

Intranasal Immunization Antibody Response

Having shown previous success with a single dose intranasal nanoparticle immunization inducing protective immunity against *Yersinia pestis* (80), the present studies outline experiments that were performed to evaluate the intranasal immunization of mice with recombinant PspA loaded into 50:50 CPTEG:CPH nanoparticles in order to effect protective immunity against *Streptococcus pneumoniae*. Seven weeks after intranasal vaccination (see Table 1), mice that were immunized three times with the PspA plus MPLA formulation developed anti-PspA antibody titers that were approximately 100,000 to 200,000. In contrast, the anti-PspA antibody response measured at seven weeks in the mice receiving the single dose nanoparticle formulation only reached a maximal titer of 1,000 to 2,000 that was significantly less ($p < 0.05$) than that induced by the triple dose MPLA regimen (Figure 4, part A). To assess whether the mice receiving the nanoparticle formulation had been primed to respond with an anamnestic response, the nanoparticle treated mice, as well as the PspA only mice, received a subcutaneous booster immunization (5 μg PspA). After the booster dose, the resultant IgG1 produced by the two previously mentioned treatment groups showed a marginal increase at day 66 compared to the response at day 49 (Figure 4, part C). While the titer in the PspA only group increased by more than 10-fold, the total IgG (H+L) titer in the mice immunized with the 50:50 CPTEG:CPH formulation increase only 2- to 3-fold. With respect to the quality of the antibody response (i.e., avidity), the anti-PspA antibody induced following immunization with the nanoparticle regimen showed higher avidity at all time points measured while it took longer for the avidity to reach a similar value in the treatment groups immunized with PspA plus MPLA (Figure 4, part B).
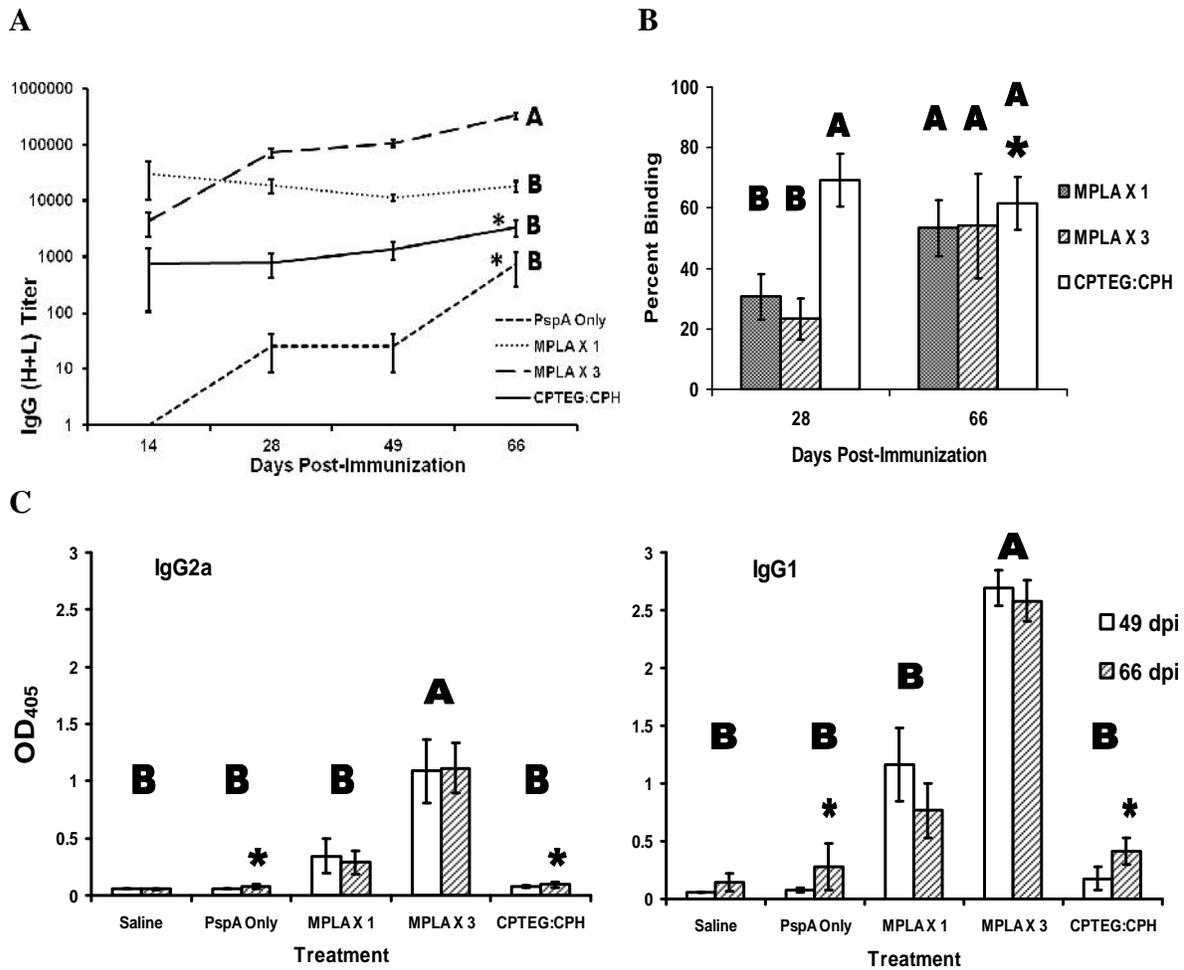
Figure 4. Assessment of the serum IgG responses following intranasal immunization of CBA/N mice with PspA. A) Using serial two-fold dilutions, serum antibody titers were measured by ELISA. B) Post-immunization anti-PspA antibody avidity was measured at days 28 and 66. C) Isotype switching was assessed by measuring the IgG1 and IgG2a PspA-specific antibody in serum samples collected at days 49 and 66. All serum samples were collected prior to *Streptococcus pneumoniae* A66.1 challenge. Full procedures are detailed in the Materials and Methods section. n = 8, except for MPLA X 3 where n = 6. Statistics were calculated with SAS JMP using Student's t-test, p $\leq$ 0.05. * = group boosted with 5 μg PspA in phosphate buffered saline (pH 7.4) at 53 days post-immunization. IN = intranasal immunization, PspA = pneumococcal surface protein A, MPLA = monophosphoryl lipid A, CPTEG:CPH = 50:50 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane: 1,6-bis(p-carboxyphenoxy)hexane.

Bacterial Challenge of Intranasally Immunized CBA/N

To test the protection of the humoral immunity induced by the intranasal

immunizations, CBA/N mice were challenged with 3.75 x 10^5 CFU of *S. pneumoniae* strain

A66.1. As can be seen in Figure 5, the triple dose MPLA treatment produced 83 % survival

of the mice following intravenous challenge through the two week observation period. While

75 % of the mice that were immunized with PspA encapsulated into 50:50 CPTEG:CPH

nanoparticles survived through day five, less than 30 % of these mice survived beyond day 9

post-challenge . The mice that were immunized once with PspA adjuvanted with MPLA

succumbed to bacteremia faster than the naive control mice, with none of the mice in this

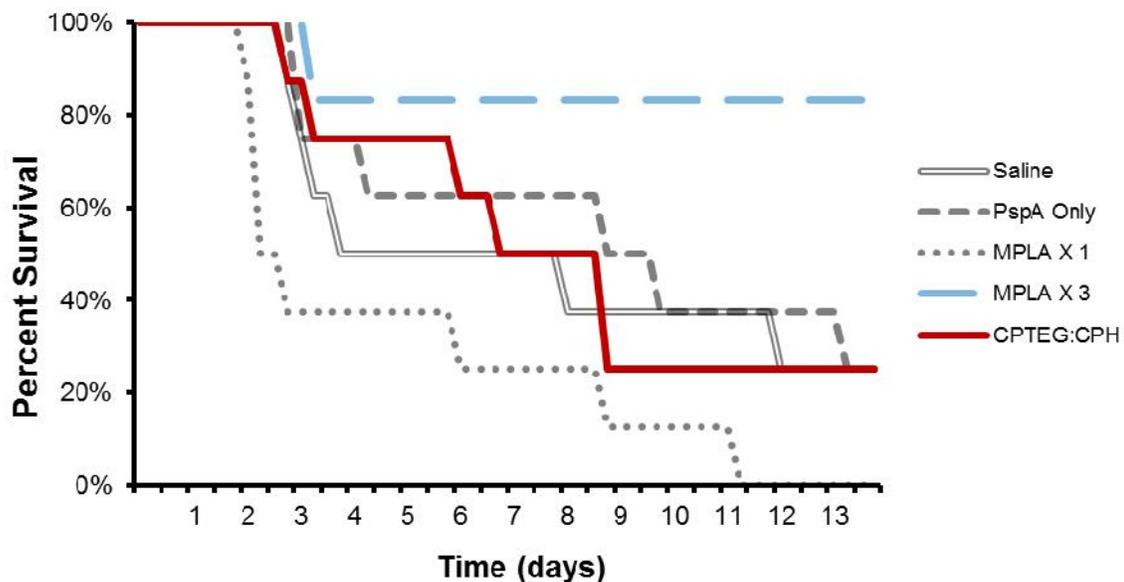group surviving after 11 days.

Figure 5. Survival of intranasally immunized CBA/N mice following challenge with *Streptococcus pneumoniae* A66.1. Mice were intravenously challenged with 3.75 x 10^5 CFU of strain A66.1 in a 200 μL volume 70 days after the initial immunization. The mice were monitored daily for clinical signs of infection as described in the Materials and Methods of the Appendix. n = 8, except for MPLA X 3 where n = 6. PspA = pneumococcal surface protein A, MPLA = monophosphoryl lipid A, CPTEG:CPH = 50:50 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane: 1,6-bis(p-carboxyphenoxy)hexane.

Subcutaneous Immunization Antibody Response

Based on the poor protective response induced following intranasal immunization,

CBA/N mice were subcutaneously immunized with the same vaccine regimen as above with

the addition of the 20:80 CPH:SA PspA loaded nanoparticles. Because of the benefits

detected with the inclusion of MPLA in the IN vaccine regimen, the effect of MPLA added along with the nanoparticle treatments was also examined for subcutaneously vaccinated mice (e.g., CPTEG:CPH + MPLA and CPH:SA + MPLA). Following subcutaneous immunization, the 50:50 CPTEG:CPH nanoparticle vaccination groups produced anti-PspA IgG titers 10 times higher than those induced by intranasal administration (Figure 6, part A). Of note, the mice immunized once with the MPLA plus PspA regimen developed a titer of 10,000, which was similar in magnitude to that induced by the same immunization given intranasally. The inclusion of MPLA in the soluble portion of the vaccine regimen did double the overall anti-PspA titer induced by the mice immunized with the nanoparticle treatments (e.g., 50:50 CPTEG:CPH + MPLA and 20:80 CPH:SA + MPLA), but did not contribute to higher IgG2a:IgG1 isotype ratios (Figure 7). The mice immunized subcutaneously with the triple dose of PspA plus MPLA produced antibody titers that were about 2- to 3-fold greater than that induced by intransal immunization with the same treatment. At 8.5 weeks post-immunization, there was no statistical difference in avidity between any of the nanoparticle immunized mice regardless of the addition of MPLA (Figure 6, part B).

Bacterial Challenge of Subcutaneously Immunized Mice

Eight and a half weeks after the initial subcutaneous immunization, CBA/N mice were challenged intravenously with 1.01×10^7 CFU of *S. pneumoniae* strain A66.1. Stunningly, all of the treatment groups, regardless of their immune status, began to succumb on day two post-challenge. By 4 days post-infection, mouse survival had dropped to about
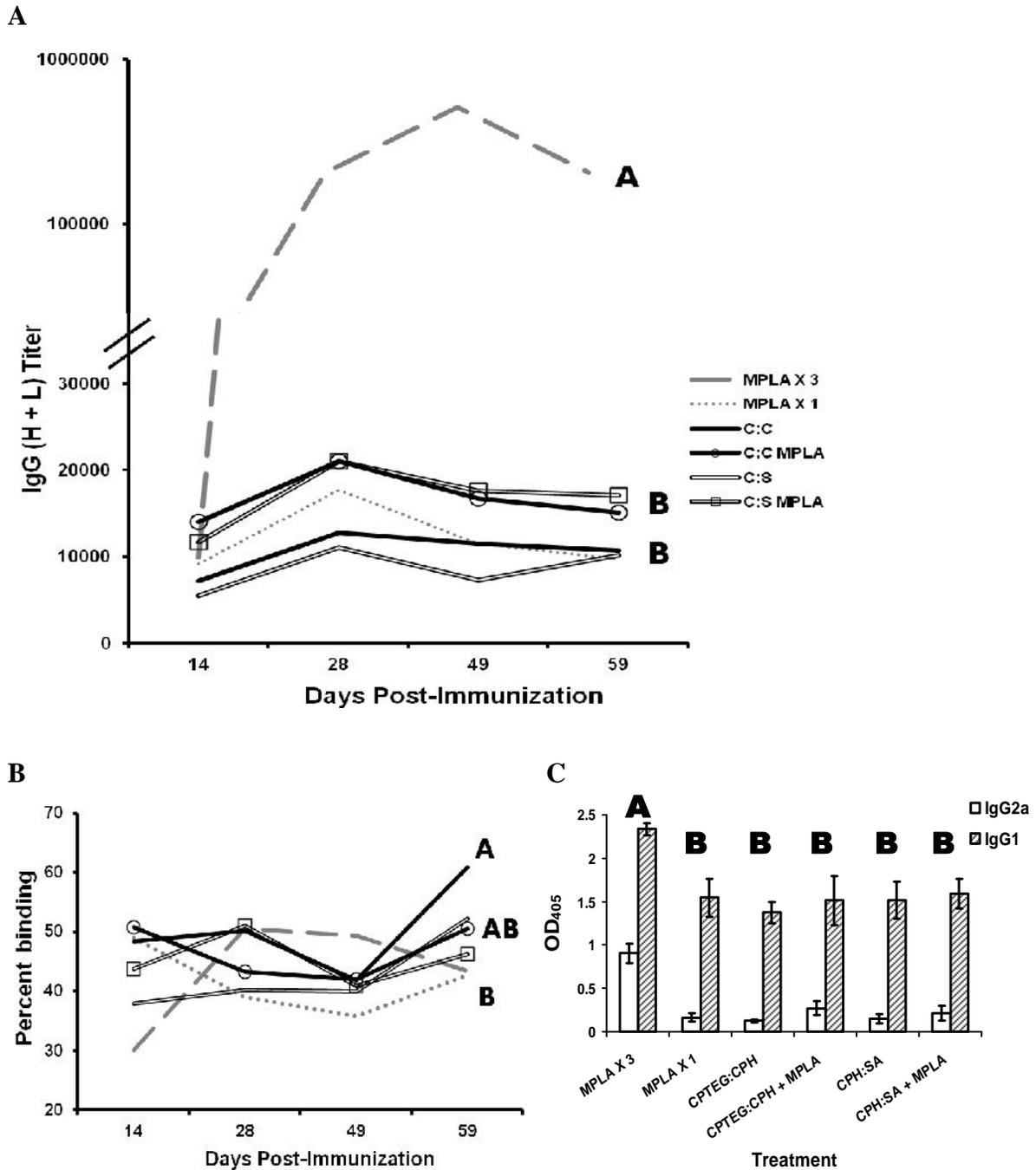
A

B

C

Figure 6. Assessment of serum IgG responses following subcutaneous immunization of CBA/N mice with PspA. A) Collected serum was serially diluted two-fold and assessed by ELISA. B) Anti-PspA antibody avidity measured post-immunization. The treatment identification is the same as in part A. C) Anti-PspA antibody isotype switching assessed by IgG1 and IgG2a amount in serum 59 days post-immunization. Full procedures are detailed in the Materials and Methods section. Statistics were calculated with SAS JMP using Student's t-test, $p \leq 0.05$. SC = subcutaneous immunization, PspA = pneumococcal surface protein A, MPLA = monophosphoryl lipid A, CPTEG:CPH = 50:50 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane: 1,6-bis(p-carboxyphenoxy)hexane, CPH:SA = 20:80 1,6-bis(p-carboxyphenoxy)hexane: sebacic acid.

25-33 % across all vaccinated groups while 50 % of the non-immunized mice survived (Figure 8).

Immunization Route Comparison

From the previously described bacterial challenges administer to PspA-immunized mice, protein delivered with MPLA in a three dose regimen showed better protection (e.g. survival) when administered intranasally compared to subcutaneous vaccination. The treatment produced similar isotype ratios in both routes though, with just a 2- to 3-fold difference in pre-challenge titer (Figure 7).
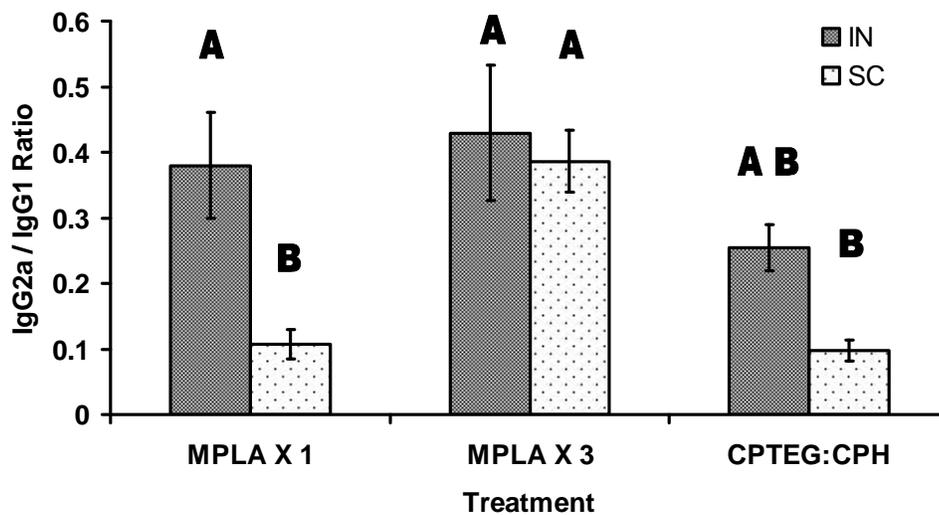

Figure 7. Comparison of the IgG2a:IgG1 ratios induced following intranasal or subcutaneous immunization with PspA. Pre-bacterial challenge serum samples from 66 days post-immunization (intranasally immunized) and 59 days post-immunization (subcutaneously immunized) were analyzed and IgG2a:IgG1 ratios compared. n = 8, except for MPLA X 3 where n = 6 (intranasally immunized), or n = 6 (subcutaneously immunized). Statistics were calculated with SAS JMP using Student's t-test, p ≤ 0.05. IN = intranasal immunization, SC = subcutaneous immunization, PspA = pneumococcal surface protein A, MPLA = monophosphoryl lipid A, CPTEG:CPH = 50:50 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane: 1,6-bis(p-carboxyphenoxy)hexane, CPH:SA = 20:80 1,6-bis(p-carboxyphenoxy)hexane: sebacic acid.
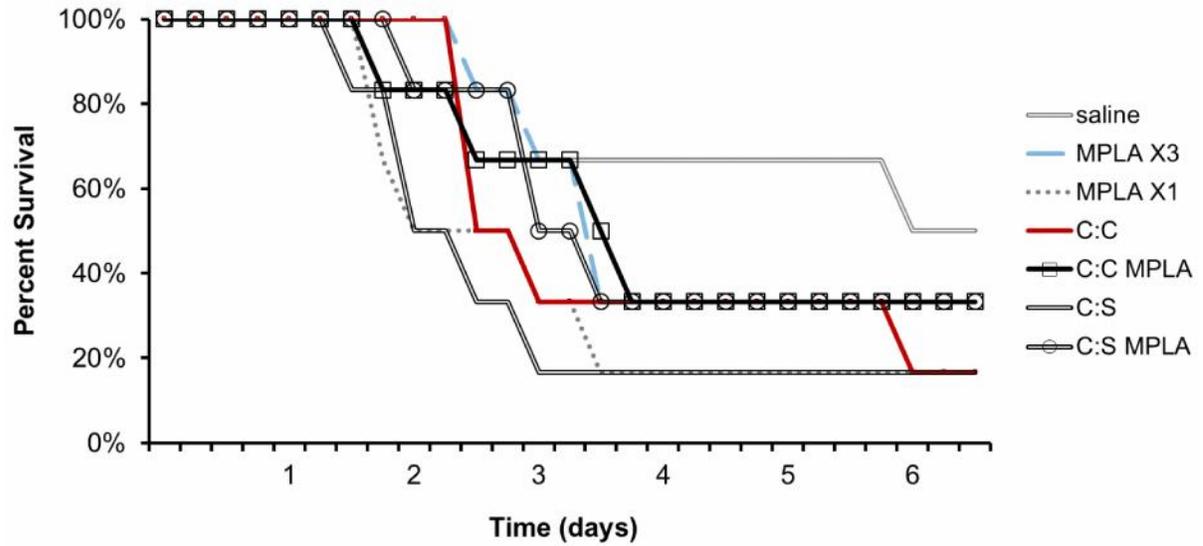
Figure 8. Survival of subcutaneously immunized CBA/N mice following challenge with *Streptococcus pneumoniae* A66.1. Intravenous challenge with 1.01 x 10^7 CFU of strain A66.1 in a 200 μL volume was administered 63 days post-initial immunization. The mice were monitored daily for clinical signs of infections as described in the Materials and Methods of the Appendix. n = 6. PspA = pneumococcal surface protein A, MPLA = monophosphoryl lipid A, CPTEG:CPH = 50:50 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane: 1,6-bis(p-carboxyphenoxy)hexane, CPH:SA = 20:80 1,6-bis(p-carboxyphenoxy)hexane: sebacic acid.

CHAPTER 4. DISCUSSION

Streptococcus pneumoniae is the primary cause of bacterial pneumonia worldwide with over 90 serotypes that have been identified (1, 11, 64). The capsule polysaccharide based 23-valent PPSV23 and 7-valent PCV vaccines have shown beneficial results due to worldwide use (59) but obstacles remain, especially in the limited number of serotypes targeted within the vaccines and the use of poorly immunogenic polysaccharide antigen to which the most at risk populations cannot mount good immune responses against without a multiple dose vaccine regimen (55, 70, 75). With the help of recombinant protein technology pneumococcal surface protein A has become a well-studied surface protein of *S. pneumoniae* and has shown protective immunity in adjuvanted multi-dose regimens in many mouse strains, inducing demonstrable antibody-mediated protection across multiple capsule serotypes characterized by IgG isotype switching (30, 34, 51, 53, 62). However, multiple-dose immunization schedules in humans pose the risk that doses will be missed due to poor patient compliance. To overcome this, nanotechnology offers the possibility to design efficacious, single dose vaccines against a variety of pathogens including *S. pneumoniae* by using polyanhydride nanoparticles, which can gradually release intact protein antigen over a period of weeks in mice (44, 66). Previous work from this laboratory has demonstrated that a 50:50 CPTEG:CPH vaccine with encapsulated F1-V antigen from *Yersinia pestis* was able to provide 100 % protection in mice 23 weeks after a single immunization (80); the protective immune response was characterized by a high titer and high avidity serum antibody that persisted for the length of the study. These findings provided the basis for the PspA-loaded polyanhydride nanoparticle vaccine formulation used in these studies.

Survival of Intranasally Immunized Mice to Lethal Bacterial Challenge

Based on the previous results obtained with F1-V antigen encapsulated into

nanoparticles (80), CBA/N mice were intranasally immunized with PspA-loaded 50:50

CPTEG:CPH nanoparticles or PspA adjuvanted with MPLA to test for the production of a

protective immune response. As shown in Figure 5, the mice immunized with three doses of

PspA adjuvanted with MPLA showed 83 % survival following an intravenous challenge with

S. pneumoniae A66.1. However, the percentage of mice surviving the challenge in all

remaining immunization groups was 12-25 % by day 14 post-infection. The protection

induced by the three doses of PspA adjuvanted with MPLA appeared to be mediated by a

combination of a high titer,   100,000 IgG (H+L), anti-PspA response (Figure 4) along with

a more balanced IgG2a:IgG1 antibody response (Figure 7) which has been demonstrated

previously (62). It is reasonable to hypothesize that the higher circulating antibody titer

present in the mice immunized three times would have opsonized more of the bacterial cells

injected into the bloodstream, thus, clearing a majority of the *S. pneumoniae* early in the

infection. The mice immunized with the nanoparticles, on the other hand, had 100 times

lower anti-PspA antibody titer and would not have been able to effectively opsonize as many

bacterial cells for clearance by phagocytes, thus, allowing *S. pneumoniae* to avoid

elimination resulting in tissue damage (based on gross anatomy observation at necropsy) and

death of the mice.

Based on the host responses in the mice immunized once with PspA adjuvanted with

MPLA, it is apparent that the MPLA + PspA vaccine administered once was not sufficient to

produce protective immunity against a *S. pneumoniae* challenge. In fact, the weak immune

response induced by a single immunization with MPLA + PspA proved to be detrimental to

survival, with all the mice succumbing to the bacterial infection by day 11 post-inoculation. Even with a IgG antibody titer 10 times higher than that induced by the nanoparticle regimen and the induction of both IgG1 and IgG2a anti-PspA responses, it proved not to be protective. The mice immunized with the 50:50 CPTEG:CPH nanoparticle regimen did receive a subcutaneous booster immunization two weeks prior to bacterial challenge, and, as seen in Figure 4 part C, this did boost the serological anti-PspA IgG1 response, while the single dose MPLA IgG1 levels were waning. This result may hint at the necessity of multiple immunizations for the mucosal route of vaccination in order for the protective immunity to be induced (20, 34, 53). Immunoglobulin G1 is a complement activating antibody, so higher levels of this antibody in the blood should help to kill extracellular bacteria more efficiently, but could also promote a more vigorous complement-induced inflammatory response. For high capsule producing serotypes of *S. pneumoniae* that can prevent efficient antibody binding to their surface (85), it is reasonable to hypothesize that the bacteria would also be able to protect their cell membrane from damage induced by the complement's membrane attack complex. If the levels of IgG2a, which binds to phagocytic cell Fc receptors to promote bacterial uptake (55) are low or unable to bind due to the capsule, then the complement activating IgG1 may promote more local tissue damage than protection.

       While the single dose, encapsulated-protein nanoparticle vaccine did not induce protection, the increase in the mean survival time suggests that an efficacious nanoparticle vaccine could be developed. In comparison to the intranasal immunization, previous work by this group utilizing several protein immunogens has demonstrated that more robust antibody titers are induced following subcutaneous administration; therefore, mice were immunized by the subcutaneous route to assess the ability to induce protective immunity.

Subcutaneously immunized CBA/N mice fail to produce a protective humoral immune response

It was an unanticipated outcome of these studies that CBA/N mice receiving the subcutaneous immunization failed to survive more than 4 days post-inoculation (Figure 8). Of the subcutaneously immunized mice, 66 - 82 % of the mice succumbed to the infection while 50 % of the non-vaccinated mice survived. The addition of MPLA to the nanoparticle regimen appeared to be advantageous with respect to the increase in total anti-PspA IgG titer, but this improvement in the titer failed to contribute to protection. The addition of MPLA appears to have detrimental effects with respect to the maintenance of the long-lived antibody response as evidenced by the more rapid decrease over time in the overall IgG titer in these mice (Figure 6, part A). What is most striking was how rapidly the immunized mice succumbed to the infection. This precipitous onset of death within two days post-inoculation suggests a possible immune-enhancement of pathogenicity of the bacteria, perhaps similar to that previously seen with syncital respiratory virus vaccines (31, 41). In the subcutaneoulsy immunized mice, there were also observations of neurological side-effects (e.g., hind-limb paralysis, inability to sit or stand upright, or maintain balance) in some of the mice, hinting at an increased potential for the systemic infection to spread to the meninges. These side-effects were most prominent in the mice receiving the single dose MPLA and 50:50 CPH:SA treatment groups, with 33% of the mice from each group displaying a neurological disorder within 2-3 days post-infection (bacterial challenge clinical signs of infection log observations).

Despite the more robust IgG (H+L) titers produced by all the treatment groups following the subcutaneous route of immunization, these titers proved more detrimental than

protective upon bacterial challenge. The IgG2a:IgG1 ratios of the single dose subcutaneous

vaccinations were equivalent to one another with a value of approximately 0.15 that signifies

a dominant IgG1 response. Of interest, this low ratio is nearly half of that induced by the

intranasal immunizations suggesting that an IgG1 skewed isotype response is detrimental

(31). Surprisingly, the IgG isotype values and overall ratio of IgG2a:IgG1 for the

subcutaneous, triple dose MPLA vaccination regimen were the same as those induced by the

protective intranasal immunization, yet, following subcutaneous administration, these

antibody characteristics were not associated with survival.

CHAPTER 5. CONCLUSIONS

Based on the experiments described herein, it is clear that the single dose, PspA-loaded polyanhydride nanoparticle vaccine used to immunize mice requires further optimization in order to induce protective immuity. While intranasal vaccination with the nanoparticle regimen was moderately successful with respect to inducing anti-PspA serum antibody, it was not capable of protecting mice from *S. pneumoniae* infection in comparison to the mice multiply immunized with PspA plus MPLA. Even with the great difference in antibody production between these two vaccine formulations, the similarities in the anti-PspA IgG responses between these two groups does show promise. The PspA booster immunizaton given to the nanoparticle treated mice may have serendipitously shown a need for more than one immunization by the intranasal vaccination route in order to induce an efficacious immune response. Although a single nanoparticle immunization of 50:50 CPTEG:CPH has been successful in protecting mice against pathogens such as *Y. pestis* (80), these recent results remind us of the need to tailor each vaccine for each different antigen because the nature of the protective immune response may be as disparate as the pathogens being studied.

The failure to induce protective immunity following subcutaneous immunization was unexpected, especially considering the higher IgG (H+L) titers induced by the MPLA X 3 immunization regimen was protective when administered intranasally. Because of the rapid onset of death in the immunized mice, it appears that the subcutaneous immunizations induced an immune-enhanced pathogenicity. The best known case of a vaccine inducing immune-enhanced pathogenicity was with a formalin-inactivated respiratory syncytial virus (RSV) in the 1960's, when immunized children showed greater disease development and

hospitalization after vaccination. Investigations as to the cause of this outcome suggested that immune complexes activating the complement cascade, in combination with a predominantly T_H2 T-cell response, promoted a high granulocytic cell infiltration and inflammatory activation in the lungs (31). A recent study using MPLA incorporated into virosomal RSV vaccine showed promise in promoting a more balanced IgG2a:IgG1 ratio and T_H1 skewed adaptive response to a two-dose intramuscular immunization regimen (41). The mucosal route of vaccination appears to promote a better protective immune response against *S. pneumoniae* infection compared to a parenteral route of immunization. This suggests that further studies are warranted to develop an efficacious vaccine regimen using the polyanhydride nanoparticle delivery platform.

The polyanhydride single dose pneumococcal vaccine formulation that was tested did not induce comparable immune responses relative to those induced by a more conventional, multiple dose vaccine regimen. While less than optimal, these results have provided invaluable insight as to how to tailor a vaccine to induce the desired immunity against an important respiratory pathogen such as *S. pneumoniae*. These results give hope for the possibility of developing a minimal dose pneumococcal vaccine for the future.

Future Directions

The single intranasal nanoparticle vaccination, followed by one protein only booster roughly 2 months post-immunization, provided valuable results suggesting that an antigen-loaded polyanhydride nanoparticle induced protective immunity against bacterial challenge is possible. Further experimentation with this prime/boost regimen may give insight as to the need for how many immunizations are required in order to produce effective mucosal

immunity against *S. pneumoniae*. Booster immunizations can be given at different time points after the initial immunization (at 2 and 4 weeks, or 3 and 6 weeks), given by different routes (intranasal or repeat the subcutaneous booster route), with or without additional adjuvants, and with different polyanhydride chemistry ratios or combinations. Additional experiments can also be performed to investigate the protein dose needed to promote higher anti-PspA antibody titers. If the quality (i.e., avidity) of the humoral response can be enhanced while maintaining a balanced IgG2a:IgG1 profile, there is a better likelihood of creating a successful nanoparticle-based intranasal vaccine.

When a protective protein-based pneumococcal vaccination regimen has been developed, experiments testing pneumococcal carriage in immunized and non-immunized mice can be conducted. With the potential risks of pneumonia and invasive pneumococcal disease from bacterial colonization, there are many published reports evaluating the carriage of *S. pneumoniae* in the respiratory tract and nasopharynx (16, 36, 40, 61, 83). If it can be confirmed that therapeutic vaccination can lower the carriage rate of the nasopharyngal-localized *S. pneumoniae*, that may provide proof-of-principle that the concept of herd immunity could be employed to reduce the public health threat associated with pneumococcal colonization.

Investigation into the induction of PspA-specific cell-mediated immunity is warranted, especially in light of the results of the apparent immune-enhanced pathogenesis observed following subcutaneous immunization. Polyanhydride nanoparticles have been shown to promote good cell-mediated immune responses (i.e., CD8$^+$ T cells), and the polyanhydride chemistries possess the potential to be modified to facilitate targeting of specific immune cell populations. These functionalized nanoparticles may then promote

better protective immunity against an extracellular, mucosally-associated pathogen like *S. pneumoniae* (22, 24).

APPENDIX: SYSTEMIC PNEUMOCOCCAL MOUSE INFECTION MODEL

Introduction

Inbred laboratory mice have been a popular animal model for investigating mammalian organ systems and human disease for decades. With their small size and rapid reproductive rate, mice can economically be housed and bred while allowing *in vivo* experiments to be conducted in a matter of months rather than years. With multiple mouse varieties having fully sequenced genomes the creation of knockout strains further enhances their usefulness in research by allowing investigations into how and why certain phenotypes or genotypes influence a response to a procedure, environment, or diet.

The C57BL/6 mouse strain is a popular and affordable mammalian model in small animal research. Being well characterized many knockout strains of this mouse variety exist including strains with immunological deficiencies, which creates the potential for in depth elucidations of the mechanisms associated with the host response to a vaccine. For these reasons, we tested the potential of the C57BL/6 mouse strain for use in a mouse model of pneumonia.

The CBA/N mouse strain contains a tyrosine kinase deficiency in B cells which affects normal B cell development and prevents the mice from effectively mounting antibody-mediated immune responses to T-cell independent antigens, in particular, to polysaccharide antigens (55, 65). This is important because the polysaccharide capsule produced by *Streptococcus pneumoniae,* which helps the bacterium to evade opsonization and killing by phagocytic cells within the mouse, cannot be effectively targeted in the

CBA/N mice. Without antibody to bind the polysaccharide capsule, it is difficult for immune cells (e.g., macrophages, neutrophils) to effectively phagocytose *S. pneumoniae*; additionally, deposition of proteins of the complement cascade on the bacterial surface, that would otherwise be detrimental to the cell membrane, are also impeded (71). The use of the CBA/N mouse strain for modeling pneumonia can be considered as analogous to young children and the elderly in humans, who are also poor responders to polysaccharide antigens produced by a variety of mucosal pathogens. The increased susceptibility of these age groups to encapsulated pathogens relates to either their underdeveloped or waning immune systems, respectively (6, 55).

To establish a bacterial challenge model female C57BL/6 and CBA/N mice were inoculated intravenously or intraperitoneally with infectious doses of *S. pneumoniae* strain A66.1 ranging from 10^2 to 10^7 colony forming units (CFUs) in 100 μL or 200 μL volumes. Previously published literature (16, 21, 25, 36, 39, 40, 61) varies widely on the number of CFUs administered for a systemic infection model due to factors such as pneumococcal strain, route of administration, and mouse strain; therefore, the experiments detailed below utilized a broad range of CFU doses in order to establish the bacterial challenge model. Once defined this systemic pneumococcal infection model can be used to evaluate the efficacy of an anti-pneumococcal vaccine.

Materials and Methods

Animals

Eight to nine week old female CBA/CaHN-*Btk^{xid}*/J mice and C57BL/6 were obtained from Jackson Laboratory (Bar Harbor, ME) or Harlan Laboratories (Haslett, MI), respectively, and were housed in specific pathogen-free conditions where bedding, cages, and feed were sterilized before use. All procedures performed were approved by the Iowa State University Institutional Animal Care and Use Committee.

Recombinant protein and Bacterial Stocks

All *S. pneumoniae* serotype strains used in these studies were generously provided by Dr. David Briles of the University of Alabama in Birmingham. *Streptococcus pneumoniae* was cultured on trypticase soy agar petri plates supplemented with 0.5 % yeast extract and 5 % sheep blood (blood agar) or in brain heart infusion (BHI) broth medium supplemented with 0.5% yeast extract at 37 $^{\circ}$C overnight.

Bacteriological medium and Taxo P-discs containing optochin, for *S. pneumoniae* verification, were purchased from BD Biosciences (Franklin Lake, NJ) and fresh whole sheep blood (with acid citrate dextrose to prevent clotting) was obtained from the USDA National Animal Disease Center facility (Ames, IA). Gentamicin sulfate salt used in blood agar plates, to select for Gram + bacteria like *S. pneumoniae*, for colony forming unit (CFU) determination was purchased from Sigma-Aldrich (St. Louis, MO).

Measurement of Bacterial Colony Forming Units (CFU)

Bacterial culture was grown overnight, and 1.0 mL of the overnight culture was used to inoculate 10 mL of fresh broth; this was performed in duplicate. An aliquot of culture was taken hourly, one aliquot was used to measure culture turbidity at OD_{600} and 100 µL added to a tube of 900 µL phosphate buffer saline (PBS, 0.05 M, pH 7.4, Mediatech, Inc., Manassas, VA) to create a 1:10 dilution. After measuring the culture turbidity, 100 µL of the primary dilution was serially diluted into subsequent tubes containing 900 µL PBS to a final dilution of 1×10^{-6}. For each dilution, 15 µL aliquots were spotted in triplicate onto separate section of the same blood agar plate; and all six dilution aliquots were replicated on blood agar plates with 4.0 µg/mL gentamicin (Sigma-Aldrich, St. Louis, MO). When the liquid had absorbed into the agar, the plates were incubated at 37 ^{o}C overnight. The *S. pneumoniae* colonies on both plates were counted, averaged, and CFU/mL calculated for samples collected at each time point and were then equated back to the corresponding OD_{600}. These results were graphed (Figure 9) and used to estimate the CFU/mL for cultures of *S. pneumoniae* used to challenge mice.

Bacterial Challenge

Streptococcus pneumoniae strain A66.1 was grown overnight on blood agar plates and 15 to 25 colonies were used to inoculate 10 mL of BHI broth medium. The cultures were incubated until an OD_{600} of 0.45-0.50 was reached. Using the graph in Figure 9, CFU/mL was calculated for the culture and an aliquot of the bacterial culture was diluted in PBS to create the inocula. One hundred microliters of each inoculum was serially diluted and plated
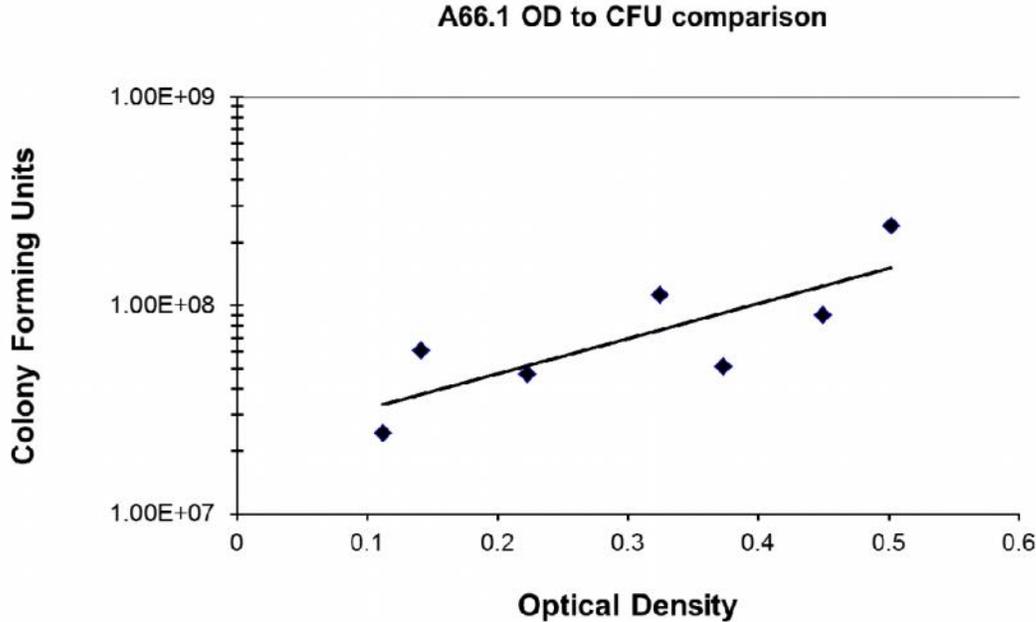
Figure 9. Standard curve establishment based on optical density and colony forming units. Seeded bacterial cultures of *Streptococcus pneumoniae* strain A66.1 were sampled every hour to read an OD_{600} and dilute a sample of culture 1:10, then serially dilute to 1.0×10^{-6}. These dilutions were plated on blood agar and incubated at $37^{o}C$ for 12 to 14 hours. The CFUs grown were counted, and CFU/mL of culture was calculated for each OD reading.

(described above) to get inoculum dose CFU counts. To prepare mice for challenge, they were placed under a heat lamp fitted with a 40 to 60 Watt bulb for three to five minutes to dilate the tail veins for intravenous administration. Female C57BL/6 were challenged intravenously with bacterial doses ranging from 244 to 3.5×10^6 CFU, and CBA/N mice were challenged intravenously with 222 to 1.0×10^6 CFU. All intravenous challenge inocula were administered in a 200 μL volume via the tail vein using a 26 gauge needle. The inocula given intraperitoneally to C57BL/6 mice ranged from 222 to 1.0×10^6 CFU and were administered in 100 μL volumes. Mice were observed three times per day for clinical signs of infection as described in Table 2. Body temperature was monitored once daily using a Fluke-566 infrared thermometer (Fluke Corporation, Everett, WA). Mice that became

moribund (Table 2) were euthanized by CO_2 asphyxiation and necropsied. After euthanasia, blood was collected via cardiac puncture, and the right lung and spleen were collected to measure bacterial burden.

Results and Discussion

Bacterial Challenge in C57BL/6 Mice

The *S. pneumoniae* strain A66.1 showed an expected increased rate of mortality with higher bacterial CFU inoculations in the C57BL/6 mice, but a challenge dose of 3.5 x 10^6 CFU was required to induce greater than 50 % mortality (Figure 10, part A). Based on these results, as well as the variability observed following intravenous inoculation of C57BL/6 mice with *S. pneumoniae* strain TIGR4 (data not shown), lethal CFU dose titration inoculations were also performed by challenging mice via the intraperitoneal route.

Challenging the C57BL/6 mice via the intraperitoneal route proved to induce 100 % lethality in all CFU doses within 48 hours of infection (Figure 10, part B). This data indicates that C57BL/6 mice are much more sensitive to an intraperitoneal challenge that they are to an intravenous challenge. Studies have shown that injection with fungal zymosan polysaccharide mixtures induce increased fluid permeability across peritoneal membranes and soluble bacterial components such as lipopolysaccharide from Gram-negative bacteria can reach the blood stream and promote amplified cytokine production (57) and greater systemic immune responses in C57BL/6 mice (47, 57). It can then be hypothesized that capsule producing bacteria, from the combination of its secreted polysaccharide capsule and various protein components, may induce a more vigorous and potentially tissue damaging inflammatory response in the mouse. This route of infection also poses a problem of reliably

Table 2. Clinical signs of infection scoring system for *Streptococcus pneumoniae* infection in mice. Post-inoculation mice were monitored three times per day for outward signs of infection. Mice were handled in a biosafety cabinet for daily temperature readings and responsiveness assessment when higher infection scores were given. Mice were removed from the study and euthanized by CO_2 asphyxiation and necropsied upon receiving a score of 5. Mice with fever readings received a "+" in addition to their recorded score for the specified time. Protocol was approved by the Iowa State University Institutional Animal Care and Use Committee

Clinical Signs of Infection Scoring System		
Score	Physical Signs	Assessment Method
1	Normal appearance and movement	Visual
2	Ruffled fur	Visual
3	Hunched back	Visual
4	Lethargic movement but responsive	Visual Response to new stimuli, e.g.: ▪ food on cage floor ▪ reorientation in cage
5	Moribund, unresponsive, unable to right itself	Visual Response to new stimuli, e.g.: ▪ food on cage floor ▪ reorientation in cage Body temperature $\leq 25^{o}C$
+	Fever	Infrared thermometer reading on back ▪ $+ 2^{o}C$ above pre-infection reading

producing such a low CFU dosage repeatedly over time, increasing the likelihood of unintentionally inducing overt and lethal inflammatory responses in the mice in future experiments.

Bacterial Challenge in CBA/N Mice

Due to the inconsistencies observed using C57BL/6 mice in intravenous and intraperitoneal bacterial challenges, the use of CBA/N mice was pursued for the remaining experiments. The CBA/N mice were inoculated intravenously by the tail vein and observed for 7 - 14 days post-infection (DPI). In this mouse strain, a predictable increase in mortality

was seen with increasing the CFU of the challenge dose (Figure 11), and the 1×10^6 CFU

dose was 100 % lethal within 5 DPI.   The time it took for mice to succumb verified that the

1×10^6 CFU was not overwhelming (which results in a quick death) but was enough of a
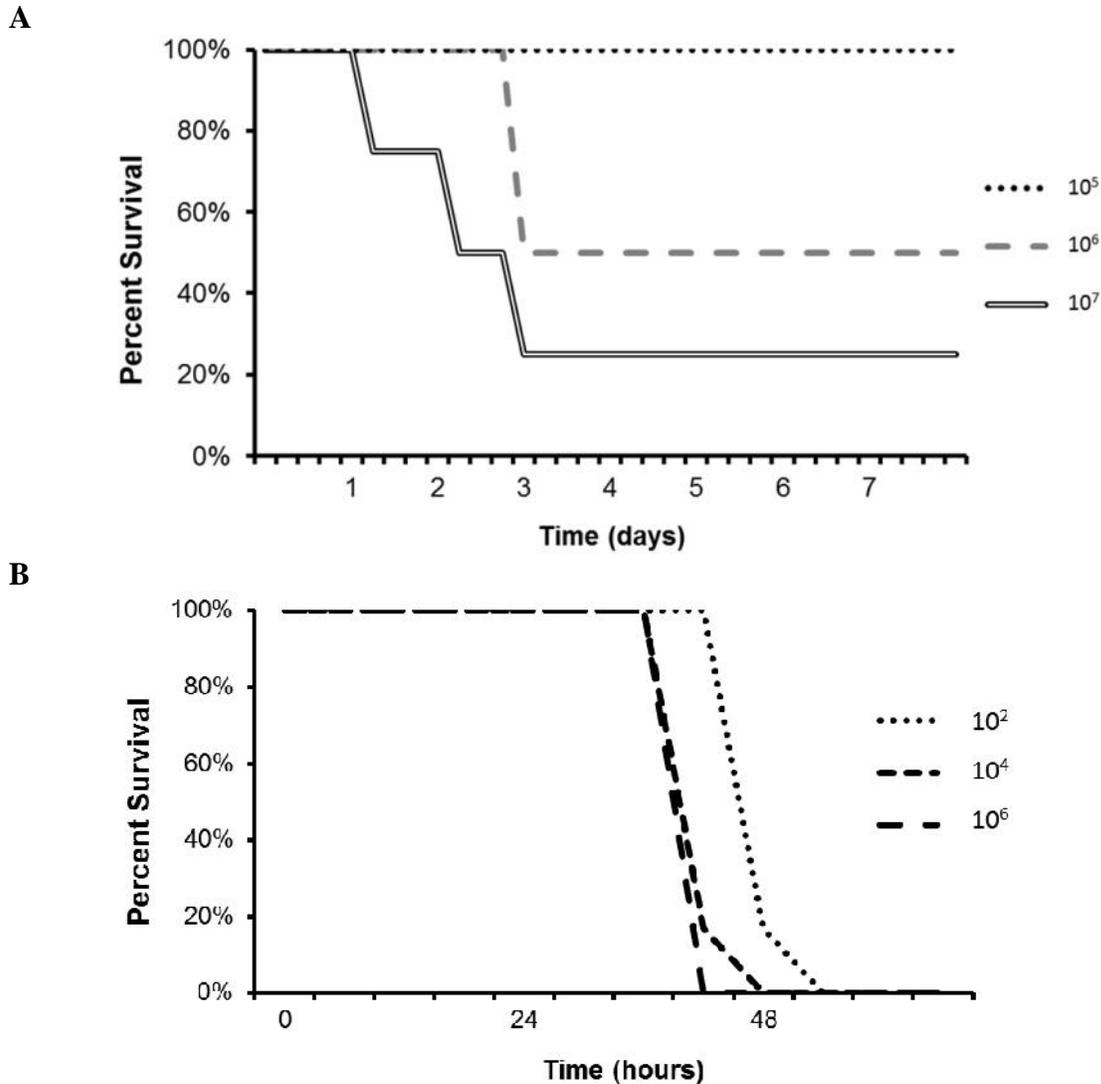
bacterial challenge to use for the infection model.

A

B

Figure 10. *Streptococcus pneumoniae* **strain A66.1 dose titration challenge in C57BL/6 mice.** Mice were inoculated with increasing doses of *S. pneumoniae* A66.1 by A) intravenous administration of 200 μL containing between 200 and 3.5×10^6 CFU into the tail vein, or B) intraperitoneal administration 100 μL containing between 200 and 1.0×10^6 CFU. Bacteria were cultivated overnight in BHI broth supplemented with 5% yeast extract to an OD_{600} of 0.45-0.50 and diluted in PBS. The mice were monitored daily for clinical signs of infection as described in the Appendix Materials and Methods.   Mouse numbers n = 8 per group for the intravenous challenge (A) and n = 6 per group for the intraperitoneal challenge (B).
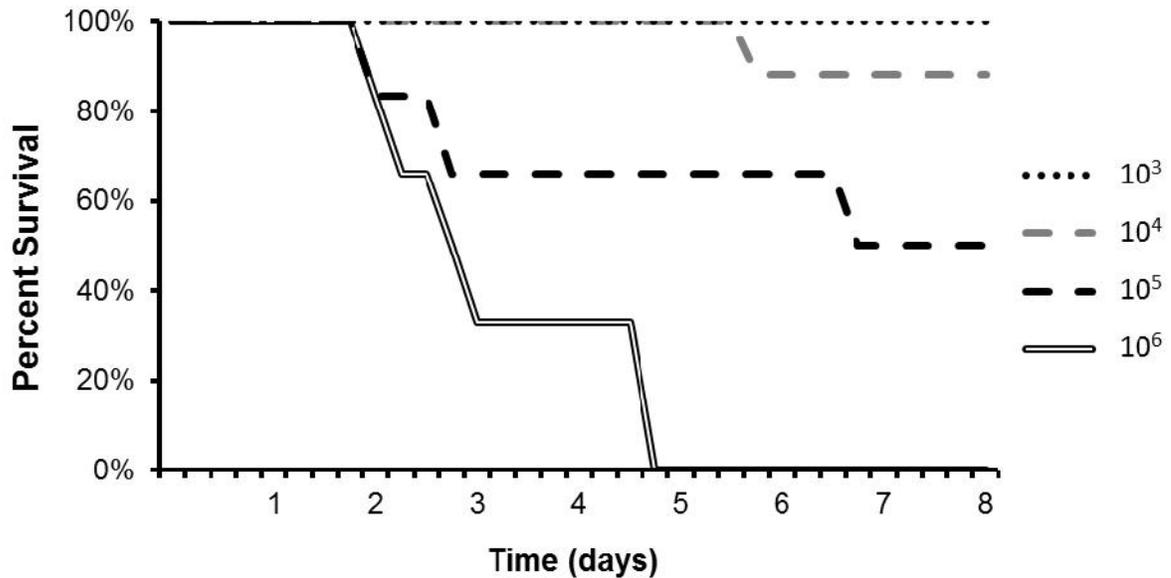
Figure 11. Intravenous challenge of CBA/N mice with *S. pneumoniae* strain A66.1 dose titration. Mice were inoculated with *S. pneumoniae* doses ranging from 200 CFU to 1.0 x 10^6 CFU. Bacteria used for the challenge experiments was cultivated as described in the Appendix Materials and Methods section. Briefly, *S. pneumoniae* A66.1 was incubated overnight in BHI broth supplemented with 5% yeast extract to an OD_{600} of approximately 0.45-0.50 and diluted in PBS. The mice were infected intravenously in a 200 μL volume into the tail vein. The mice were monitored daily for signs of infection and clinical disease. Mouse numbers n = 4 to 9 per group.

Conclusions

The results obtained following the intravenous infection of C57BL/6 mice with *S. pneumoniae* strain A66.1 were inconsistent with previously published reports (16, 17, 36, 84) in that the survival rate was high for mice challenged with large numbers of *S. pneumoniae* (e.g., > 1.0 x 10^6 CFU). Because of these inconsistencies, it was determined that a model employing an intravenous challenge of *S. pneumoniae* A66.1 in C57BL/6 mice would not be optimal to evaluate the efficacy of an anti-pneumococcal vaccine. In contrast, infecting C57BL/6 mice via the intraperitoneal route proved overly sensitive as evidenced by the fact that as little as 222 CFU of *S. pneumoniae* A66.1 proved overwhelmingly fatal within 48 hours of inoculation. Finally, these studies demonstrated that CBA/N mice provided a more

reproducible *S. pneumoniae* systemic challenge model and, therefore, these mice were

chosen for the vaccine studies described previously.

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