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# Analysis of the human variable gene repertoire in response to pneumococcal polysaccharides

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Analysis of the Human Variable Gene Repertoire in Response to Pneumococcal Polysaccharides

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## DEDICATION

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# INTRODUCTION

*Streptococcus pneumoniae* (*S. pneumoniae*) is a major human pathogen and leading cause of morbidity and mortality worldwide. It is a gram positive bacterium that grows in pairs or short chains (Jedrzejas, 2001). This bacterium is often enclosed in a polysaccharide (PS) capsule, which has long been recognized as a major virulence factor. Pneumococci are categorized into more than 90 serotypes based on differences in the structure of the capsular polysaccharides (Henrichsen, 1995). *Streptococcus pneumoniae* colonizes the nasopharynx and subsequent invasion causes otitis media, pneumonia, meningitis and bacteremia, and results in more than 40,000 deaths per year in the United States alone (Nuorti, 1997). Pneumonia has been the seventh leading cause of death for the past five years and age-adjusted death rates for this disease continue to climb (Kochanek and Smith, 2004). Pneumococcal disease is most prevalent at the extremes of age, i.e., children less than 2 years and elderly adults over the age of 65. Numerous antibiotics have been used for the treatment of pneumococcal disease; however, the emergence of antibiotic-resistant strains is on the rise. The frequency and severity of pneumococcal infections, combined with the high cost of treatment and emergence of drug-resistant strains, led to the development of pneumococcal vaccines.

The pneumococcal polysaccharide vaccine (PPV) is composed of purified capsular polysaccharide antigens of 23 serotypes of *S. pneumoniae* which are responsible for 88% of bacteremic pneumococcal disease (Nuorti, 1997). This vaccine is based on the observation that antibodies directed at the polysaccharide capsule initiate complement-dependent opsonophagocytosis as a means to protect against disease. The

PPV acts as a T cell independent (TI) antigen, and therefore, was previously assumed to elicit a *de novo* antibody response. However, several studies have demonstrated that pneumococcal polysaccharide (PPS)-specific antibodies isolated 5-10 days post-vaccination have undergone significant somatic mutation, suggesting that immunological memory is induced by previous exposure to *S. pneumoniae* or cross-reactive antigen (Baxendale, 2000; Zhou et al., 2002, 2004). These extensive mutations, occurring one week post-vaccination, are indicative of a recall response in which memory B cell clones become activated and expand, suggesting the involvement of T cell help. In addition, several studies have demonstrated an important role for IgM memory cells and natural antibody produced by B1 cells in the response to *S. pneumoniae* (Haas et al., 2005; Mi et al., 2000). Further investigation of the mechanisms involved in the host response to polysaccharide antigens would be of value.

The 23-valent pneumococcal polysaccharide vaccine is highly effective in young adults (Rubins, 1998); however, it fails to protect children less than 2 years of age likely due to its TI-2 nature. The PPV is recommended for elderly adults and other high-risk populations yet recent meta-analyses on the vaccine's efficacy have concluded that its protective capacity in older populations is markedly reduced (Cornu et al., 2001; Fine et al., 1994; Moore et al., 2000). While polysaccharide-specific antibody concentrations in elderly are similar to those of young adults, antibody functional activity is decreased considerably (Romero-Steiner et al., 1999). The exact mechanisms for this decline in functional activity have yet to be elucidated.

The poor efficacy of the PPV in infants and children has led to the development of pneumococcal conjugate vaccines. The 7-valent pneumococcal conjugate vaccine (CV) consists of purified capsular polysaccharide from 7 serotypes which account for approximately 80% of invasive pneumococcal disease in children (Darkes and Plosker, 2002). These polysaccharide antigens are individually conjugated to the carrier protein, diphtheria toxoid $CRM_{197}$. The CV elicits a T cell-dependent (TD) response and has proven safe and immunogenic in children less than 5 years of age (Black et al., 2000b). Despite its TD nature, administration of the CV to elderly adults offers no increased immunogenicity over the PPV (Powers et al., 1996; Shelly, 1997).

With multiple vaccines offering varying degrees of protection, it is necessary to standardize the methods used to assess vaccine efficacy. In the past, vaccine evaluation has been limited to serological assessment, with anti-pneumococcal polysaccharide (PPS) antibody concentrations serving as the main surrogate marker of protection. More recent studies have demonstrated that antibody avidity, not concentration, is a more reliable predictor of functional antibody activity (Sun et al., 2001; Usinger and Lucas, 1999). Furthermore, variation in antibody avidity in response to polysaccharide antigens has been correlated with differences in antibody molecular structure (Lucas et al., 1998; Nicoletti et al., 1991). Thus, understanding the molecular diversity of PPS-specific antibodies has become increasingly important in predicting the human immune response against pneumococcal antigens.

Several studies have been performed to characterize the immune response to polysaccharide antigens on a molecular level. Previous data have established that the

anti-PS antibody variable gene repertoire is limited (Abadi et al., 1998; Baxendale, 2000; Lucas et al., 1994, 1998). In addition, studies have shown that the PS-specific gene repertoire is antigen and serotype-specific and undergoes significant changes with increasing age (Kolibab et al., 2005a; Nicoletti et al., 1991; Smithson et al., 2005). The underlying cause(s) of altered V gene expression in the aged, however, remains to be elucidated. Numerous factors may influence the expressed B cell repertoire in response to pneumococcal vaccination, including pre-existing antibody, T cells, accessory cells and cytokine environment (Aydar et al., 2004; Baxendale, 2000; Chelvarajan et al., 2005; McNerlan et al., 2002; Sandmand et al., 2002; Saurwein-Teissl et al., 2002; Zhou et al., 2004).

The experiments presented here were performed to increase our understanding of the human immune response to PPS by analyzing variable gene usage pre- and post-vaccination and factors which may influence it. The hypotheses of these studies are as follows:

1. Elderly B cells can be stimulated to express a gene repertoire resembling that of young B cells in the presence of a similar cytokine environment.

2. The nature of the immunizing agent (TI versus TD) is capable of influencing variable gene usage of B cells stimulated in identical environments.

3. Pre-existing memory cells contribute to the PPS-specific antibody response post-vaccination.

# LITERATURE

## *History*

*Streptococcus pneumoniae* has been the subject of extensive study since its initial isolation over a century ago. The organism was first reported in 1881 by two microbiologists, Louis Pasteur (Pasteur, 1881) and George Sternberg (Sternberg, 1881). Both researchers independently isolated pairs of elongated coccoid bacteria in human saliva which they then used to inoculate uninfected rabbits. Pasteur and Sternberg both subsequently identified the same organism in the blood of the infected rabbits. By 1886 the organism was identified as Pneumococcus, due to its ability to cause pulmonary disease (Fraenkel, 1886). Its causative role in pneumonia was well-established by the 1880's and evidence of its involvement in otitis media and meningitis soon followed (Watson et al., 1993).

Agglutination experiments by two French researchers, Benzançon and Griffon, led to the discovery that different strains of pneumococci existed and by 1910, the need for serum treatment for all types of the organism had been established by German researchers Neufeld and Handel (White, 1938). Together, Neufeld and Handel conducted mouse protection experiments using highly potent sera from immunized rabbits, donkeys and horses (Henrichsen, 1999) and within the same year had established protocols for type-specific serum therapy. Within the early 20$^{th}$ century, researchers continued to perfect serum therapy treatments and by the late 1930's numerous studies had demonstrated that serum derived from rabbits served as a more effective treatment compared to serum derived from horses (Henrichsen, 1999). This rabbit, type-specific

serum therapy was first used to treat pneumococcal pneumonia in Denmark in 1937; however, the development of other anti-pneumococcal treatments eventually resulted in its discontinued use.

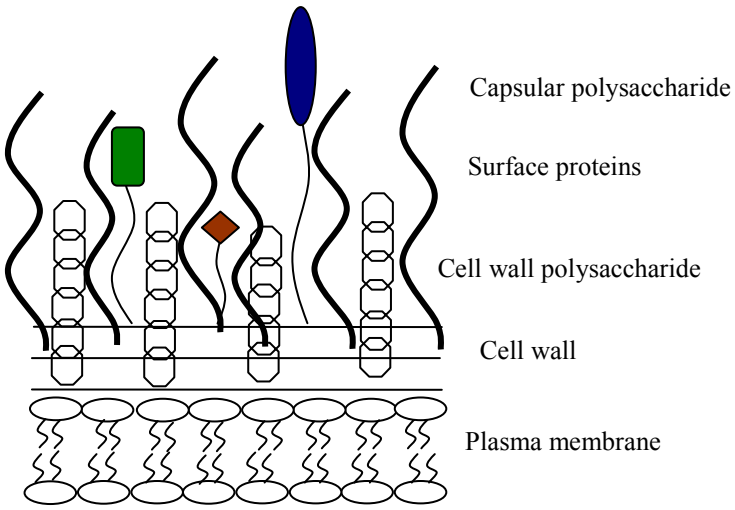
The use of optochin and sulfonamides to treat pneumococcal infections proved somewhat successful initially, however, by the mid-1900's resistance to such antimicrobial agents was increasing (Austrian, 2004). The discovery of penicillin as an antimicrobial drug in 1945 led to its use as the preferred method of treatment for *S. pneumoniae*-induced illnesses. By the 1970's, numerous antibiotic strains had surfaced, and the need for an effective pneumococcal vaccine was at its greatest (Kazanjian, 2004). This led to the development of the first, 14-valent polysaccharide vaccine in 1977 and 23-valent polysaccharide vaccine in 1983; followed by the 7-valent conjugate vaccine in 2000.

*Structure and Virulence Factors*

*Streptococcus pneumoniae* is a gram-positive, non-motile, non-spore forming bacterium. This organism grows in pairs or short chains and generally requires complex culture media for growth. Under a microscope, it is usually lancet shaped and appears as a 0.5-1.25 μm diplococcus (Jedrzejas, 2001). The three major surface layers are plasma membrane, cell wall and capsule, illustrated in Figure 1. The cell wall consists of three layers of peptidoglycan that acts to anchor the capsule, cell wall polysaccharides (CPS) and surface proteins. The capsule is the densest layer and is made of units of repeating oligosaccharides. Ninety serotypes of *S. pneumoniae* have been defined, based on the

structure of the capsular polysaccharides (Henrichsen, 1995). In contrast, cell wall polysaccharide is common to all serotypes.

Figure 1. Surface Structure of *S. pneumoniae*.




Cell wall polysaccharide has been shown to induce inflammation, however antibodies specific for CPS generally do not elicit the same protection as those directed at the capsular polysaccharides. While not all strains of *S. pneumoniae* are encapsulated, those that do have a capsule are considerably more virulent (Watson and Musher, 1990). The capsule is considered the main virulence factor of the bacteria due to its ability to block the deposition of C3b, and therefore prevent complement mediated opsonophagocytosis (Yother, 2004).

Other important virulence factors include choline binding proteins, pneumolysin and autolysins, pneumococcal surface antigen A and hyaluronate lyase. The main

virulence factors of *S. pneumoniae* and their mechanisms of action are summarized in Table I.

Table I. Important Virulence Factors for *S. pneumoniae* and Their Modes of Action.

| **Virulence factor** | **Mode of action** |
|---|---|
| Capsule (PPS) | Prevents complement mediated opsonophagocytosis |
| Cell wall | Induces inflammation<br>Attachment to epithelial cells |
| Choline Binding Protein A (CpBA) | Adherence to host tissues |
| Pneumococcal surface protein A (PspA) | Prevents complement mediated opsonophagocytosis |
| Pneumococcal surface antigen A (PsaA) | Metal binding protein aids bacterial growth |
| Pneumolysin (Ply) | Slows ciliary bacterial clearance<br>Inhibits phagocyte function,<br>Inhibits lymphocyte production |
| Autolysin | Degrades bacterial cell wall to release pneumolysin |
| Hyaluronate lyase (Hyl) | Facilitates tissue invasion |

Choline binding proteins are distinctive in that they are attached to the surface of the bacteria via a unique choline binding motif (Jedrzejas, 2001). This binding segment consists of approximately 10 repeating regions of 20 amino acids and acts as the anchoring module for a variety of proteins such as choline binding protein A (CbpA),

pneumococcal surface protein A (PspA) and N-acetylmuramoyl-L-alanine amidase (LytA amidase) (Jedrzejas, 2001). Choline binding protein A is a major surface-exposed protein with a mass of 75 kDa. Its main function is to adhere to host tissues during colonization; however, other functions may include blocking the cell surface from interaction with host immune cells (Jedrzejas, 2001). The structure of CbpA is closely related to that of PspA, which has been examined more extensively. PspA is an elongated surface protein which has been the subject of recent studies as a possible protein vaccine candidate (Briles, 2004, 2000b). It ranges between 67 to 99 kDa in size and is found on the surface of all clinically relevant strains of *S. pneumoniae* (Crain et al., 1990). Its main function is to protect pneumococci from complement by emitting an electrostatic charge to prohibit binding of the complement C3 molecule and thereby preventing opsonophagocytosis (Cundell et al., 1995; Jedrzejas et al., 2000).

Pneumococcal surface antigen A (PsaA) was once thought to contribute to the virulence of *S. pneumoniae* due to adhesion properties (Berry and Paton, 1996), however, more recent investigations have concluded it works through other mechanisms. This protein has a unique lipid component which acts to anchor it to the cell membrane. This secure linkage allows PsaA to take on a variety of shapes and aids in its role as a metal binding protein. This protein transports $Mn^{2+}$ and $Zn^{2+}$ into the cytoplasm of the bacteria to support its growth (Dintilhac et al., 1997) and consequently contributes to its virulence.

Another important surface protein is hyaluronate lyase (Hyl) which likely plays an important role in the virulence of *S. pneumoniae* and could be a potential vaccine candidate. Hyaluronate lyase has a molecular weight of 107 kDa and is expressed by

most strains of the bacteria. It uses an enzyme mediated method of tissue invasion to break down the extracellular matrix of host cells (Jedrzejas, 2001). This allows for increased tissue permeability as well as the host's increased susceptibility to pneumococcal disease.

Not all *S. pneumoniae* virulence factors are surface exposed. Pneumolysin (Ply) is a 53 kDa cytoplasmic enzyme with a variety of functions which contribute to the bacteria's pathogenesis (Jedrzejas, 2001). Pneumolysin acts to slow ciliary beating and therefore hinders the clearance of mucus produced by the host's cilia. Other functions include the disruption of alveolar epithelial cells which lead to hemorrhaging associated with pneumonia, as well as the inhibition of the production of lymphocytes and immunoglobulins and subsequent suppression of the host's immune response (Jedrzejas, 2001). Pneumolysin's ability to hinder the clearance of mucus, disrupt bronchial epithelial cells and inhibit the host's immune response render this enzyme essential to the bacteria's virulence. However, Ply is only released following its activation by autolysins. Autolysins are located in the cell envelope and act as cell wall degrading enzymes (Tomasz, 1981). These proteins contribute to the bacteria's virulence by altering the cell wall to release compounds capable of inducing inflammation as well as other virulence factors such as pneumolysin (Jedrzejas, 2001). Collectively, these numerous and unique virulence factors are what make *S. pneumoniae* a significant pathogen in immune competent and compromised hosts.

*Epidemiology*

*Streptococcus pneumoniae* was first reported as causing disease of epidemic proportions during the late 1800's when it was isolated from the lungs of gold miners following their death from a respiratory illness (Kazanjian, 2004). Since then, *S. pneumoniae* has been identified as a serious human pathogen worldwide and the causative agent for pneumonia, meningitis, otitis media and bacteremia (Watson et al., 1993). Pneumococcal disease is the seventh leading cause of death in the United States and accounts for an estimated 40,000 deaths annually (Kochanek and Smith, 2004; Nuorti, 1997). Each year, *S. pneumoniae* is responsible for approximately 7 million cases of otitis media, 500,000 cases of pneumonia, 50,000 cases of bacteremia and 3,000 cases of meningitis (Nuorti, 1997). The pneumococcus is the most frequent cause of community acquired pneumonia and the second leading cause of bacterial meningitis and otitis media following *Neisseria meningitidis* and *Haemophilus influenzae* type b, respectively (AlonsoDeVelasco et al., 1995).

Pneumococcal disease is most prevalent at the extremes of age, children less than 2 years old and elderly adults over 65 years old and selected populations including African Americans, Native Americans and immunocompromised hosts. A recent study at the Centers for Disease Control and Prevention determined the overall incidence of *S. pneumoniae*-caused invasive pneumococcal disease to be 23.2 cases per 100,000 persons, or 62,840 cases in the United States (Robinson et al., 2001). Overall, incidence was highest among children less than 2 years of age and elderly adults over the age of 65 with approximately 167 and 60 cases per 100,000 persons, respectively. In addition, the

incidence of disease among African Americans was 2.6 times higher than that of Caucasians (Robinson et al., 2001).

The frequency of pneumococcal disease in children has declined significantly in recent years, largely due to the advent of pneumococcal conjugate vaccines. However, its incidence in elderly populations has remained significant; pneumonia ranks as the fourth leading cause of death in this age group (2006). *Streptococcus pneumoniae* is the most frequent cause of pneumonia in elderly populations and persons over 65 are 3 to 5 times more likely than young adults to die from such infections (2006). The rate of pneumococcal pneumonia increases among elderly adults living in long-term care facilities, and can often lead to other complications such as bacteremia and meningitis.

Immunocompromised patients face significant risk of developing pneumococcal disease. Patients with asplenia, such as those with sickle cell disease or splenectomy, are at the greatest risk for pneumococcal infection (Nuorti, 1997). *Streptococcus pneumoniae* accounts for over 50% of all bacteremia cases in asplenic individuals (Wara, 1981). Other types of immunocompromised persons include those with a genetic immunodeficiency, HIV, leukemia, lymphoma, etc. Such conditions often lead to a decrease in serum antibody response which in turn results in serious and sometimes fatal pneumococcal infection. For example, in HIV-positive persons, the frequency of *S. pneumoniae*-caused bacterial pneumonia can reach up to 25 times of that of uninfected persons (Feikin et al., 2004).

*Host Response*

In order to determine the best methods for combating pneumococcal disease, it is important to first understand the mechanisms involved in host-pathogen interactions. Natural exposure to *S. pneumoniae* occurs via colonization of the human nasopharynx or oropharynx. Pneumococcal colonization, or carriage, frequently occurs early in childhood (Faden et al., 1997) although it often does not result in invasive disease (Garcia-Rodriguez and Fresnadillo Martinez, 2002). One study demonstrated that as many as 54% of children are colonized with *S. pneumoniae* by their first birthday (Faden et al., 1997). Frequency of colonization varies; however, children are often carriers for a longer duration than adults. Pneumococci are often identified as part of the local flora of the respiratory tract, and can be isolated from 5-70% of healthy adults (Crook et al., 2004). A number of factors can play a role in colonization, including geographical location, housing, family size, access to health care, personal hygiene and day care contact (Garcia-Rodriguez and Fresnadillo Martinez, 2002). Colonization varies widely by serotype and multiple serotypes may colonize one individual at different times (Bogaert et al., 2004a). Although persons colonized with *S. pneumoniae* are often asymptomatic, the organism can evade the host's defenses and subsequently initiate respiratory or systemic disease (Bogaert et al., 2004a).

Asymptomatic colonization involves the bacteria's adherence to non-inflamed epithelial layers of the respiratory tract, via pneumococcal surface adhesins mentioned previously. Colonization has been shown to elicit both innate and humoral immunity. Studies have demonstrated that pneumococcal colonization is sufficient to elicit mucosal

IgA and serum IgG antibodies specific for PspA, PsaA and pneumolysin in children (Rapola et al., 2000; Simell et al., 2001). Despite these findings, such antibody responses have not been adequately proven to be protective. More recent studies have concluded that immunity to pneumococcal colonization may be induced in the absence of antibody and instead requires the presence of CD4+ T cells (Malley et al., 2005; van Rossum et al., 2005).

Colonization is the first step in all types of pneumococcal infection and its invasion of the host's defenses leads to serious disease. Humans can employ both innate and adaptive immune responses to eliminate pathogens from their system. If the bacteria are able to evade the host's first line of defense, i.e., the mucosa and epithelial layers, then alternative mechanisms are needed to protect against disease. Like many microorganisms, *S. pneumoniae* has highly conserved pathogen-associated molecular patterns, or PAMPs, which allow for its identification by the innate immune system (Majcherczyk and Moreillon, 2004). These PAMPs are recognized by pattern recognition receptors or Toll-like receptors (TLRs) which in turn activate a signaling cascade used to activate the pro-inflammatory transcription factor, NF-kappaB. Gram positive bacteria such as *S. pneumoniae* are often recognized specifically by TLR2, via the adapter protein, MyD88 (Khan et al., 2005). However, recent evidence indicates that other TLR pathways likely play a role in host defense (Khan et al., 2005). The TLR-signaling cascade results in the activation of dendritic cells and phagocytes used to ingest bacteria. Following TLR activation, dendritic cells migrate to the lymphoid tissue, where

they act as antigen presenting cells (APC) to T cells and B cells, thereby contributing to the initiation of an adaptive immune response (Majcherczyk and Moreillon, 2004).

While innate immunity is activated immediately upon antigen recognition, an adaptive immune response occurs when the innate defenses are not sufficient to clear an infection. An adaptive immune response is one in which antigen-specific lymphocytes are used to eliminate pathogens and to induce immunological memory. Antigen presenting cells such as dendritic cells, macrophages and B cells are key players in adaptive immunity. These cells encounter antigen in the tissues or blood and can then present bacterial peptides on their surface via major histocompatibility complex II (MHC II) molecules. Such antigen presentation often takes place in the lymphoid tissues, where the presented peptides are recognized by $CD4^+$ T cells. The $CD4^+$ T cells then proliferate and differentiate into either T helper cells type 1 ($T_H1$) or type 2 ($T_H2$). Whereas the main effector function of $T_H1$ cells is to carry out cell mediated immunity via the activation of macrophages, $T_H2$ cells are predominantly responsible for the activation of B cells and subsequent antibody production (Curtis, 2005). This T cell involvement in the production of antibody is what classifies antigens as being T cell dependent (TD) in nature (Table II). The TD antigens were originally classified as such due to their inability to induce antibody responses in animal models lacking T cells. In addition, TD antigens induce immunological memory, affinity maturation and immunoglobulin class switching (Laman and Claassen, 1996).

In contrast, T cell independent (TI) antigens induce antibody production in the absence of T cells and do not stimulate immunological memory (Lesinski and Westerink,

2001). These TI antigens are capable of stimulating antibody responses in athymic individuals (Laman and Claassen, 1996). T cell independent antigens are further divided into TI antigens type 1 (TI-1) and type 2 (TI-2) (Table II). The TI-1 antigens are B cell mitogens, which, at high concentrations, are capable of polyclonal activation of B cells. The TI-2 antigens, however, consist of repeating epitopes and only activate mature B cells. Capsular polysaccharides on the surface of bacteria such as *S. pneumoniae* are TI-2 antigens which activate B cells by extensively cross-linking their receptors, resulting in the production of polysaccharide-specific antibodies (Lesinski and Westerink, 2001).

Table II. Properties of T Cell Dependent and T Cell Independent Antigens

| | TD antigens | TI-1 antigens | TI-2 antigens |
|---|---|---|---|
| Antibody production in athymic individuals | No | Yes | Yes |
| Antibody response in absence of all T cells | No | Yes | No |
| Primes T cells | Yes | No | No |
| Polyclonal activation of B cells | No | Yes | No |
| Requires repeating epitopes | No | No | Yes |
| Antibody response in infants | Yes | Yes | No |
| Examples | Diphtheria toxin | Lipopolysaccharide | Pneumococcal PS |

TI-2 antigens are not immunogenic in all individuals. The immune response to TI-2 antigens is age-dependent and children < 2 years of age do not respond to these antigens. In addition, individuals who are asplenic or otherwise immunocompromised, as well as

elderly adults over 65, generally do not respond well to TI-2 antigens (Lesinski and Westerink, 2001). The capsular polysaccharide of *S. pneumoniae* is often regarded as the organisms' primary immunogen and is traditionally classified as a TI-2 antigen. The activation of B cells by TI antigens predominantly occurs in the marginal zone (MZ) of the spleen (Carsetti et al., 2004; Kruetzmann et al., 2003; Martin et al., 2001). A study by Kruetzmann et al. (2003) was conducted to define which B cell subsets are responsible for protection against *S. pneumoniae* infection. The authors concluded that IgM memory B cells are required for protection against *S. pneumoniae* and require the spleen for their generation and/or survival (Kruetzmann et al., 2003). These IgM memory B cells produce natural antibody and are similar in function to B-1a B cells. Murine studies have shown B-1a B cells also depend on the spleen (Wardemann et al., 2002) and rapidly produce natural antibody in response to encapsulated bacteria (Forster and Rajewsky, 1987). In addition, previous studies have shown that B-1a and B-1b B cell play key roles in innate and adaptive immunity to *S. pneumoniae* (Briles et al., 1981; Haas et al., 2005) and further analysis of the contribution of all B cells subsets will be beneficial.

Due to the classification of polysaccharide antigens as T-cell independent, it was assumed that the PPV elicits a *de novo* antibody response. However, several studies have demonstrated that polysaccharide-specific antibodies isolated 5-10 days post-vaccination have undergone significant somatic mutation, suggesting that immunological memory is induced by previous exposure to *S. pneumoniae* or cross-reactive antigen (Baxendale, 2000; Zhou et al., 2002, 2004). These extensive mutations, occurring one week post-vaccination, indicate a recall response in which memory B cell clones become activated

and expand. Furthermore, recent evidence suggests a key role for T cell mediated immunity in the clearance of *S. pneumoniae*. A study by Malley et al. (2005) demonstrated that clearance of pneumococcal colonization does not require antibody, and protection from subsequent challenge requires the help of CD4+ T cells. In addition, a study by Jeurissen et al. (2004) reports that the antibody response to capsular polysaccharides is largely T cell dependent, and requires the involvement of the CD40-CD40 ligand interaction. Nevertheless, this concept remains controversial (Snapper, 2004) and the mechanisms involved in the host response to polysaccharide antigens are still under investigation.

*Treatment of Pneumococcal Disease*

Various anti-pneumococcal treatments have been developed over the years, beginning with the serotherapy developed in the early 1900's. During this time, the first chemotherapy treatments were introduced. Sulfonamides were among the first anti-pneumococcal drugs used, however, resistance to these antimicrobial agents quickly evolved (Austrian, 2004). Penicillin treatment in humans begun in the 1940's and proved highly effective against pneumococcal infections. Penicillin is a β-lactam antibiotic which is widely used to treat bacterial infections caused by gram-positive organisms. These antibiotics work by inhibiting peptidoglycan formation in the cell wall and causing cell death during bacterial cell division (Popham and Young, 2003). Use of penicillin reduced the case fatality rate of pneumococcal pneumonia to 5-8% (Austrian, 2004) and thus it served as the main anti-pneumococcal treatment for the next 3 decades. Resistance to penicillin led to the development of a number of derivatives, including

ampicillin and amoxicillin (Paradisi et al., 2001). Despite the advent of numerous antibiotics, bacterial resistance continued to rise. Various strains of *S. pneumoniae* began to develop mutations in their penicillin binding proteins allowing them to circumvent penicillin-based therapy (Austrian, 2004). By the 1960's, pneumococcal research had shifted from treatment to prevention and focused more on the development of effective pneumococcal vaccines (Austrian, 2004).

*Pneumococcal Polysaccharide Vaccines*

The first pneumococcal polysaccharide vaccine proven safe and effective was developed in 1944 by Colin MacLeod and colleagues (MacLeod et al., 1945). This vaccine consisted of purified capsular polysaccharides from four serotypes of *S. pneumoniae* (1, 2, 5, and 7) based on the prevalent strains from the previous winter's outbreak (Kazanjian, 2004). Polysaccharide vaccines were designed based on the observation that antibodies directed at the capsule initiate phagocytosis and protect against disease (Musher et al., 1990a). This vaccine was 90% effective in preventing pneumonia caused by the four included serotypes and no serious side effects were observed (Kazanjian, 2004). However, the popularity of penicillin as an anti-microbial drug overshadowed vaccine design until the development of a 14-valent pneumococcal polysaccharide vaccine in the 1970's. This vaccine was developed by Robert Austrian, a physician and long-time advocate of pneumococcal vaccine development (Kazanjian, 2004). This vaccine proved to be nearly 80% effective in preventing pneumococcal pneumonia or bacteremia caused by the following serotypes: 1, 2, 3, 4, 6, 7, 8, 9, 12, 14, 18, 19, and 25 (Austrian, 1977). By 1977, the Food and Drug Administration (FDA)

licensed a 14-valent polysaccharide vaccine for use in adults over the age of 50 as well as patients with chronic disease (Austrian, 1977).

The currently licensed pneumococcal polysaccharide vaccine (PPV) was introduced in 1983 and consists of purified capsular polysaccharide of 23 serotypes of *S. pneumoniae*. The serotypes included in the vaccine are 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F. Together, these serotypes account for approximately 88% of bacteremic pneumococcal disease in adults (Nuorti, 1997). The Advisory Committee on Immunization Practices recommends the use of this vaccine in persons who are over the age of 65, immunocompromised or who are over the age of 2 and at high risk for developing pneumococcal disease (Nuorti, 1997). Numerous studies have shown that the PPV is highly effective in preventing pneumococcal disease in normal healthy adults; however, vaccine efficacy is often reduced in the elderly. A study performed by Rubins et al. assessed the immune responses of young and elderly adults following vaccination with PPV (Rubins, 1998). Their results demonstrated an increase in antibody concentration as measured by enzyme-linked immunosorbent assay (ELISA) as well as an increase in antibody functional activity as measured by avidity and opsonization assays. This increase in magnitude of response was observed for both young and elderly adults when assessed on a population level. However, individual data from the elderly group strongly suggests that a subset of this population have very poor immune responses following vaccination and likely are not protected (Rubins, 1998). In support of these findings, a more recent study was performed using a large cohort of older adults to assess the effectiveness of PPV (Jackson

et al., 2003). The authors conclude that while vaccination is associated with a significant decrease in cases of pneumococcal bacteremia, there is insufficient evidence to suggest that it prevents non-bacteremic pneumonia in adults over the age of 65. Despite these findings, other studies have shown that the PPV is sufficient to elicit a protective immune response in elderly adults (Koivula, 1997). Several meta-analyses have been performed to evaluate data from multiple clinical trials involving the use of the pneumococcal vaccine with elderly and immunocompromised persons (Cornu et al., 2001; Fine et al., 1994; Moore et al., 2000; Watson et al., 2002). A study by Moore et al. (2000) evaluated 13 randomized trials which included more than 45,000 subjects total. The authors conclude that although the vaccine is relatively safe and inexpensive, it offers no significant benefit to populations at high risk, i.e., elderly adults and immunocompromised individuals. Other meta-analyses conducted have generated similar conclusions (Cornu et al., 2001; Fine et al., 1994; Watson et al., 2002). Despite these seemingly discouraging results, the Centers for Disease Control and Prevention (CDC) continue to recommend vaccination of immunocompromised persons due to its safety and cost-effectiveness (Fedson and Liss, 2004). Although some evidence demonstrates that PPV can prevent bacteremia in these high risk populations (Jackson et al., 2003), possible alternatives, such as conjugate or protein-based vaccines, should be explored.

*Immunosenescence*

The decreased vaccine efficacy observed in elderly adults parallels with the increased susceptibility to infections in the same age group. Immunosenescence is

defined as the state of dysregulated immune function among elderly individuals which contributes to increased frequency of infections (Pawelec, 1999). Susceptibility to infectious diseases may not be the only consequence of immunosenescence. An altered immune system likely plays an important role in age-related illnesses such as dementia, atherosclerosis, osteoporosis and cancer (Castle, 2000). Extensive studies have been conducted using animals and elderly adults that have shown that changes in innate and adaptive immunity occur with increasing age (Castle, 2000); however, precisely how they relate to the increase in infections has yet to be fully elucidated. The cell types and mechanisms which undergo changes with age will be discussed in more detail in a later section.

Several investigators have shown that age plays an important role in susceptibility to *S. pneumoniae*. Jokinen et al. (2001) demonstrated that pneumococcal infection occurred more frequently in populations over 60 years of age compared to populations under 60 years of age. This study also reports that elderly patients are four times more likely than young patients to develop pneumococcal infections. In addition, the *S. pneumoniae* mortality rate is highest among the elderly with nearly one in five cases resulting in death (Butler and Schuchat, 1999). Consequently, *S. pneumoniae* remains a very important and costly pathogen among elderly populations, despite advances in treatment and vaccination.

*Polysaccharide Vaccine Cost Effectiveness*

Due to the high incidence of pneumococcal disease and limited vaccine efficacy in elderly populations, numerous studies have investigated the cost effectiveness of the

PPV. A study by Sisk et al. (1997) examined the cost effectiveness of vaccinating elderly adults $\geq$ 65 in the prevention of pneumococcal bacteremia. The authors conclude that the 23-valent PPV is effective in both preventing bacteremia and saving medical costs. They recommend an increase in vaccination rates among this age group. Other studies suggest that vaccination is cost-effective in not only preventing bacteremia, but non-bacteremic pneumonia as well (Ament et al., 2000, 2001). While PPV may be effective at treating bacteremia in elderly persons, research in support of its effectiveness in preventing the more prevalent pneumococcal pneumonia is limited (Waugh, 2005). Additional evidence in support of the protective abilities of the PPV will strengthen its cost effectiveness.

*Pneumococcal Conjugate Vaccines*

The pneumococcal polysaccharide vaccine's limited effectiveness in protecting young children and elderly adults has led to investigation of alternative vaccine strategies. Due to the polysaccharide vaccine's TI nature, it is not effective in children under the age of 2, while this age group accounts for 80% of pneumococcal invasive disease in children (Black et al., 2000b). Efforts to use this vaccine for the prevention of otitis media in children have also been unsuccessful. The ineffectiveness of PPV in children under 2 years of age led to the development of pneumococcal conjugate vaccines. Conjugate vaccines consist of purified capsular polysaccharides of different pneumococcal serotypes individually conjugated to a carrier protein such as the diphtheria toxoid protein, $CRM^{197}$. Various studies have been conducted to test the immunogenicity of 7-valent (Black et al., 2000b), 9-valent (Cutts et al., 2005) and 11-valent (Dagan et al., 2004) pneumococcal conjugate vaccines. Currently, the 7-valent

conjugate vaccine (CV) is licensed for use in infants and toddlers (2000) and contains 7 of the most prevalent serotypes in North America, namely, 4, 6B, 9V, 14, 18C, 19F and 23F. These 7 serotypes account for approximately 80-90% of invasive pneumococcal disease in children $\leq$ 5 years of age (Darkes and Plosker, 2002).

The conjugate vaccine differs from the polysaccharide vaccine in its ability to induce a T cell dependent immune response in young children. The B cells recognize and bind the polysaccharide subunit of the conjugate vaccine and then internalize and process both the polysaccharide antigens and protein carrier. Following internalization, the B cells display the toxoid-derived peptides on their surface via MHC class II presentation, thereby inducing T cell help. In turn, the T cells initiate antibody production, class switching and immunological memory resulting in a long-lasting protective response. The design of the conjugate vaccine has proven highly effective in children under 2. A large study involving more than 37,500 children was conducted to determine the efficacy, safety and immunogenicity of the 7-valent CV against invasive pneumococcal disease caused by the serotypes included in the vaccine (Black et al., 2000b). The results of this study show that the CV reduces invasive pneumococcal disease in infants and toddlers by 89%. In addition, vaccination significantly reduced the number of cases of otitis media and no severe side effects were observed (Black et al., 2000b). Conjugate vaccination has also resulted in a significant decease in vaccine type nasopharyngeal carriage in infants (Dagan et al., 1996, 1997) and adults (Hammitt et al., 2006).

*Conjugate Vaccine Cost-Effectiveness*

The 7-valent pneumococcal conjugate vaccine is highly effective at preventing invasive pneumococcal disease, pneumococcal otitis media and carriage with vaccine related serotypes. Therefore its widespread use could dramatically reduce costs associated with hospital visits and other medical expenses. However, such a benefit may require a decrease in vaccine costs, presently $46 per dose (Black et al., 2000a). The pneumococcal conjugate vaccine remains one of the most expensive vaccines available, with four doses needed for children under 2 and each dose resulting in a total patient charge of $360 (Butler et al., 2004). Further analysis involving the contribution of herd immunity in the prevention of pneumococcal disease (Dagan and Fraser, 2000) may result in an increase in cost-effectiveness of the conjugate vaccine.

*Conjugate Vaccine Efficacy in Adults*

The limited efficacy of the polysaccharide vaccine in the elderly has led to an increased interest in the use of the conjugate vaccine for adult populations. A study by Whitney et al. (2003) assessed the rate of pneumococcal invasive disease in children and adults following conjugate vaccination of children under the age of 5. The authors observed a decrease in invasive pneumococcal disease in children consistent with previous studies (Black et al., 2000b, 2002). Additionally, they suggest that conjugate vaccination plays an indirect role in preventing pneumococcal disease in adults. The reduction in pneumococcal carriage and infection in children may result in reduced transmission to adults, therefore extending the protective abilities of the conjugate vaccine. Nevertheless, studies analyzing the direct effect of conjugate vaccination in adults, specifically the elderly, demonstrate no advantage over the PPV in older adults

(Powers et al., 1996; Shelly, 1997). A study by Shelly et al. (Shelly, 1997) compared the immunogenicity of PPV to that of a 5-valent conjugate vaccine in young and elderly adults. The results demonstrated that conjugation of the polysaccharide resulted in an increase in immunogenicity compared to the un-conjugated polysaccharide vaccine formulation in young adults. In contrast, the conjugate vaccine offered no increase in immunogenicity over the PPV in elderly adults (Shelly, 1997). These results are consistent with those of Powers et al. (1996) who observed no advantage of the pneumococcal conjugate vaccine over the polysaccharide vaccine in healthy adults ≥ 50 years old. Therefore, development of an effective vaccine for elderly adults may require investigation of alternative vaccine strategies.

*Protein Based Vaccines*

Although conjugate vaccines have thus far proven highly immunogenic, their design is limited to a restricted number of serotypes. They do not take into account serotype replacement or the emergence of other prevalent strains. Additionally, conjugate vaccines are expensive and may not be practical for use in developing countries. To combat these issues, researchers have investigated the use of pneumococcal proteins as potential vaccine candidates. Studies have shown that the surface protein PspA holds great promise as a potential vaccine candidate against *S. pneumoniae*. Pneumococcal surface protein A is structurally variable (Jedrzejas et al., 2000), yet common to all clinically relevant strains (Crain et al., 1990). Studies in mice have demonstrated that antibody responses to PspA are broadly cross-reactive to heterologous PspA proteins found on other strains (McDaniel et al., 1991). Nabors et al.

(2000) conducted the first phase 1 clinical trial in humans using a recombinant PspA vaccine preparation. Their results demonstrate that the recombinant PspA is safe, immunogenic and broadly cross reactive. Moreover, sera from this trial were capable of protecting mice from pneumococcal disease which suggests its potential as an effective vaccine (Nabors et al., 2000). Other protein antigens have shown potential as possible vaccine candidates including PsaA, pneumolysin and PspC (Bogaert et al., 2004b). A vaccine developed by Briles et al. (2000a) including PspA and PsaA was shown to elicit better protection against pneumococcal carriage than PspA alone. Therefore, a combination vaccine incorporating both protein antigens may provide optimal protection against carriage and invasive disease (Briles et al., 2000a). Further analysis of PspA and other alternatives will provide insight on their true potential as effective vaccines.

*Measuring Vaccine Efficacy*

With the wide variety of vaccines being developed and evaluated, consistency and accuracy when measuring vaccine effectiveness is essential. In the past, vaccine evaluation has been limited to serological assessment, with anti-pneumococcal polysaccharide (PPS) antibody concentrations serving as the main surrogate marker of protection. Due to the fact that antibodies against the capsular polysaccharides provide the most consistent immunity against infection, these antibodies have been quantified as a way to predict vaccine effectiveness. Enzyme-linked immunosorbent assay (ELISA) is the most widely used method for measuring antibody concentration. Numerous studies have been performed in an effort to standardize the methods of measuring serotype specific antibody via ELISA. A study by Musher et al. (1990c) revealed that cell wall

polysaccharide (CPS) is detected by ELISA but offers little to no protection. Therefore, pneumococcal ELISAs were developed to include the removal of these non-immunogenic antibodies by pre-incubating sera with CPS (Musher et al., 1990b). These ELISAs were further refined with the use of reference serum 89SF for cross-standardization (Concepcion, 1998) and additional serum absorption with pneumococcal polysaccharide 22F (PPS22F) for the absorption of antibodies cross-reactive to non-polysaccharide components of the polysaccharide preparation (Concepcion, 2001).

Despite the extensive utilization and standardization of pneumococcal ELISAs, measuring antibody concentration is no longer considered sufficient to accurately assess vaccine efficacy (Jodar et al., 2003). The World Health Organization recommends other criteria be considered when measuring vaccine effectiveness, such as antibody avidity, functional activity and the induction of immunological memory (Jodar et al., 2003). Furthermore, previous work has demonstrated that antibody avidity as measured by opsonophagocytic assays, not concentration, is a more reliable predictor of functional antibody activity (Sun et al., 2001; Usinger and Lucas, 1999). Antibody avidity is defined as the equilibrium association constant of the whole antibody and multivalent antigen. A study by Usinger and Lucas (1999) demonstrated that anti-PPS antibodies vary in avidity and these differences are directly related to differences in functional activity and protective capabilities. Their data show that high avidity antibodies more effectively mediate protection than the low avidity antibodies, and therefore avidity can be used as a surrogate for protective efficacy (Usinger and Lucas, 1999). In agreement with this study, Sun et al. (2001) measured the avidity, opsonizing capacity and

protective capacity of monoclonal antibodies (mAbs) specific for PPS6B. This study demonstrated that antibody avidity strongly correlated with functional activity and *in vivo* protective immunity (Sun et al., 2001). Similar correlations between avidity and functional activity have been shown in response to other PS antigens as well (Schlesinger and Granoff, 1992).

Antibody avidity can be measured by a variety of assays. Two common methods used to measure antibody avidity are radioimmunoassay (RIA) and avidity ELISA. Radioimmunoassay is a technique in which radiolabeled polysaccharide is used to measure the avidity of anti-PPS antibody in the serum (Usinger and Lucas, 1999). This technique is highly sensitive and specific, but due to its relatively high cost and need for radioantigens, alternative methods are often used. Antibody avidity can also be determined by avidity ELISA. Avidity ELISAs use sodium thiocyanate (NaSCN), a chaotropic agent, to interfere with the antigen-antibody interaction (Pullen et al., 1986). Avidity is determined by measuring the amount of NaSCN needed to inhibit 50% of bound serum antibody (Romero-Steiner et al., 1999).

Due to the strong relationship established between antibody avidity and functional activity, opsonophagocytic assays are frequently used to determine vaccine efficacy. Opsonophagocytic assays measure the functional activity of anti-PPS antibodies in vitro by determining their ability to opsonize live bacteria and induce phagocytosis (Romero-Steiner et al., 2006). Previous studies have used opsonophagocytic assays and found them to be a reliable predictor of protective capacity (Johnson et al., 1999; Romero-Steiner et al., 1999). These assays have been recently standardized (Romero-Steiner et

al., 2003) and are regarded as the principal test used to measure the immunogenicity of pneumococcal vaccines (Romero-Steiner et al., 2006).

The reduced PPV efficacy among the elderly has resulted in numerous studies designed to characterize the PPS-specific immune response within these individuals. Previous studies have shown that anti-pneumococcal antibody concentrations are similar between young and elderly adults (Musher et al., 1993; Ruben and Uhrin, 1985; Sankilampi et al., 1996). However, more recent evidence has demonstrated a reduction in antibody functional activity associated with aging. A study by Romero-Steiner et al. (1999) compared the anti-pneumococcal immune response of elderly subjects to that of young adults following vaccination with PPV. They observed no significant differences in post-vaccination antibody concentration between young and elderly adults for 3 of the 5 serotypes tested. However, opsonophagocytic activity was significantly reduced in elderly compared to young for all 5 serotypes (4, 6B, 14, 19F and 23F) but to a lesser extent for PPS14. This overall decrease in functional activity correlated with low antibody avidity and protective ability. Furthermore, this reduction in antibody functionality among the elderly became more apparent with increasing age (Romero-Steiner et al., 1999).

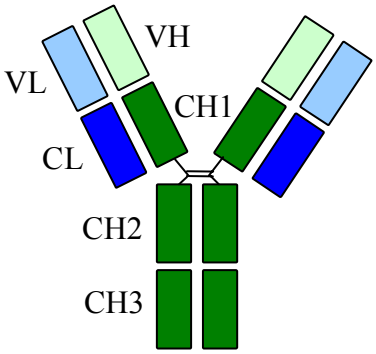
The discrepancy between antibody concentration and functional activity in the elderly raises pertinent questions regarding variation between antibodies, i.e., how do anti-pneumococcal antibodies differ between young and elderly adults, and what contributes to these differences? To improve our understanding of age-related differences in antibody function, investigators have begun to characterize the structure of

anti-PS antibodies on a molecular level. An increased understanding of the antibody structure-function relationship will be beneficial in elucidating the mechanisms involved in antibody variation.

*Antibody Structure*

The general structure of an antibody, or immunoglobulin (Ig), is illustrated in Figure 2. Antibodies are multi-functional proteins that are composed of 2 identical heavy chains and 2 identical light chains. Each heavy and light chain consists of constant regions (CH1, 2, etc. or CL) and one variable region (VH or VL). The constant regions are responsible for effector functions including the initiation of phagocytosis and the activation of complement whereas the variable regions are responsible for antigen binding and antibody specificity.

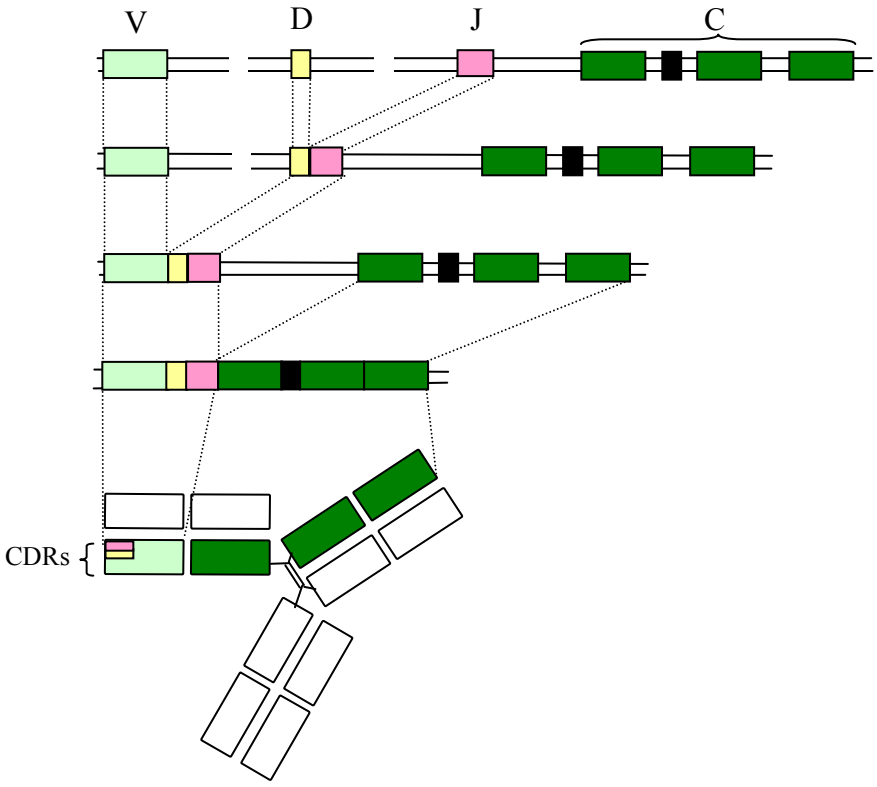
Figure 2. Antibody Structure.




Differences between heavy chain constant regions separate antibodies into 5 isotypes: IgM, IgG, IgA, IgD, and IgE. The heavy chains pair with one of two light chain

isotypes, kappa or lambda. The heavy and light chain variable regions are the main source of antibody diversity. They are encoded by multiple gene segments and are assembled during B cell development. The variable regions of heavy chains are made up of 3 segments, variable (V), diversity (D) and joining (J). The D gene is located between the V segment and J chain, and the J chain joins the variable region to the constant region. The rearrangements that lead to the formation of a complete heavy chain are illustrated in Figure 3. The D and J segments join first, followed by the joining to the V segment. Once the V, D and J segments have been assembled, they join with the constant region segments to complete the heavy chain variable region.

Figure 3. Immunoglobulin Gene Rearrangement.



Light chains are assembled in the same manner; however, they lack a D gene. The V gene segment encodes the majority of the variable region and consists of 3 highly conserved framework regions and 3 highly variable complementary determining regions (CDRs) which form the antigen binding site. All immunoglobulin gene segments are organized into three genetic loci, the heavy chain locus and kappa and lambda loci, all of which are located on different chromosomes. The numbers of functional gene segments for the heavy and light chain variable regions differ by loci and are illustrated in Table III. Due to the large number of human variable gene segments, they have been divided into gene families based on sequence similarity. There are 7 heavy chain, 7 kappa and 8 lambda chain gene families.

Table III. Number of Gene Segments in Human Immunoglobulin Loci.

| Number of gene segments in human Ig loci | | | |
|---|---|---|---|
| | Heavy chain | Light chain | |
| | | kappa | lambda |
| Variable (V) | 65 | 40 | 30 |
| Diversity (D) | 27 | 0 | 0 |
| Joining (J) | 6 | 5 | 4 |

*Generation of antibody diversity*

Within each locus, gene segments undergo a process of rearrangement, or somatic recombination, as a way to create a functional V, D or J gene and add diversity to the immunoglobulin repertoire. Furthermore, the combination of separate V, D and J segments to form a complete variable region contributes to the total diversity. Due to the

large number of individual gene segments in each Ig locus, antibodies could, theoretically, be comprised of more than 3 x $10^6$ unique V region rearrangements. Additional antibody diversity can result from the insertion or deletion of nucleotides between gene segments. These mutations often occur in the CDR3 region, resulting in a significant increase in CDR3 diversity. Further antibody variation arises from the association of different heavy and light chains, and by somatic hypermutation. Somatic hypermutation is a process which occurs after the whole B cell receptor has been assembled and upon antigenic stimulation. This process introduces mutations into the variable region and often increases the antibody's affinity for a particular antigen. Together, these mechanisms create the extensive antibody diversity used in the humoral immune response to a wide array of antigens.

*Variable Gene Usage Analysis*

Advances in molecular biology have allowed investigation of the genetic diversity involved in the humoral immune response to polysaccharide (PS) antigens such as *Neisseria meningitidis* serotype C polysaccharide (MCPS), Hib and PPS. Studies designed to examine antibody structure and variable gene usage can be performed using single cell polymerase chain reaction (Wang and Stollar, 2000), combinatorial libraries (Lucas et al., 2001; Zhou et al., 2002), generation of heavy and light chain libraries (Kolibab et al., 2005a; Smithson et al., 2005), human mAbs (Baxendale, 2000; Park et al., 1996; Shaw et al., 1995) or mAbs derived from transgenic mouse strains (Chang et al., 2002; Russell et al., 2000). Using these techniques, numerous studies have analyzed antibody variable gene usage in response to polysaccharide antigens. Although there are

7 variable heavy chain (VH) gene families, gene usage in response to PS antigens is often limited to the largest family, VH3 (Abadi et al., 1998; Baxendale, 2000; Lucas et al., 1994, 1997). Similarly, several studies characterizing the anti-Hib-PS antibody response have demonstrated limited variable heavy and light chain gene usage (Adderson et al., 1991, 1992; Scott et al., 1989; Silverman and Lucas, 1991). Previous work by Scott et al. (1989) has shown that anti-Hib-PS antibodies isolated post-immunization predominantly express VH genes belonging to the VH3 gene family. The variable light chain gene usage was less restricted, with antibodies expressing up to 4 different light chain families overall. Despite the variety of VL genes used, the VL response to Hib-PS is often dominated by the variable kappa 2 (Vκ2) gene, A2 (Lucas et al., 1991; Scott et al., 1989). This preferential gene usage has also been observed in response to pneumococcal antigens. A study by Zhou et al. (Zhou et al., 2002) examined the molecular immune response to PPS23F following pneumococcal vaccination. The authors demonstrated highly restricted V gene usage of both heavy and light chains within individuals, with one or two unique gene loci accounting for the majority of the expressed repertoire. In addition, biased gene usage was observed at the population level (Zhou et al., 2002), although this may be an antigen-specific phenomenon (Zhou et al., 2004). Taken together, these studies confirm the importance of analyzing antibody molecular structure to advance our understanding of the immune response to polysaccharide antigens.

In addition to observing restricted antigen-specific gene usage, studies have begun to demonstrate correlations between antibody molecular structure and functional activity in response to PS antigens (Lucas et al., 1998; Nahm et al., 1995). A study by Nahm et

al. (1995) used anti-Hib antibodies to show that antibody molecular structure correlates with avidity, which in turn, correlates with antibody bactericidal activity. Furthermore, work by Lucas et al. (1998) demonstrated an important role for the light chain CDR3 region in determining the affinity of anti-Hib-PS antibodies. Thus, understanding the molecular diversity of PPS-specific antibodies is becoming increasingly important in predicting the human immune response against pneumococcal disease.

*Variable Gene Usage and Aging*

Previous work has demonstrated that immunization with PPV results in decreased protection in elderly adults compared to young adults. This decline in vaccine efficacy increases with age and is likely due to a decrease in antibody avidity and thus functional activity. Evidence has shown that changes in antibody function are related to differences in antibody molecular structure. Therefore studies investigating the effect of aging on antibody molecular structure may explain the decrease in functional immune response to pneumococcal vaccination in the elderly. The first studies performed to examine changes in variable gene usage with aging were carried out in young and aged mice in response to phosphorylcholine. The results of these studies demonstrate that changes in VH and VL gene usage occur with increasing age, and are associated with a decrease in antibody functional activity (Nicoletti et al., 1991, 1993; Riley et al., 1989; Yang et al., 1994). In addition, a study by Lucas et al. (1993) investigated changes in human VL gene usage in response to a Hib PS conjugate vaccine. In this study, the authors monitored expression of 3 predominant VL genes in subjects of different ages. Their results clearly demonstrate age-related differences in gene expression (Lucas et al., 1993). In support of

these findings, a study by Adderson et al. (1998) determined the variable region gene sequences of infants and adults following vaccination with a Hib PS conjugate vaccine. Their data show that the gene repertoire of immunized infants differs from that of immunized adults and is associated with a low affinity, poly-reactive antibody response. Furthermore, age-related changes in variable gene mutational frequency have been observed independent of antigenic stimulation, and may contribute to differences in antibody affinity between age groups (Chong et al., 2003). The results of these studies demonstrate that variable gene usage can change with age and such changes may be directly related to antibody avidity and functional activity.

To explore this concept further, studies performed in our laboratory compared the young versus elderly immune response following vaccination with the 23-valent pneumococcal polysaccharide vaccine (Kolibab et al., 2005a, 2005b; Smithson et al., 2005). Twenty young (20-30 years old) and 20 elderly adults ($\geq$65 years old) were vaccinated with PPV. Antibodies specific for PPS4 and PPS14 were isolated 6 weeks post-vaccination and PPS-specific antibody concentration, avidity, opsonophagocytic activity (Kolibab et al., 2005b) and VH (Kolibab et al., 2005a) and VL (Smithson et al., 2005) gene usage were determined for all volunteers. Serum analysis demonstrated a significant increase in PPS-specific antibody concentration and opsonophagocytic activity in both young and elderly adults (Kolibab et al., 2005b). However, when elderly adults were further subdivided by age, those adults > 77 years of age had opsonophagocytic titers significantly lower than young adults (Kolibab et al., 2005b), in agreement with data reported previously (Romero-Steiner et al., 1999). Overall, serum

analysis of the young and elderly adults suggest a decline in the functional antibody response with increasing age which could be associated with changes in antibody molecular structure.

To define potential differences in variable gene usage between young and elderly adults, the PPS-specific VH and VL gene repertoires were characterized by the generation of heavy (Kolibab et al., 2005a) and light chain (Smithson et al., 2005) libraries. Analysis of the post-vaccination VH gene response to PPS4 and PPS14 demonstrated that changes in heavy chain gene usage occurs as a function of age (Kolibab et al., 2005a). The dominant heavy chain gene family expressed in response to both polysaccharides was VH3, however significant differences in VH3 gene loci were observed between age groups. In addition, the results from this study showed that elderly adults demonstrated a significant loss in oligoclonality, defined as one gene locus representing $\geq$ 50% of isolated sequences, when compared to young adults (Kolibab et al., 2005a). Analysis of the light chain PPS-specific repertoire resulted in the identification of further age-related changes in V gene usage (Smithson et al., 2005). The light chain libraries generated from young and elderly adults displayed gene expression from 6 different VL families, Vλ1, Vλ3, Vκ1, Vκ2, Vκ3 and Vκ4 (Smithson et al., 2005). The VL gene usage in response to PPS4 and PPS14 showed marked diversity on a population level, yet gene expression was more restricted within individuals. Overall, significant differences in PPS-specific VL gene expression were observed between young and elderly adults. These comprehensive studies characterizing the young versus elderly immune response to pneumococcal polysaccharides suggest that differences in antibody

molecular structure occur as a function of age and are likely responsible for altered antibody functional activity. Further elucidation of the relationship between aging and antibody molecular structure should lead to a better understanding of the underlying mechanisms responsible for differences in vaccine efficacy.

*Age-Related Changes of the Immune System*

Numerous studies have demonstrated that significant changes in immune cell populations and functions occur with aging. It has been well-documented that alterations in cellular immunity occur with increasing age (Chakravarti and Abraham, 1999; Globerson, 1995; Goidl et al., 1976; Jackola et al., 1994; Ruiz et al., 1995). Elderly individuals experience a decline in T cell production (Min et al., 2005) accompanied by an overall reduction in T cells (Rea et al., 1996; Xu et al., 1993). Studies have also associated aging with altered T cell subpopulations (Goidl et al., 1976) and a limited response to T cell mitogens (De Greef et al., 1992; Hallgren et al., 1988). In addition to T cells, accessory cells such as dendritic cells, macrophages and natural killer cells (NK cells) undergo significant changes with age (Aydar et al., 2004; Bondada et al., 2001; Chelvarajan et al., 2005; Garg et al., 1996; Lloberas and Celada, 2002; Takeda and Dennert, 1993). Previous studies have demonstrated age-related accessory cell defects such as impaired regulation of follicular dendritic cell receptors (Aydar et al., 2004) in concert with decreased NK cell production are likely related to an increase in autoimmunity in the elderly (Takeda and Dennert, 1993). Changes involving macrophages have been observed on a developmental and functional level. A study by Sadeghi et al. (1999) revealed that monocytes, the precursors of macrophages,

experienced irregular expansion and cytokine secretion in the elderly. As a result, these monocytes were defective in their development, and therefore unable to become mature and fully functional macrophages (Sadeghi et al., 1999). Chelvarajan et al. (2005) demonstrated that murine macrophages were functionally impaired in aged mice and exhibited a diminished response to TI antigens. The authors conclude that these defects were caused by altered cytokine secretion in the form of a decrease in interleukin 1 (IL-1) and IL-6, and a concomitant excess of IL-10 (Chelvarajan et al., 2005). Further investigation of accessory cell defects with aging were performed using young and aged mice vaccinated with a 23-valent pneumococcal polysaccharide vaccine (Garg et al., 1994, 1996; Garg and Subbarao, 1992). These studies investigated the cellular basis of the decreased immune response to PPV both *in vivo* and *in vitro*. The authors concluded that the decrease in the immune response of aged mice to pneumococcal vaccination is not a result of T cell mediated suppression; rather it was due to a lack of accessory cell function (Garg et al., 1996). They suggested that the defects in accessory cell function were brought about by a decrease in cytokine secretion, specifically IL-1, IL-4 and IL-5. Moreover, supplementing accessory cells with these cytokines resulted in an enhanced response to pneumococcal vaccination in the aged (Garg et al., 1996). The authors concluded that cytokine-based adjuvants should be considered as a potential method used to increase the immunogenicity of PPV in the elderly (Bondada et al., 2001; Garg et al., 1996).

Several studies investigated cytokine levels in young versus elderly populations and revealed changes that occur as a function of age (Mascarucci et al., 2001; McNerlan

et al., 2002; O'Mahony et al., 1998; Sandmand et al., 2002; Saurwein-Teissl et al., 2002). McNerlan et al. (2002) used flow cytometry to measure intracellular levels of tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and IL-2 in whole blood taken from young and elderly subjects. Their data demonstrated that although IL-2 levels were similar between age groups, there was a significant increase in TNF-α and IFN-γ expression in the elderly. In support of these findings, O'Mahony et al. (1998) measured proinflammatory cytokine production of monocytes and T cells in young and elderly adults and observed significant increases in TNF-α and IL-6 with advanced age. Such age-related imbalances in cytokine production are not surprising, given the observed differences that arise in cell populations between young and elderly adults.

The significant changes in cytokine environment likely play a key role in the impaired response to PS antigens in the elderly. A recent study by Saurwein-Teissl et al. (2002) suggested that imbalances in cytokine production resulted in an increase in $T_H1$ responses. Their data showed an increase in IFN-γ, but not IL-5 production in elderly persons who failed to respond to an influenza vaccine (Saurwein-Teissl et al., 2002). Therefore, the authors hypothesized that shifts in cytokine profile with advanced age likely contribute to an increase in $T_H1$ response associated with a decrease in antibody production. In contrast, a study by Sandmand et al. (2002) observed a shift towards a dominance of $T_H2$ cytokine levels in the elderly, but only in subjects over 100 years of age. Overall, the precise changes in cytokine profile that occur with increasing age remain controversial and further analysis will be beneficial in understanding the diminished response to vaccination in the elderly.

Various studies have examined cytokine profile as a way to further characterize the immune response to PS antigens. A study by Kemp et al. (2002) was designed to determine which cytokines were produced in response to *S. pneumoniae* infection. The authors examined the cytokine profiles of patients with current *S. pneumoniae* infections and healthy adults as controls. Their data show that a decrease in $T_H1$ cytokines, namely IL-2, TNF-α and IFN-γ, was observed during acute pneumococcal infection, however, these cytokines partly reappeared after 1 week's treatment. In addition, there was no change in levels of the $T_H2$ cytokine IL-4; hence, these results suggest the significant involvement of $T_H1$ cytokines in combating pneumococcal infections (Kemp et al., 2002). In agreement with this study, Khan et al. (2002) demonstrated that the proinflammatory cytokines TNF-α, IFN-γ, IL-1, IL-6 and IL-12 play an essential role in the response to *S. pneumoniae* and act as an important link between the innate and adaptive humoral immune response to this pathogen. The identification of the predominant cytokines involved in the immune response to pneumococcal infection is of great value due to their potential use as vaccine adjuvants.

*Interleukin-12*

The cytokine IL-12, in particular, plays a key role in boosting the immune response to pneumococcal polysaccharides. Interleukin-12 is produced by B cells dendritic cells and macrophages and plays a key role in enhancing cell-mediated immunity in the form of a $T_H1$ response (Cooper et al., 2004; Khan et al., 2002; Metzger et al., 1995; Romani et al., 1997). It acts as a proinflammatory cytokine in response to infection by increasing IFN-γ production, and therefore activating macrophages and Ig

class switching (Romani et al., 1997). Buchanan et al. (1998) examined the effect of IL-12 on antibody responses to PPV in the absence of T cells and NK cells. The authors report that IL-12 significantly enhanced the antibody response, and therefore conclude that IL-12 may be a valuable adjuvant for increasing the protective capacity of the pneumococcal vaccines (Buchanan et al., 1998). Other studies have also supported the use of IL-12 as an adjuvant to heighten the immune response vaccination (Arulanandam et al., 1999, 2000; Bliss et al., 1996; Lynch et al., 2003).

*B Cells and Aging*

It was previously thought that the immune senescence seen in the elderly was largely due to changes in cellular immunity (Goidl et al., 1976). It has been well-established that factors such as T cells, accessory cells and cytokine environment undergo significant changes with advanced age, and therefore may be associated with the age-related changes observed in B cell variable gene usage and functional activity. However, studies have demonstrated that the effects of aging on the immune system extend beyond T cells and accessory cells and affect B cells as well (LeMaoult et al., 1997; Linton and Dorshkind, 2004; Paganelli et al., 1994). Age-related changes in B cells have been observed when examining antibody subclass (Lottenbach et al., 1999) and B cell subpopulations (LeMaoult et al., 1997). The percentage of CD5+ B1 cells (LeMaoult et al., 1997; Linton and Dorshkind, 2004) and marginal zone B cells (Johnson et al., 2002) increases in older populations. Despite these altered B cell populations, the amount of B cells in the periphery remains constant with increasing age (Kline et al., 1999). Studies performed in mice have demonstrated a decrease in B cell proliferation in the bone

marrow with advanced age (Szabo et al., 1998). These developmental changes may be associated with the changes observed in B cell repertoire with aging. Evidence has shown that the B cell gene repertoire changes with age and is related to a shift in antibody specificity (Hu et al., 1993). A study by Hu et al. (1993) demonstrated that the B cell repertoire shifts with aging, resulting in more antibodies specific for autologous antigens compared to foreign antigens. These observations and those of others (Kolibab et al., 2005a; Lucas et al., 1993; Nicoletti et al., 1991; Smithson et al., 2005) demonstrated intriguing differences in the antibody gene repertoire that occur as a function of age. It is unknown whether such alterations in the expressed B cell repertoire occur intrinsically, or are a reflection of age-related modifications in T cells or accessory cells. As previously discussed, numerous age-related changes of the immune system have been identified which could influence B cell variable gene usage and functional activity. Factors which may influence antibody gene usage and functional activity included extrinsic stimuli such as T cells, accessory cells and cytokine environment and/or the intrinsic immunosenescence of B cells themselves. An increased understanding of the mechanisms underlying altered gene expression will be highly beneficial in establishing strategies to enhance elderly immune function.

*Development of the SCID Mouse Model*

Studies designed to investigate the influence of extrinsic factors on B cell molecular structure and function have been limited in number due to a lack of suitable experimental models. The development of a severe combined immunodeficient (SCID) mouse model has allowed for the manipulation of the human immune system to study

these concepts. Severe combined immunodeficient mice are so named due to their inability to successfully complete lymphocyte gene rearrangement which results in a failure to produce functionally mature B and T cells (Bosma et al., 1983). Due to this deficiency, SCID mice can be engrafted with human peripheral blood lymphocytes (PBL) (Mosier et al., 1988), human adult bone marrow (Lubin et al., 1991) or human hematopoietic fetal tissue (McCune et al., 1988) without rejection (Kamel-Reid and Dick, 1988). Methods using human bone marrow or fetal tissue for engraftment, although relatively reliable, are often unfeasible due to procedural or ethical restraints (Amadori et al., 1996). Therefore, most studies used to evaluate human lymphocyte function *in vivo* have been performed using SCID mice engrafted with human PBL (hu-PBL-SCID).

The hu-PBL-SCID model was first developed by Mosier et al. (1988) using human PBL injected intraperitoneal (i.p.). The authors demonstrated that their model represents a stable, long-term reconstitution of a functional human immune system. Their results revealed that normal human lymphocytes survive, expand and recirculate in the SCID mice with little evidence of graft rejection or development of B cell lymphomas (Mosier et al., 1988). Moreover, human lymphocytes were capable of mounting a specific antibody response following immunization with tetanus toxoid. All major cell populations were represented in the blood and lymphoid tissue of the reconstituted SCID mice. However, the relative proportions of macrophages and B and T cell subsets became skewed over time (Mosier et al., 1988). In addition to skewed cell populations, other limitations have been demonstrated, thus caution should be used in the application of this model. Some SCID mice have been classified as leaky, following the detection of

murine IgM (Bosma et al., 1988; Carroll and Bosma, 1988), however, most murine B cells do not expand due to the lack of T cell help (Aaberge et al., 1992). Other drawbacks to this model include possible variation that can arise between multiple donors as well as between each individual graft recipient (Aaberge et al., 1996; Greenwood, 1993; Somasundaram et al., 1995). Despite the limitations of hu-PBL-SCID mice, this model has been frequently used to study human lymphocyte function and lymphomagenesis (Amadori et al., 1996).

Several studies have investigated the use of the hu-PBL-SCID model in studying the human immune response to polysaccharide antigens (Aaberge et al., 1992, 1996; Lucas et al., 1992; Reason et al., 1994; Smithson et al., 1999; Westerink et al., 1997). A study by Lucas et al. (1992) was performed to test the ability of the hu-PBL-SCID model to respond to Hib PS vaccination. In this study, hu-PBL-SCID mice produced human Ig one week post-reconstitution and maintained peak levels in the serum for 2-4 weeks, consistent with the observations made by Mosier et al. (1988). In addition, the Hib-PS-specific antibodies were functional and idiotypically characteristic of those isolated in humans. Furthermore, a study by Reason et al. (1994) demonstrated that anti-Hib-PS antibodies recovered from hu-PBL-SCID mice were capable of inducing a protective response against Hib bacteremia following their passive transfer to neonatal rats. Additional investigation of this model's response to PS antigens involved use of the pneumococcal polysaccharide vaccine (Aaberge et al., 1992). A study by Aaberge et al. (1992) demonstrated that an anti-PPS IgG response could be induced in up to 78% of hu-PBL-SCID mice following vaccination with PPV. Subsequent challenge with *S.*

*pneumoniae* resulted in a decrease in bacteremia and a survival rate of 45-60% (Aaberge et al., 1992). These data, along with that of others (Lucas et al., 1992; Reason et al., 1994), support the use of the hu-PBL-SCID model for the development of a functional human antibody response to PS antigens.

Once the effectiveness of hu-PBL-SCID mice had been demonstrated, a study by Aaberge et al. (1996) was conducted to determine whether this model served as an accurate representation of the human immune response *in vivo*. The results of this study demonstrated that antibody levels varied between donors and consequently not all donors should be used for reconstitution. Furthermore, the level of human IgG in the hu-PBL-SCID mice varied between and within donors and was not an accurate predictor of the donor's ability to produce PPS-specific antibodies (Aaberge et al., 1996). These findings could be explained by detectable levels of anti-PPS antibodies observed in the donors prior to transplant, which are likely the result of previous exposure to pneumococcal antigens. Such previous exposure would suggest that the antibodies isolated from hu-PBL-SCID mice do not represent the induction of a primary immune response, and consequently, would not necessarily mimic the *in vivo* immune response. A study by Saxon et al. (1991) was performed to characterize the variable gene usage of human PBL recovered from hu-PBL-SCID mice. The authors demonstrated that this system resulted in a restricted B cell repertoire (Saxon et al., 1991), likely due to the presence of NK cells (Carlsson et al., 1992) or failure to induce a primary immune response. Other studies have shown that use of the hu-PBL-SCID model does not result in the production of a primary immune response (Markham and Donnenberg, 1992; Mazingue et al., 1991;

Nonoyama et al., 1993). To this purpose, Westerink et al. (1997) developed an adapted hu-PBL-SCID mouse model to induce a primary immune response following immunization with meningococcal capsular polysaccharide (MCPS). The results of this study demonstrated that a primary immune response can be achieved using the SCID mouse model supplemented with human IL-2 and IL-12. Furthermore, treatment of PBL with L-leucine methyl ester hydrochloride allowed for the removal of NK cells and encouraged the expression of a broad B cell repertoire as previously shown (Carlsson et al., 1992). The idiotypic expression of the anti-MCPS antibodies from hu-PBL-SCID mice was not significantly different from those of human volunteers following MCPS immunization (Westerink et al., 1997). Few studies have been performed to examine heavy and light chain variable gene expression of the hu-PBL-SCID model (Saxon et al., 1991; Smithson et al., 1999), however, further use of this model may provide insight on mechanisms that influence antibody variable gene usage.

Other SCID mouse models more recently developed include recombination activating gene (RAG) deficient strains (Goldman et al., 1998; Shultz et al., 2000), however, their engraftment with human cells has resulted in limited success (Greiner et al., 1998). The use of nonobese diabetic severe combined immunodeficient (NOD/SCID) mice, however, has shown promise as an effective model for the engraftment of human cells (Greiner et al., 1995). Recently, NOD/SCID mice with a complete null mutation of the cytokine receptor gamma chain (NOD/SCID/IL2r$\gamma^{null}$) (Cao et al., 1995) were successfully used to support the development of the human hemato-lymphoid system

(Ishikawa et al., 2005). Future use of similar SCID models should prove highly successful in the investigation of the human immune system.

## MANUSCRIPT 1

**Title:** Analysis of the Young and Elderly Variable Gene Repertoire in Response to Pneumococcal Polysaccharides Using a Reconstituted SCID Mouse Model

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**Abstract**

Significant changes in anti-pneumococcal polysaccharide (PPS) variable gene usage occur with aging and may be influenced by changes in cytokine environment. Severe combined immunodeficient (SCID) mice were engrafted with B cells obtained from young and elderly donors, supplemented with human cytokines and immunized with the pneumococcal polysaccharide vaccine. B cells specific for PPS serotypes 4 and 14 were isolated from mice and immunized donors, and variable region sequences analyzed. Significant differences in variable heavy and light chain gene usage were observed between young and elderly adults despite a more constant cytokine environment. Due to the limitations of the hu-PBL-SCID model, the use of alternative systems would be beneficial in the elucidation of mechanisms underlying the reduced vaccine efficacy in the elderly.

**Key words:** aging, cytokines, gene expression, pneumococcal polysaccharides

**Abbreviated Title:** *S. pneumoniae* Response Using SCID Model

## 1. Introduction

*Streptococcus pneumoniae* is a major human pathogen worldwide. The organism colonizes the nasopharynx and subsequent invasion can result in otitis media, pneumonia, meningitis or bacteremia [1,2]. *S. pneumoniae* is a significant cause of morbidity and results in over 40,000 deaths in the United States each year [3]. Pneumococcal disease has a characteristic age distribution with the highest incidence occurring at the extremes of age. The currently licensed pneumococcal polysaccharide vaccine (PPV), Pneumovax, (Merck & Co., Inc.) is based on the observation that antibodies directed at the capsule initiate complement-dependent opsonophagocytosis and protect against disease [1,2]. This vaccine consists of the capsular polysaccharide of 23 serotypes and is recommended for adults over the age of 65 and other high-risk populations by the Centers for Disease Control and Prevention. Although effective in young adults, numerous studies have shown that vaccine efficacy is diminished in the elderly [4,5]. While polysaccharide-specific antibody concentrations in elderly are similar to those of young adults, antibody functional activity, as measured by opsonophagocytic activity, is markedly decreased [6]. Studies have shown that the reduction in functional antibody activity is likely related to a decrease in antibody avidity [7,8] and therefore, may correlate with differences in antibody molecular structure [9]. The importance of changes in VL repertoire on antibody functional activity in response to *Haemophilus influenzae* type b (Hib) has been reported previously [10-13]. Additionally, these studies [10,11,13], and others [14,15], have demonstrated that changes in anti-polysaccharide gene expression occur as a function of age. The underlying cause of altered V gene expression in the elderly

however, remains to be elucidated. Numerous factors may influence the expressed B cell repertoire in response to T-independent type 2 (TI-2) antigens, including T cells [16], accessory cells [17,18], auto-anti-idiotypic antibodies [19] and cytokines [20,21]. Previous studies have shown not only that cytokines can influence gene expression [22], but that cytokine environment can undergo significant changes with increasing age [20,21]. We investigated whether the differences in variable gene expression noted between young and elderly adults would disappear if young and elderly B cells are stimulated in similar cytokine environments.

To this purpose, we have used severe combined immune deficient (SCID) mice reconstituted with peripheral blood lymphocytes (PBL) obtained from un-immunized young and elderly adults. We hypothesized that elderly B cells in the SCID mouse model can be stimulated to express a gene repertoire resembling that of young B cells in the presence of the same soluble regulatory factors. We have compared the V region sequences of antibodies to pneumococcal polysaccharide serotype 4 (PPS4) and 14 (PPS14) generated by B cells derived from elderly and young donors in the SCID mouse model to those generated by the donor's B cells *in vivo* following immunization. We chose to study the anti-PPS4 and anti-PPS14 response as previous studies demonstrated that a significant decrease in anti-PPS4 antibody opsonophagocytic activity occurs with aging, while in contrast, the immune response to PPS14 appears well conserved in the elderly [6].

The results of this study indicate that significant differences in B cell variable gene usage remain between young and elderly adults, despite stimulation in a more

controlled cytokine environment. However, similar shifts in gene usage are noted between donors and SCID mice reconstituted with donors' PBL in response to PPS4 and PPS14, indicating the importance of cytokine environment on gene expression.

## 2. Materials and Methods

*2.1*

*Animals.* Homozygous CB-17 *scid/scid* (SCID) 21 day-old mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Mice were maintained in the Animal Care Facility at the University of Toledo. All mouse experiments were reviewed and approved by the Institutional Animal Care and Use Committee.

*2.2*

*Human volunteers.* A total of five young (<30 years old) and five elderly (≥65 years old) volunteers were included in each experimental group. Each individual was screened for past illness and present health as described previously [15]. Blood samples were collected pre-immunization and 6 weeks post-immunization for immunological analysis and B cell selection. All protocols and literature were reviewed and approved by the Institutional Review Board of the University of Toledo.

*2.3*

*Preparation of human PBL*. Peripheral blood lymphocytes (PBL) were obtained from the volunteers via leukapheresis performed at the Toledo Red Cross on day 0. Following leukapheresis, the volunteers were immunized with the 23-valent pneumococcal polysaccharide vaccine (Pneumovax, Merck & Co., Inc., Whitehouse Station, NJ). Peripheral blood lymphocytes were isolated from leukapheresis samples using

lymphocyte separation medium (Mediatech, Herndon, VA). Cells were treated with L-leucine methyl ester hydrochloride (Sigma, St. Louis, MO) to reduce the number of natural killer cells present [23]. The cells were washed three times with culture medium containing 2% human serum, suspended in RPMI-1640 and 50% T cell replacement factor (TRF), to provide human cytokines, especially IL-2 [24] and immediately used for reconstitution. T cell replacement factor is defined as the supernatant of a 24-hour culture of irradiated T cells supplemented with 5% monocytes and stimulated with pokeweed mitogen [24].

*2.4*

*Reconstitution/Immunization of SCID mice.* All SCID mice subjected to cell transfer were treated with recombinant human IL-12 (rhIL-12) (kind gift from Wyeth, Madison, NJ) according to the following protocol. The animals received 1µg of rhIL-12 i.p. on day -1, day 0 (day of reconstitution), day 1 and day 14. The mice were reconstituted i.p. with $1 \times 10^8$ PBL in 0.3 mL total volume of RPMI-1640 and 50% TRF. Hu-PBL-SCID mice were immunized i.p. with either 5µl of the pneumococcal polysaccharide vaccine (Pneumovax, Merck & Co., Inc. Whitehouse Station, NJ), corresponding to 0.25µg of each polysaccharide, or with sterile PBS at the time of cell transfer (day 0). The mice were euthanized at week 4 and human lymphocytes were recovered by washing the peritoneum and perfusing the spleens with 10 mL Hanks Buffered Salt Solution (HBSS) (Sigma, St. Louis, MO). The reconstitution and immunization schedule is illustrated in Table 1. Lymphocytes from each group of six mice were pooled for gene analysis.

*2.5*

*Affinity selection of PPS-specific B cells.* The selection of PPS-specific B cells was performed as described previously [15]. Briefly, blood was obtained from the human volunteers by venous puncture 6 weeks post-vaccination. Lymphocytes from human blood and SCID mice were isolated using lymphocyte separation medium (Mediatech, Herndon, VA). Biotinylated PPS4, PPS14 and an irrelevant polysaccharide, PPS23F, were bound to streptavidin-coated immunomagnetic beads (Dynal Biotech, Oslo, Norway). Beads coated with PPS23F were added to the lymphocytes to remove non-specific binding B cells. Bound B cells were separated from the population using a magnet and discarded. The remaining cell population was used to select for PPS4 or PPS14 specific B cells and cells were co-incubated with free (unbound) cell wall polysaccharide to remove additional non-specific antibodies.

*2.6*

*mRNA extraction, cDNA preparation and amplification.* B cells specific for PPS4 or PPS14 were used to prepare cDNA as previously described [15]. Synthesis was performed using Dynabeads Oligo$(dT)_{25}$ (Dynal Biotech, Oslo, Norway) according to the manufacture's instructions and samples were PCR-amplified using V family specific primers [14,15].

*2.7*

*Sequence analysis of VH and VL chain gene usage.* PCR products were ligated into the TOPO TA vector (Invitrogen, Carlsbad, CA), transformed into Top 10 *E. coli* and sequenced. Fifty to one hundred clones were screened for each sample and 35 sequences were analyzed. Variable region sequences were compared to the database of human Ig

by using VBASE DNA PLOT (http://vbase.mrc-cpe.cam.ac.uk). All sequences are available from GenBank under accession numbers: DQ322724 - DQ323030, DQ187638 - DQ187645, DQ187658 - DQ187666 and DQ187672 – DQ187677.

*2.8*

*Statistical analysis.* Statistical differences in antibody concentration were determined using Student's paired t-test (SPSS 11.5.1 software). Statistical differences in gene usage were determined using Poisson regression models (GENMOD procedure in SAS version 9.1). These models take into account the possible skewing by one or more individuals within our populations. P values $< 0.05$ were considered to be significant.

*2.9*

*Identification of human anti-PPS4 antibodies by ELISA.* Sera obtained from human donors were subjected to ELISA to detect the presence of anti-PPS4 specific human antibodies as described previously [25]. Serum antibody concentrations (μg/ml) were calculated based on a standard curve generated with reference serum 89SF.

## 3. Results

*3.1*

*Donors' serum antibody response*

To confirm the presence of a PS-specific serum antibody response following immunization, we measured PPS4 and PPS14-specific Ig levels of all donors pre- and post-vaccination. Both young and elderly adults demonstrated increases in IgM and IgG

antibody concentration from pre- to post-vaccination in response to PPS4 and PPS14. Donors' serum antibody response data is summarized in Table 2.

*3.2*

*Young versus Elderly Donors V gene usage*

We compared the expressed V gene repertoire of five young and five elderly volunteers in response to PPS4 and PPS14 to determine whether the age-related differences in gene usage observed in our previous studies [14,15] were maintained, despite the smaller group size (data not shown). Polysaccharide-specific lymphocytes were isolated from 5 young volunteers yielding 75 variable heavy chain (VH) sequences and 100 variable light chain (VL) sequences for both PPS4 and PPS14. In addition, 60 VH and 80 VL sequences specific for PPS4 and 75 VH and 80 VL sequences specific for PPS14 were isolated from 5 elderly volunteers. We previously performed B cell expansion studies in order to confirm that our selection method accurately selects B cells producing anti-PPS specific antibody [15]. Heavy chain gene analysis was limited to the VH3 gene family based on previous studies [26,27] indicating predominance of this family in response to pneumococcal polysaccharides. We examined the expressed V gene repertoire expressed between young and elderly donors in response to PPS4 and PPS14 and observed significant differences in gene usage, in response to both polysaccharides (data not shown). The predominant VH loci used (VH3-07, VH3-11, VH3-21, VH3-30 and VH3-33) and VL gene families, with the exception of λ2, (figures 1 and 2) were consistent with our previous studies [14,15].

*3.3*

*Young donors versus SCID mice reconstituted with young PBL V gene usage*

It has been demonstrated that immunosenescence results in significant alterations in cytokine environment [20,21]. To determine the potential influence of cytokine environment on anti-PPS VH and VL gene usage in aging, PBL (including accessory cells) derived from young and elderly donors were suspended in an identical cytokine cocktail and used to reconstitute SCID mice (hu-PBL-SCID). The SCID mice were immunized with PPV and human PPS-specific B cells were isolated 4 weeks post-immunization. Control groups consisted of unimmunized SCID mice given either young or elderly PBL, IL-12 and TRF. The control, unimmunized mice yielded low amounts of PPS-specific B cells resulting in very few sequences which were insufficient for statistical analysis (data not shown).

The expressed anti-PPS repertoire of B cells isolated from vaccinated young donors was compared to that of SCID mice reconstituted with young PBL (Y PBL SCID) and vaccinated with PPV (Figures 1 and 2). A total of 60 VH sequences and 100 VL sequences were isolated from Y PBL SCID specific for PPS4 and 45 VH sequences and 100 VL sequences isolated specific for PPS14. In response to PPS4, both young donors and Y PBL SCID predominantly expressed the VH3-30 locus (Figure 1). However, a shift in gene usage from VH3-21 and VH3-64 in donors to VH3-33 and VH3-72 in SCID mice was evident. In response to PPS14, both donors and Y PBL SCID predominantly expressed the VH3-30 gene locus; however, the percent usage of VH3-30 was higher in the Y PBL SCID (Figure 1). In addition, the donors' response was characterized by a

more diversified repertoire with a total of four different VH3 gene loci expressed (Figure 1).

Numerous significant differences were observed in VL gene usage between B cells isolated from young donors compared to those isolated from Y PBL SCID (Figure 2). However, similar patterns of gene expression were observed between groups in response to both polysaccharides. Both the anti-PPS4 and anti-PPS14 antibody responses were characterized by a substantial increase in κ2 gene usage and decrease in κ1 and κ3 gene expression in Y PBL SCID compared to young donors (Figure 2). Expression of λ2 and λ3 gene families increased in Y PBL SCID compared to donors, however, this increase was only significant for λ3 in response to PPS14 ($p < 0.05$). When variable gene usage was analyzed between donors and their respective SCID mice on an individual level, significant differences in gene expression persisted in response to both polysaccharides (data not shown). These results indicate that the addition of supplementary cytokines to human PBL in the SCID mouse model significantly influences gene expression.

*3.4*

*Elderly donors versus SCID mice reconstituted with elderly PBL V gene usage*

The *in vivo* expressed gene repertoire of elderly donors was compared to that of SCID mice reconstituted with elderly PBL (E PBL SCID) (Figure 1). A total of 75 VH sequences and 100 VL sequences specific for PPS4 as well as 60 VH sequences and 100 VL sequences specific for PPS14 were obtained from E PBL SCID. In response to both PPS4 and PPS14, notable shifts in gene usage were observed between elderly donors

compared to E PBL SCID despite the predominance of VH3-30 within both groups (Figure 1). The E PBL SCID light chain response to PPS4 was characterized by a dramatic increase in λ1 and κ2 with concomitant decrease in λ3 and κ1 gene usage compared to elderly donors (Figure 2). Similar patterns in λ1 and κ1 gene usage were noted in response to PPS14 (Figure 2) and these shifts in gene usage were similar to those observed between young donors and Y PBL SCID. In summary, significant differences in variable gene usage were noted between E PBL SCID and the elderly donors. In addition, the observed shifts in light chain gene usage resembled those noted in the young donors/Y PBL SCID.

*3.5*

*SCID mice reconstituted with young PBL versus SCID mice reconstituted with elderly PBL V gene usage*

We postulated that the observed age-related differences in donors' variable gene expression could be eliminated by stimulating young and elderly B cells in similar cytokine environments. To this purpose, we compared the expressed variable gene usage of Y PBL SCID and E PBL SCID (Figures 3 and 4). In response to PPS4, the VH gene usage of Y PBL SCID was characterized by the use of VH3-30, VH3-33 and VH3-72 (Figure 3), while, in contrast, the E PBL SCID demonstrated a decrease in VH3-30 and VH3-72 gene expression and an increase in VH3-11 and VH3-64. The response to PPS14 demonstrated a similar predominance of VH3-30 gene usage in E PBL SCID and Y PBL SCID (Figure 3); however, significant differences in other predominant gene loci remained. In response to PPS4 and PPS14, VL sequences isolated from E PBL SCID

demonstrated an increase in λ1 and κ3 gene families with a concomitant decrease in λ3 and κ2 gene families compared to Y PBL SCID (Figure 4). In conclusion, significant age-related differences in VH and VL gene expression remained when B cells from elderly and young donors were stimulated in similar cytokine environments.

**4. Discussion**

The decrease in pneumococcal vaccine efficacy observed in elderly adults remains poorly understood. Recent studies in our laboratory have shown significant differences in antibody V gene usage between young and elderly adults [14,15]. In the present study, we investigated the role of cytokines on age-related changes in anti-PPS VH and VL gene expression. A reconstituted SCID mouse model was used to analyze the V gene repertoire of PPS-specific young and elderly B cells in the presence of a more controlled cytokine environment.

To determine the effect of the cytokine environment on V gene expression, we analyzed the gene usage of human B cells isolated from the peritoneum and spleen of reconstituted SCID mice and compared it to that of the young and elderly donors. In response to both PPS4 and PPS14, we observed numerous significant differences in heavy and light chain usage between Y PBL SCID and young donors. Additionally, significant differences were observed between E PBL SCID and the elderly donors in response to both polysaccharides. These results suggest that cytokine environment potentially influences V gene expression and are consistent with our previous study in hu-PBL-SCID mice, which characterized the heavy chain gene usage in response to the meningococcal serogroup C polysaccharide (MCPS) [28]. Numerous differences in anti-

MCPS antibody molecular structure were observed between donor and hu-PBL-SCID mice, which may be indicative of the changes induced by the altered cytokine environment in the SCID mice.

Despite the differences in variable gene usage noted between donors and SCID mice, we observed similar trends among young donor/SCID pairs and elderly donor/SCID pairs in response to both polysaccharides. Both Y PBL SCID and E PBL SCID mice demonstrated significant shifts from κ1 to κ2 and λ1 gene families when compared to donors. These parallel changes in light chain gene usage between donors and mice support the notion that a modified cytokine environment is capable of altering V gene expression and thus potentially affect antibody functional activity.

We hypothesized that elderly B cells could be stimulated to express a gene repertoire resembling that of young B cells in the presence of the same soluble regulatory factors *in vivo*. Overall, the gene repertoire used by B cells isolated from E PBL SCID mice was significantly different than that observed in Y PBL SCID mice. Thus age-related differences in gene expression persisted in hu-PBL-SCID mice although they were not identical to those observed between the young and elderly donors. These age-related differences in gene expression may be explained by either persistent differences in cytokine environment between Y PBL SCID versus E PBL SCID or indicate that other factors are involved.

We encountered several limitations to the hu-PBL-SCID model which likely contributed to the persistent differences between young and elderly B cell V gene usage. For example, the generation of antigen-specific human antibody in the hu-PBL-SCID

mouse model used in this study requires the transfer of the donors' accessory cells as well as B cells. It has been well established that accessory cells are a substantial source of cytokines and that accessory cell function, specifically cytokine production, undergoes significant changes with age [17,18,29]. A study performed by Bondada *et al*. [29] demonstrated that, in addition to deficiencies in lymphocytes, defects in accessory cell function, specifically cytokine secretion, in the aged contributed to the impaired antibody response to TI-2 antigens. It is therefore plausible that, based on the presence of age-specific accessory cells, differences in cytokine environment in Y PBL SCID and E PBL SCID remained despite manipulation of the global cytokine milieu. These differences in cytokine environment may have been sufficient to result in age-related differences in gene expression.

In addition to the local cytokine environment, there were significant differences in available B cell populations between the donors and SCID mice. SCID mice were reconstituted with circulating lymphocytes from the peripheral blood of human donors. Consequently, lymphocytes that resided in the donors' bone marrow or spleen did not contribute to the repertoire of cells available for stimulation within the SCID mice. Thus, the B cells isolated from the peritoneum and spleen of the SCID mice represented only a fraction of the total TI-2 responsive human B cell population and may not provide an accurate representation of the *in vivo* human immune response. Furthermore, in the donors, the majority of lymphocytes retreat to the bone marrow following antigen stimulation [30,31] and therefore are not available at the time of B cell isolation. These

inherent differences in the B cell populations used for isolation of PPS-specific cells likely contributed to the variation in gene usage between donors and SCID mice.

To our knowledge, this is the first report in which gene expression between young and elderly adults has been analyzed using the SCID mouse model. We postulated that the observed age-related differences in donors' variable gene expression could be eliminated by stimulating young and elderly B cells in similar cytokine environments. Nonetheless, differences in variable gene usage between young and elderly persisted in the hu-PBL-SCID model and future use of this model should control for the transfer of age-matched accessory cells. SCID mouse models are unique in that they allow for analysis of human antibody following manipulation of the human cytokine environment *in vivo*. Therefore, experiments using alternative models, such as NOD/SCID murine systems [32] may prove valuable in exploring the relationship between antibody molecular structure and function and mechanisms underlying reduced vaccine efficacy in the elderly.

**Acknowledgements**

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Figure 1. Variable heavy chain gene usage of young and elderly donors versus Y PBL SCID and E PBL SCID mice in response to PPS4 and PPS14. (*) denotes significant difference between donors and mice. Statistical analysis performed using Poisson regression models, p values $< 0.05$ were considered significant.

Figure 2. Variable light chain gene usage of young and elderly donors versus Y PBL SCID and E PBL SCID mice in response to PPS4 and PPS14. (*) denotes significant difference between donors and mice. Statistical analysis performed using Poisson regression models, p values $< 0.05$ were considered significant.

Figure 3. Variable heavy chain gene usage for SCID mice reconstituted with young vs. elderly PBL in response to PPS4 and PPS14. (*) denotes significant difference between Y PBL SCID and E PBL SCID. Statistical analysis performed using Poisson regression models, p values $< 0.05$ were considered significant.

Figure 4. Variable light chain gene usage for SCID mice reconstituted with young vs. elderly PBLs in response to PPS4 and PPS14. (*) denotes significant difference between Y PBL SCID and E PBL SCID. Statistical analysis performed using Poisson regression models, p values $< 0.05$ were considered significant.

Table 1. Reconstitution schedule of SCID mice given PBL from Young Donor 1 and Elderly Donor 1. Young Donors 2-5 and Elderly Donors 2-5 followed the same schedule.

Table 2. Average serum PPS4 and PPS14-specific antibody concentration of donors pre- and post-vaccination. Significant increases from pre- to post-vaccination denoted by P values $< 0.05$ (in bold), as determined by Student's paired T-test.

Analysis of the Young and Elderly Variable Gene Repertoire in Response to Pneumococcal Polysaccharides using a Reconstituted SCID Mouse Model.

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Figure 1

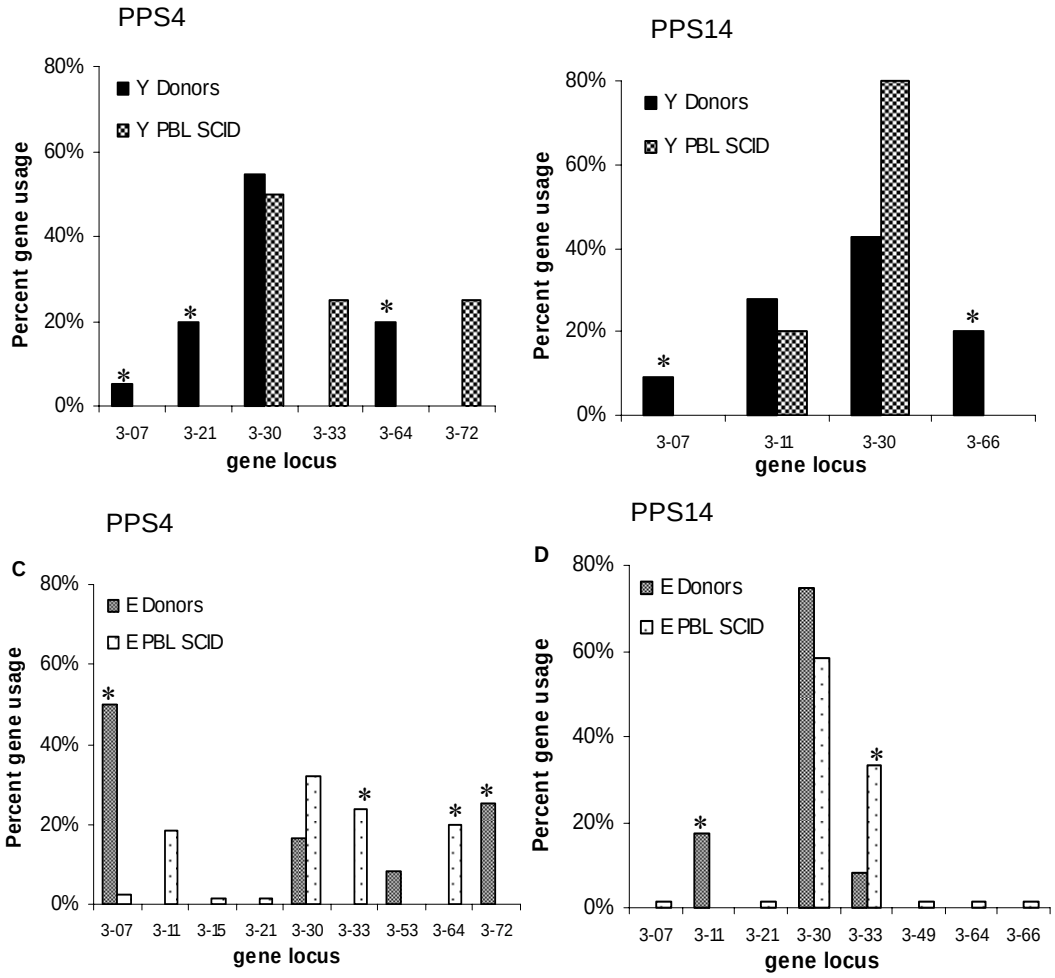

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Figure 2

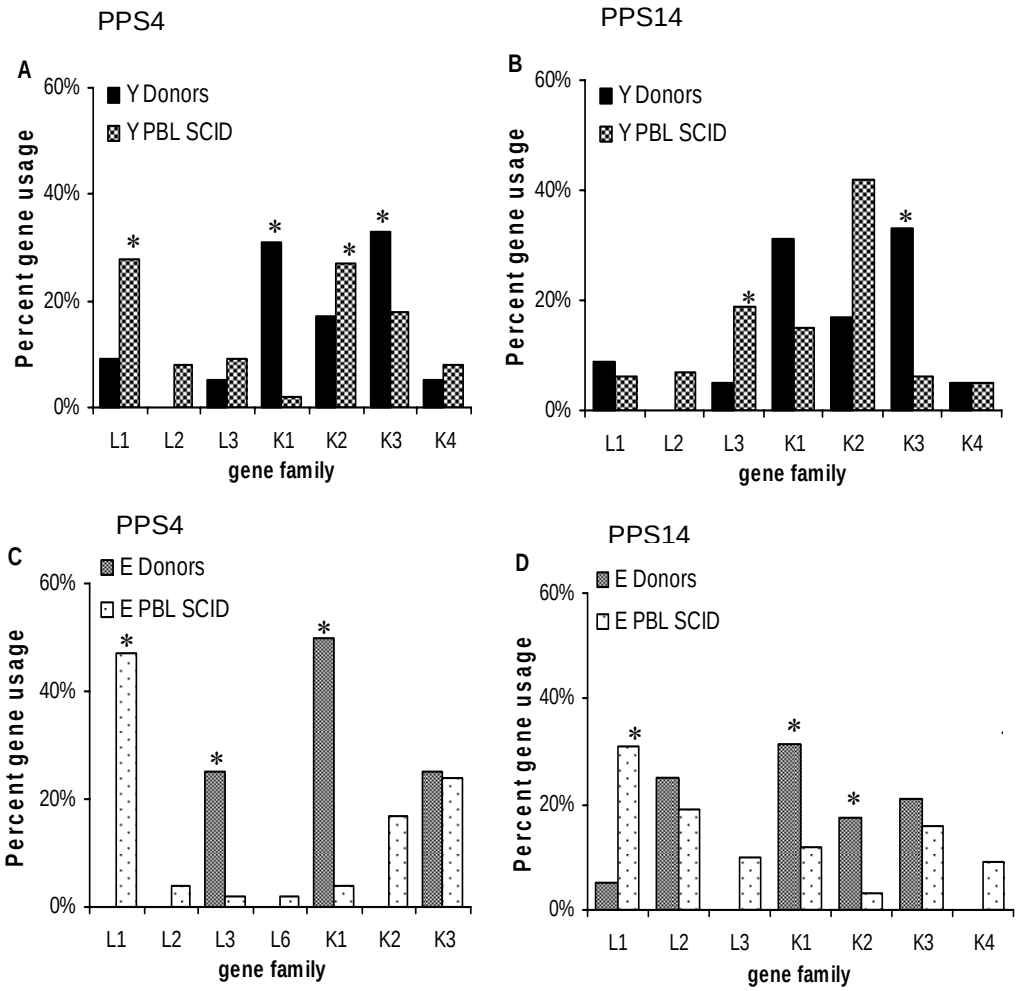

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Figure 3

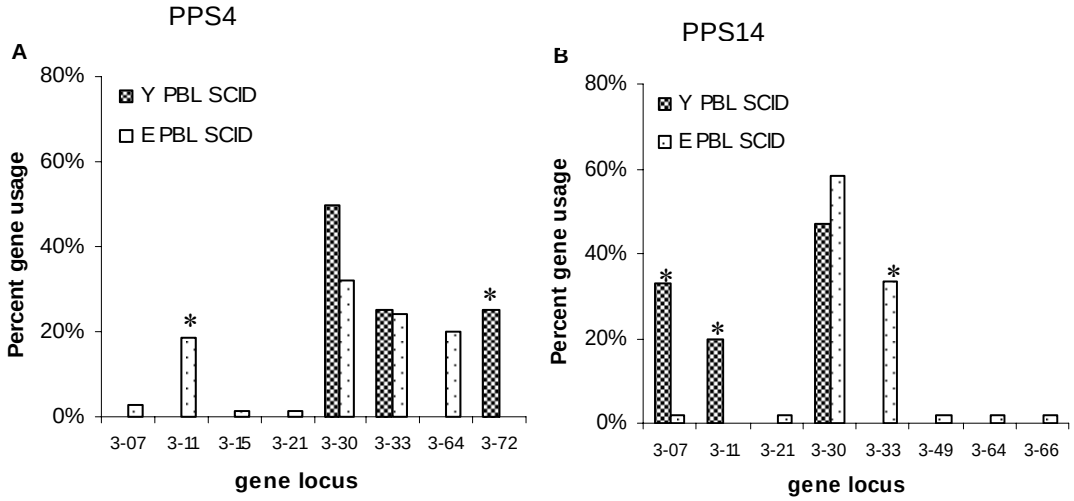

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Figure 4

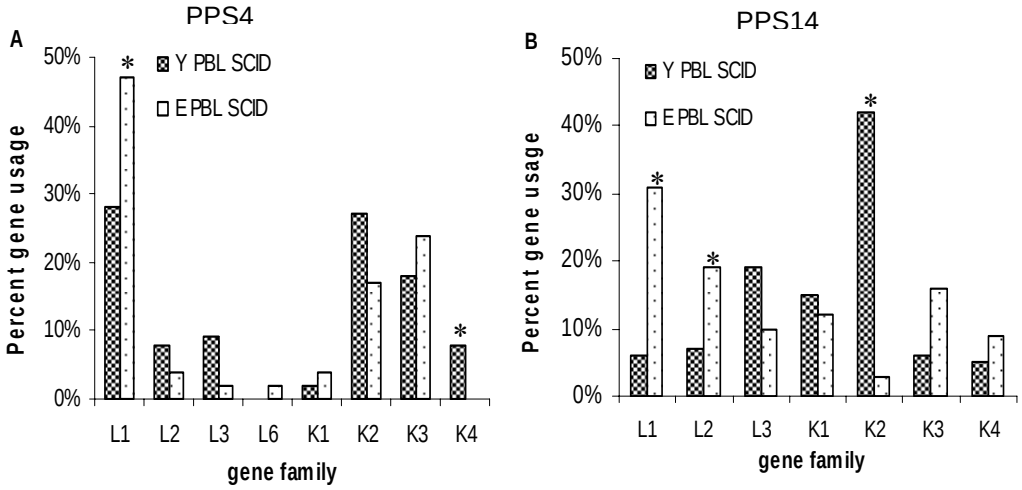

Table 1.

| | | Reconstitution schedule | | | |
|---|---|---|---|---|---|
| | | Day -1 | Day 0 | Day 1 | Day 14 |
| Young Donor 1 | PPV (n=6) | IL-12 | Y PBL, IL-12, TRF, PPV | IL-12 | IL-12 |
| | PBS (n=6) | IL-12 | Y PBL, IL-12, TRF, PBS | IL-12 | IL-12 |
| Elderly Donor 1 | PPV (n=6) | IL-12 | E PBL, IL-12, TRF, PPV | IL-12 | IL-12 |
| | PBS (n=6) | IL-12 | E PBL, IL-12, TRF, PBS | IL-12 | IL-12 |

Table 2.

| | | Donors' serum antibody concentration (ug/mL) | | | | | |
|---|---|---|---|---|---|---|---|
| | | PPS4 | | | PPS14 | | |
| | | Pre | Post | P value | Pre | Post | P value |
| Young donors | IgM | 0.97 | 1.46 | **0.03** | 0.90 | 0.92 | 0.98 |
| | IgG | 1.80 | 2.85 | 0.07 | 4.96 | 13.34 | 0.11 |
| Elderly donors | IgM | 0.16 | 0.32 | 0.08 | 0.20 | 0.27 | 0.28 |
| | IgG | 0.99 | 1.82 | **0.04** | 3.35 | 12.37 | 0.10 |

**MANUSCRIPT 2**

## Title

Comparison of the Human Immune Response to Conjugate and Polysaccharide Pneumococcal Vaccination Using a Reconstituted SCID Mouse Model

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**Abstract**

The pneumococcal conjugate vaccine (CV), although highly immunogenic in infants and young children, does not consistently demonstrate an advantage over the pneumococcal polysaccharide vaccine (PPV) in older adults. To further elucidate the adult immune response to CV, we compared its response to PPV on a molecular level using a severe combined immunodeficient (SCID) mouse model. This model allowed us to analyze a single individual's response to two different forms of antigen and define differences in gene usage elicited by these vaccines. We reconstituted SCID mice with human lymphocytes derived from an unimmunized donor; the mice were divided into two groups and immunized with either the PPV or CV. Our results demonstrate significant differences in variable gene usage in SCID mice immunized with PPV vs. CV and suggest that the nature of the immunizing agent has a significant impact on gene usage and therefore influences antibody function and vaccine efficacy.

**Key words** pneumococcal vaccines; conjugate vaccine; variable gene usage

**Abbreviated title** Pneumococcal Conjugate vs. Polysaccharide Vaccination

## 1. Introduction

The 23-valent pneumococcal polysaccharide vaccine (PPV) is highly effective in young adults [1]. However, the vaccine fails to protect children less than two years of age and it's protective efficacy is markedly reduced in elderly adults over the age of 65 [2,3]. The vaccine's poor efficacy in infants and children has led to the development of the pneumococcal conjugate vaccine (CV). The seven serotypes incorporated in the licensed vaccine account for approximately 80% of invasive pneumococcal disease in children [4]. The conjugate vaccine has been proven safe and immunogenic in children less than 5 years of age [4,5] and elicits a T cell-dependent (TD) response [6]. Despite its TD nature, administration of the CV to adults does not result in increased antibody avidity [7] or generate a significant booster response [8] as has been demonstrated in infants [9,10]. In addition, adult immunization with CV has not previously been shown to result in increased magnitude of serological response compared to PPV [8,11], although more recent data have yielded conflicting results [12].

To further elucidate the adult immune response to CV, we compared its response to that generated by PPV on a molecular level. To this purpose we used a severe combined immunodeficient (SCID) mouse model reconstituted with human peripheral blood lymphocytes. The SCID model allowed us to characterize variable (V) gene usage in response to PPV and CV using B cells derived from the same adult donors. The results of these studies indicate that significant differences in heavy and light chain variable gene usage occur between B cells stimulated with the PPV compared to those stimulated with CV. In addition, when gene usage was analyzed based on the age of the donors used to

reconstitute the SCID mice, the overall patterns of V gene usage observed in the elderly resembled those observed in the young adults. These variations in gene usage may help clarify the role of the stimulating agent (T cell independent (TI) vs. TD antigens) on variable gene expression. Our results suggest that the nature of the immunizing agent significantly influences antibody heavy and light chain variable gene usage in response to pneumococcal vaccination.

## 2. Materials and Methods

*2.1*

*Animals.* Homozygous CB-17 *scid/scid* (SCID) 21 day-old mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Mice were maintained in the Animal Care Facility at the Medical University of Ohio at Toledo. All mouse experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Toledo.

*2.2*

*Human volunteers.* Five healthy young adults (<30 years old) and five elderly adults (>65 years old) participated in this study. Each individual was screened for past illness and present health as described previously [13]. All protocols and literature were reviewed and approved by the Institutional Review Board of the University of Toledo.

*2.3*

*Preparation of human PBL*. Peripheral blood lymphocytes (PBL) were obtained from the volunteers via leukapheresis performed at the Toledo Red Cross on day 0 and purified using lymphocyte separation medium (Mediatech, Herndon, VA). Cells were treated

with L-leucine methyl ester hydrochloride (Sigma, St. Louis, MO) to reduce the number of natural killer cells present [14]. The cells were washed three times with culture medium containing 2% human serum from an unrelated donor and suspended in RPMI-1640 and 50% T cell replacement factor (TRF), to provide human cytokines, especially IL-2 [15]. T cell replacement factor is defined as the supernatant of a 24-hour culture of irradiated T cells supplemented with 5% monocytes and stimulated with pokeweed mitogen [15].

*2.4*

*Reconstitution/Immunization of SCID mice.* All SCID mice subjected to cell transfer were treated with recombinant human IL-12 (rhIL-12) according to the following protocol. The animals received 1µg of rhIL-12 (kind gift from Wyeth, Madison, NJ) i.p. on day -1, day 0 (day of reconstitution), day 1 and day 14. The mice were reconstituted i.p. with 1 x $10^8$ PBL in 0.3 mL total volume of RPMI-1640 and 50% TRF. Hu-PBL-SCID mice (n=6) were immunized i.p. with 5µl of either the pneumococcal polysaccharide vaccine (Pneumovax, Merck & Co., Inc. Whitehouse Station, NJ), corresponding to 0.25µg of each polysaccharide, or the pneumococcal conjugate vaccine (Prevnar, Wyeth Vaccines Research, Pearl River, NY), corresponding to 0.2 µg of each polysaccharide at the time of cell transfer on day 0. The mice were euthanized 4 weeks post-immunization and human lymphocytes were recovered by washing the peritoneum and perfusing the spleens with 10 cc 1x Hanks Buffered Salt Solution (HBSS) (Sigma, St. Louis, MO). Lymphocytes isolated from each group of mice (6 mice per group, 2 groups per donor) were pooled for gene analysis.

*2.5*

*Affinity selection of PPS-specific B cells.* The selection of pneumococcal polysaccharide serotype 4 (PPS4) and 14 (PPS14)-specific B cells was performed as previously described [13]. Briefly, biotinylated PPS4, PPS14 and an irrelevant polysaccharide, PPS23F, were bound to streptavidin-coated immunomagnetic beads (Dynal Biotech, Oslo, Norway). Beads coated with PPS23F were added to the lymphocytes to remove non-specific binding B cells. Bound B cells were separated from the population using a magnet and discarded. The remaining cell population was used to select for PPS4 or PPS14 specific B cells and cells were co-incubated with free (unbound) cell wall polysaccharide to remove additional non-specific antibodies.

*2.6*

*mRNA extraction, cDNA preparation, amplification and sequence analysis.* B cells specific for PPS4 or PPS14 were used to prepare cDNA as previously described [13]. Synthesis of cDNA was performed using Dynabeads Oligo$(dT)_{25}$ (Dynal Biotech, Oslo, Norway) according to the manufacture's instructions and samples were PCR-amplified 2 times using V family specific primers [13,16]. cDNA was PCR-amplified, ligated into the TOPO TA vector (Invitrogen, Carlsbad, CA), transformed into Top 10 *E. coli* and sequenced as described elsewhere [13,16]. Variable region sequences were compared to the database of human Ig by using VBASE DNA PLOT (http://vbase.mrc-cpe.cam.ac.uk) and gene family was determined. All sequences are available from GenBank under accession numbers: (currently pending).

*2.7*

*Statistical analysis.* Statistical differences in gene usage were determined using Poisson regression models (GENMOD procedure in SAS version 9.1). These models take into account the clustering of sequences within each donor. P values $< 0.05$ were considered to be significant.

## 3. Results

*3.1*

*Variable heavy chain response: PPV SCID vs. CV SCID*

We used the hu-PBL-SCID model, where lymphocytes from the same donor were transferred in identical cytokine environments, to determine potential differences in variable gene usage in response to two different forms of pneumococcal polysaccharide, namely PPV and CV. We analyzed the expressed V gene repertoire of B cells from five young and five elderly volunteers in response to PPS4 and PPS14 after vaccination with CV and compared it to the response following vaccination with PPV. Human lymphocytes were isolated from SCID mice vaccinated with PPV (PPV SCID) yielding 135 variable heavy chain (VH) sequences specific for PPS4 and 105 VH sequences specific for PPS14. Additionally, SCID mice vaccinated with CV (CV SCID) yielded 90 VH sequences specific for PPS4 and 105 sequences specific for PPS14. CV SCID mice and PPV SCID mice were further divided into two groups, those reconstituted with PBL from young donors (Y CV SCID, Y PPV SCID) and those reconstituted with PBL from elderly donors (E CV SCID, E PPV SCID). Heavy chain gene analysis was limited to the VH3 gene family based on previous studies [17,18] that demonstrated predominance of this family in response to pneumococcal polysaccharides.

In response to PPS4, lymphocytes from both Y PPV SCID mice and E PPV SCID mice expressed a gene repertoire which was significantly different from that of Y CV SCID and E CV SCID mice ($p < 0.05$). These differences were characterized by a significant increase in VH3-30 gene usage and a substantial decrease in VH3-07 and VH3-33 gene usage in PPV SCID mice compared to CV SCID mice, regardless of donors' age ($p < 0.05$) (Figure 1A and 1B). Differences remained when SCID mice with young and elderly PBL were combined, as illustrated in figure 1C. In response to PPS14, significant differences in VH gene usage occurred for 4 out of 5 loci expressed by Y PPV SCID mice compared to Y CV SCID mice (Figure 2A). In contrast, when comparing E PPV SCID mice to E CV SCID mice, the gene repertoire was characterized by the predominance of VH3-30 and VH3-33, however, no significant differences were observed between groups for these loci (Figure 2B). The percent gene usage for all PPV SCID mice compared to all CV SCID mice is shown in figure 2C.

In summary, in response to PPS4, significant differences in variable gene usage occurred between all PPV SCID mice and CV SCID mice. In response to PPS14, most significant differences were limited to sequences obtained from mice reconstituted with young PBL. Additionally, when groups were separated based on the age of the donors, the shifts in gene usage between PPV SCID and CV SCID mice in the elderly were similar to those seen in the young, however this is more apparent in response to PPS4.

*3.2*

*Variable light chain response: CV SCID vs. PPV SCID*

Lymphocytes were isolated from PPV SCID mice yielding 200 light chain (VL) sequences specific for each polysaccharide. In addition, lymphocytes were isolated from CV SCID mice yielding 160 sequences specific for PPS4 and 120 sequences specific for PPS14. The expressed VL gene repertoire in response to PPS4 demonstrated some similarities between PPV SCID mice and CV SCID mice, however significant differences in Vλ1 and Vκ1 gene families occurred regardless of the donors' age (Figure 3A and 3B). These patterns in gene usage were maintained when gene usage of young and elderly lymphocytes were combined (Figure 3C), however a significant difference in Vκ3 and Vκ4 gene usage became apparent. In response to PPS14, the expressed VL gene repertoires were characterized by substantial increases in Vλ and decreases in Vκ gene usage in PPV SCID mice compared to CV SCID mice in both age groups (Figure 4A and 4B). Furthermore, when gene usage from young and elderly lymphocytes was combined these differences were maintained (Figure 4C). When VH and VL gene expression between PPV SCID and CV SCID mice were compared on an individual level, some gene loci were shared between groups, however the sequences isolated were not identical (data not shown).

In summary, significant differences in VH and VL gene usage were observed between PPV SCID mice and CV SCID mice in response to both polysaccharides. Additionally, when SCID mice were divided based on the age of donors used for

reconstitution, notable polysaccharide-specific trends were observed between the young and elderly lymphocytes.

## 4. Discussion

The PPV vaccine, although effective in young adults, has a reduced efficacy in the elderly. Early studies have shown that in adults, the antibody concentration in response to the CV is similar to that of the PPV response, and thus by that measure alone, may offer no advantage over immunization with PPV in adults [8,11]. Previous studies have investigated antibody variable gene usage following vaccination with either PPV or CV [18-20]. However, these studies were limited by small group size and volunteers were randomly assigned to receive either PPV or CV vaccination. To our knowledge, studies that directly address the influence of antigen composition (PPV vs. CV) on V gene usage have not been performed.

The hu-PBL-SCID model has been used previously to analyze the *in vivo* human antibody response to a variety of polysaccharide antigens [21-23] as well as TD antigens [24,25]. Previous studies have shown that this model produces human lymphocytes which are functional, antigen specific and representative of all Ig isotypes [23,24,26,27]. Human cells used for reconstitution increase in number and survive for up to 6 months in the SCID mice [24]. In addition, all major human lymphocyte populations are recovered from SCID mice; however numbers can become skewed over time [24].

We have used this model to evaluate the human immune response of a single individual to two different forms of antigen, namely, the PPV and the CV. Therefore, we were able to analyze a single individual's response to two different forms of antigen and

define differences in VH/VL gene usage elicited by these vaccines. We reconstituted SCID mice with human PBL derived from an unimmunized donor; the mice were divided into two groups and immunized with either the PPV or CV. Our results demonstrate significant differences in variable gene usage in SCID mice immunized with PPV vs. CV and that these differences occur regardless of the donor's age.

Studies investigating variable gene usage of human B cells in the SCID mouse model are limited in number. In a study by Smithson *et al*. [22], the authors analyzed the variable heavy chain sequences of the meningococcal capsular polysaccharide (MCPS) antibody response generated by B cells of a single donor in the SCID mouse model and compared it to the donor's response *in vivo*. The authors demonstrated that the expressed VH repertoire in the SCID mice is markedly different from that of the donor *in vivo*. However, when comparing heavy chain gene usage of SCID mice in the current study to gene usage observed in donor sequences isolated previously [13], we observed similar predominant gene family expression in response to the same antigen. We postulate that the differences in gene usage noted in response to MCPS are antigen specific and not comparable to the observed responses to pneumococcal polysaccharides. In the present study, heavy chain and light chain gene usage demonstrated significant differences between PPV SCID and CV SCID mice in response to both polysaccharides. Of particular interest, CV SCID mice demonstrated a dramatic decrease in Vλ gene usage (specifically Vλ1) with a concomitant increase in Vκ gene usage compared to PPV SCID mice in response to both PPS4 and PPS14. Because SCID mice were reconstituted with PBL from the same donor, the accessory cells, cytokine environment and lymphocyte

populations remained constant between groups of PPV and CV SCID mice. Thus, the only difference between groups was the nature of antigen presentation (TI vs. TD). We therefore postulate that the significant shift in VL gene expression was directly related to vaccine composition and may suggest the potential importance of the VL in response to pneumococcal antigens. The significance of light chain gene expression on antibody function and specificity has been demonstrated previously in response to *S. pneumoniae* [28] and *Haemophilus influenzae* type b (Hib) [29]. Lucas *et al*. [29] reported that VL gene usage and CDR3 length play a significant role in anti-Hib antibody binding affinity. Further analysis of these relationships, particularly in response to pneumococcal antigens would therefore be of significant value. Additionally, differences in gene usage in response to PPV vs. CV have not previously been analyzed. Previous studies [20,30] have demonstrated both Vκ and Vλ gene usage in response to PPV, while others [31,32] demonstrated a dominance of Vλ genes. Similarly, we observed a predominance of Vλ gene usage in PPV SCID mice while their identical counterparts immunized with CV, demonstrated predominant Vκ gene usage. This shift from Vλ to Vκ gene expression could be related to potential differences in antibody specificity and deserves further investigation.

The observed differences in light chain gene usage between PPV and CV may be related to other factors such as differences in epitope recognition or cytokine microenvironment. In a previous study by Reason and Zhou [33], the authors linked antibody VL gene usage with specific antigenic epitopes of PPS23F. They suggest that VH and VL gene pairing is often unique for each epitope, despite the repetitive nature of

capsular polysaccharides. Therefore, variation in epitopes created by the PPV vs. CV could help explain differences in gene usage and may lead to a better understanding of how these vaccines protect against disease. Furthermore, it has been well established that antigenic stimulation with different forms of antigen create different cytokine environments (as reviewed in [34]). In addition, previous work has shown that cytokines are capable of influencing gene expression [35] and recent data from our laboratory support these findings (unpublished observations). As a result, the different mechanisms of protection elicited by these two vaccines could result in changes in cytokine environment sufficient to induce differences in gene expression. Therefore, the nature of the immunizing agent and the microenvironment it creates may influence gene usage, and in concert with recent findings [12], may account for differences in antibody functional activity.

In summary, significant differences in variable gene usage are present when lymphocytes are stimulated with two different forms of antigen regardless of the donor's age. These differences are likely due to the different mechanisms (TI vs. TD) used to elicit the anti-pneumococcal immune response. Together, these results suggest that the nature of the immunizing agent may have a significant impact on VH/VL gene usage and therefore influence antibody functionality and vaccine efficacy. To further our understanding of this phenomenon, we are currently investigating relationships between antibody molecular structure and functional activity.

**Acknowledgements**

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Figure 1. Variable heavy chain gene usage in response to PPS4 for Y PPV SCID vs. Y CV SCID (A), E PPV SCID vs. E CV SCID (B), and combined age groups PPV SCID vs. CV SCID (C). (*) denotes significant difference between PPV and CV groups. Statistical analysis performed using Poisson regression models, p values $< 0.05$ were considered significant.

Figure 2. Variable heavy chain gene usage in response to PPS14 for Y PPV SCID vs. Y CV SCID (A), E PPV SCID vs. E CV SCID (B), and combined age groups PPV SCID vs. CV SCID (C). (*) denotes significant difference between PPV and CV groups. Statistical analysis performed using Poisson regression models, p values $< 0.05$ were considered significant.

Figure 3. Variable light chain gene usage in response to PPS4 for Y PPV SCID vs. Y CV SCID (A), E PPV SCID vs. E CV SCID (B), and combined age groups PPV SCID vs. CV SCID (C). (*) denotes significant difference between PPV and CV groups. Statistical analysis performed using Poisson regression models, p values $< 0.05$ were considered significant.

Figure 4. Variable light chain gene usage in response to PPS14 for Y PPV SCID vs. Y CV SCID (A), E PPV SCID vs. E CV SCID (B), and combined age groups PPV SCID vs. CV SCID (C). (*) denotes significant difference between PPV and CV groups. Statistical analysis performed using Poisson regression models, p values $< 0.05$ were considered significant.

Comparison of the Human Immune Response to Conjugate and Polysaccharide Pneumococcal Vaccination Using a Reconstituted SCID Mouse Model

Anne K. Shriner, S. Louise Smithson, Deborah M. Prinz, Bradley Rabquer, Sadik Khuder, Roger Goomber and M .A. Julie Westerink

Figure 1

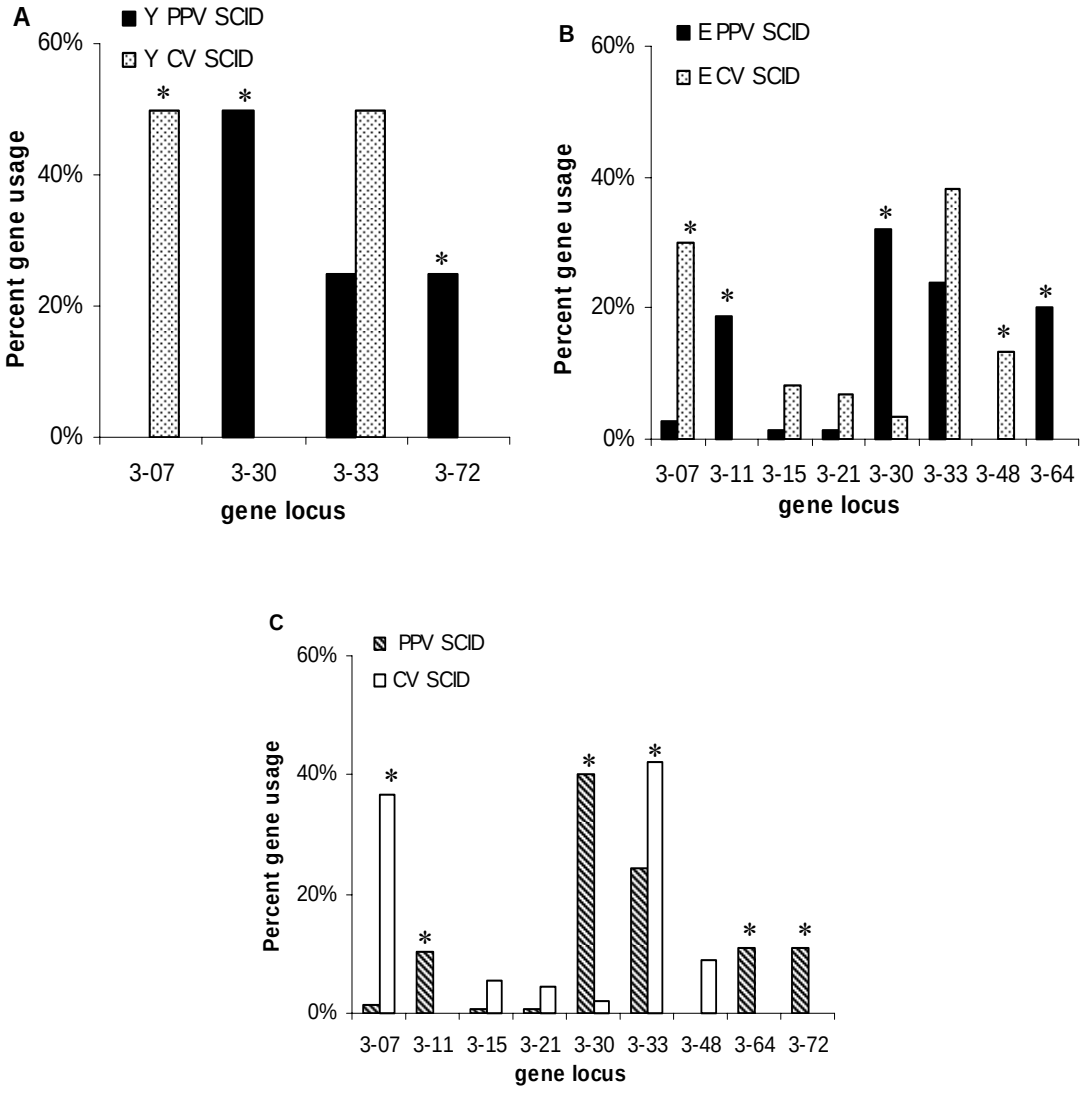

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Figure 2

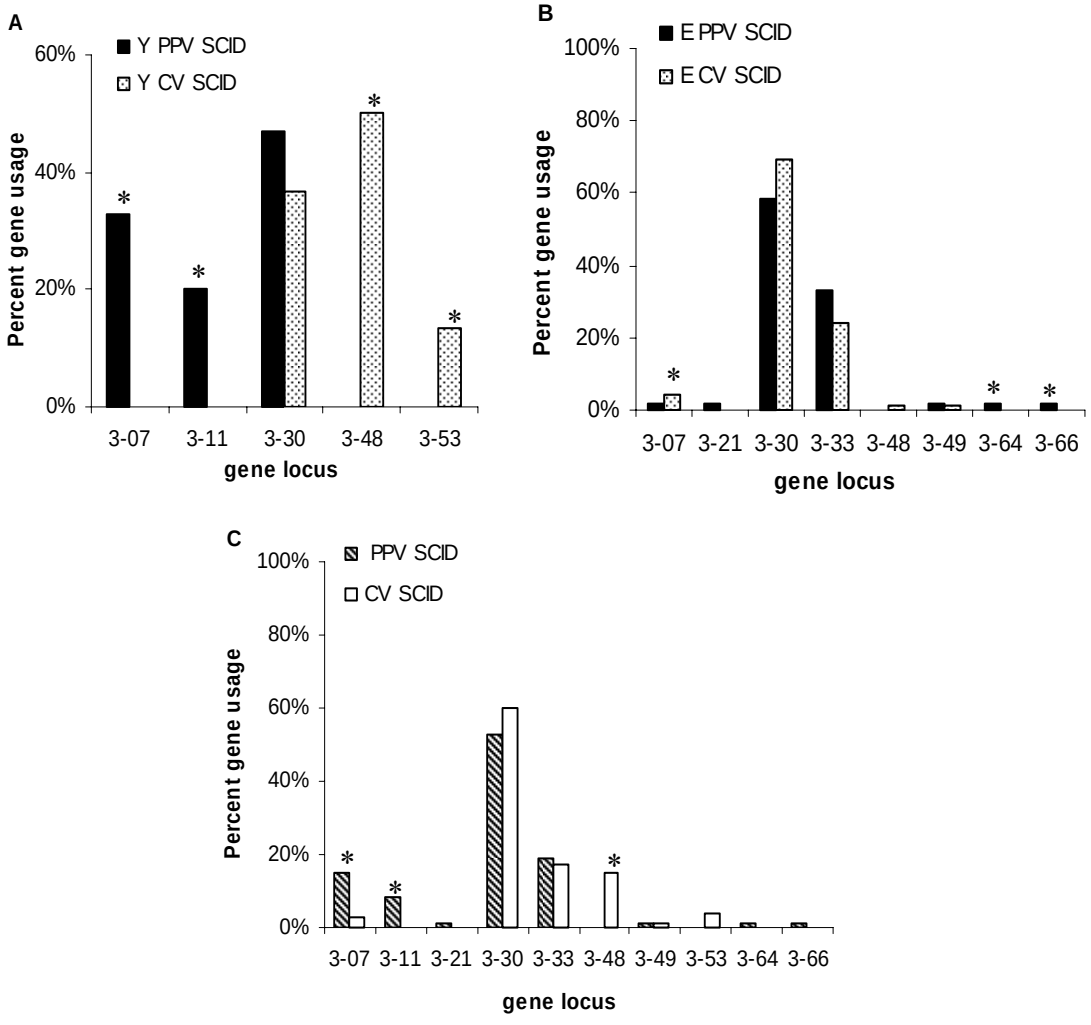

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Figure 3

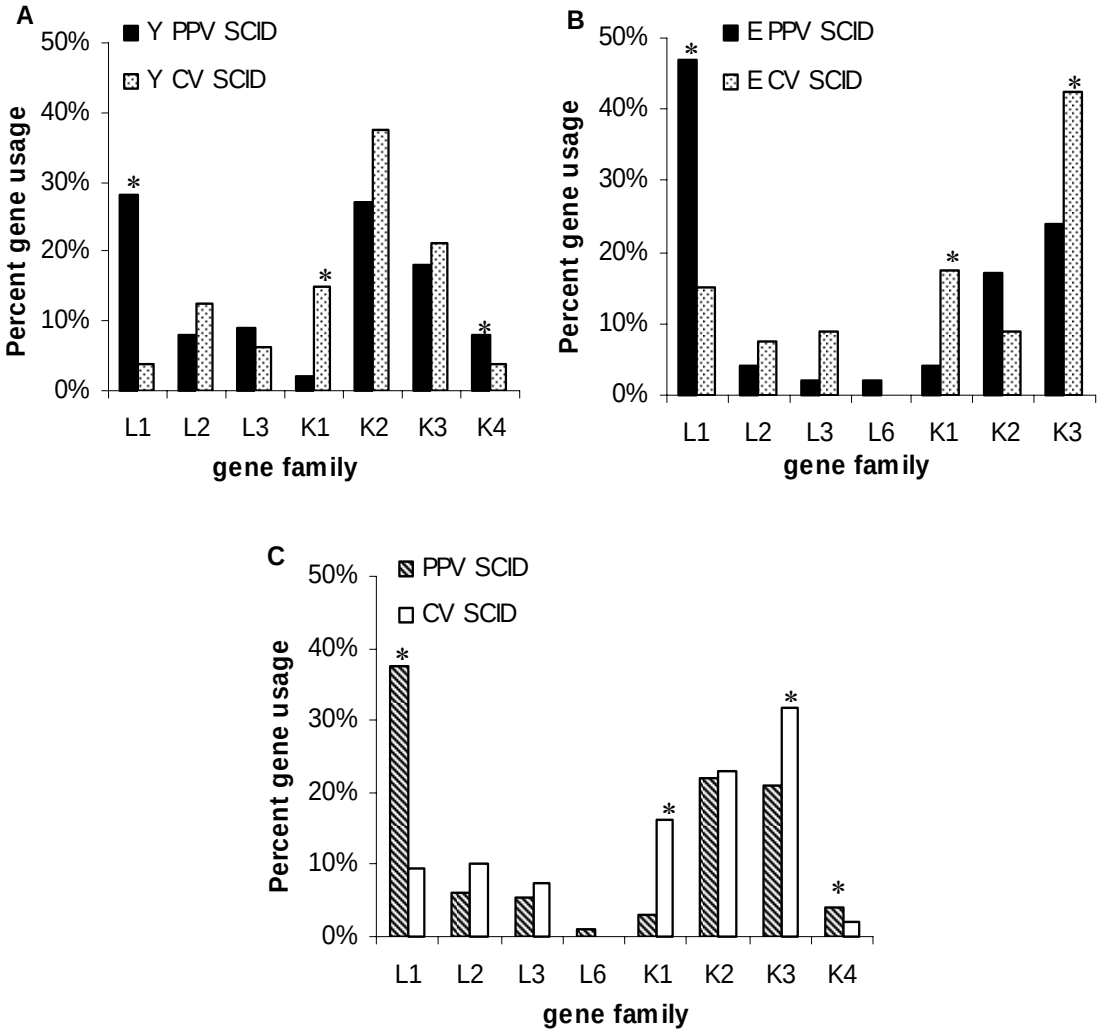

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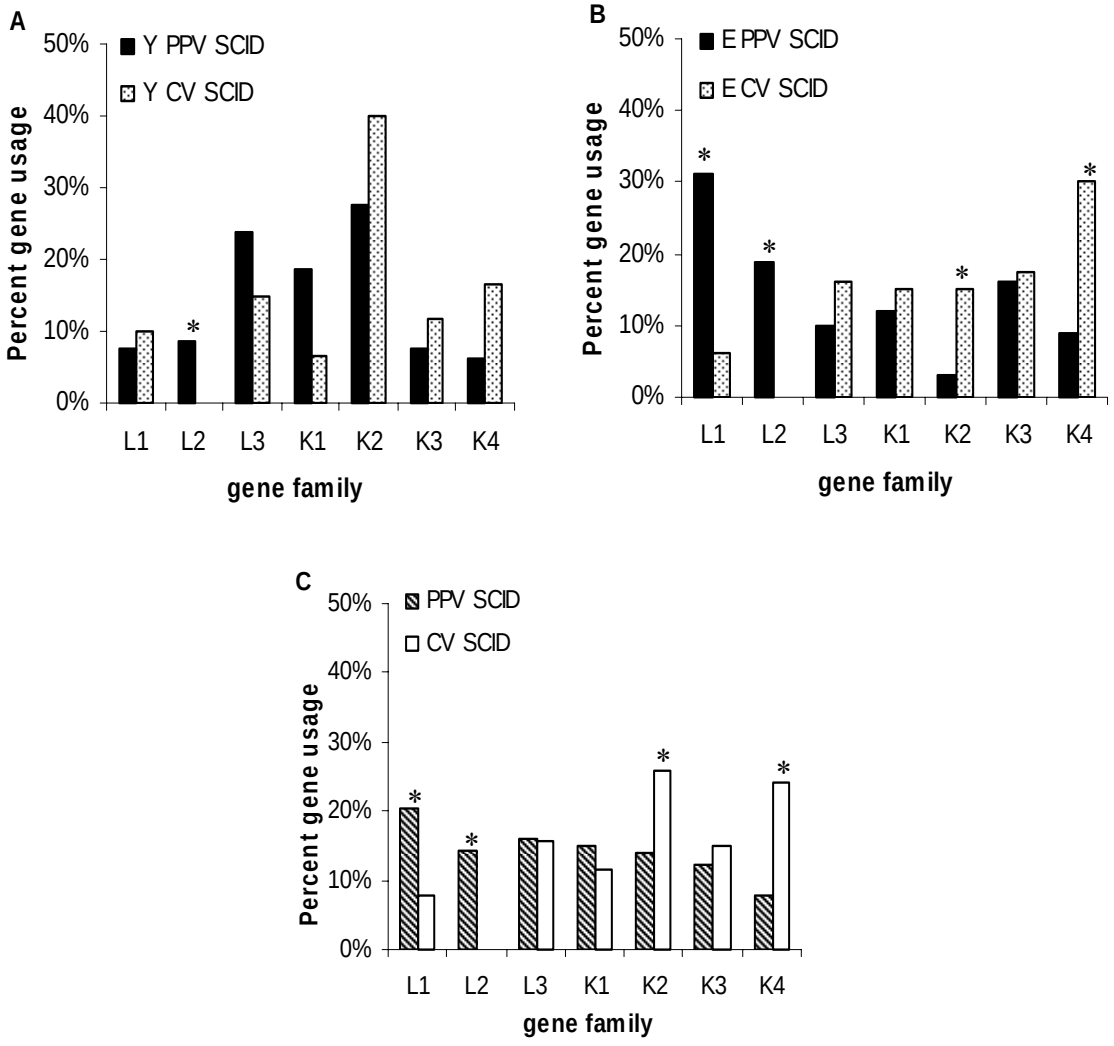
Figure 4



## MANUSCRIPT 3

**Title:** Analysis of the Pre- and Post-vaccination Pneumococcal Polysaccharide Serotype 4-Specific Antibody Repertoire

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**Key words:** Pneumococcal polysaccharide vaccine, antibody variable gene repertoire, gene expression

**Running head:** Pre- vs. Post-vaccination PPS4-specific antibody repertoire

**Abstract**

Understanding the molecular diversity of polysaccharide-specific antibodies is becoming increasingly important in predicting the human immune response against pneumococcal disease. Studies have demonstrated that polysaccharide-specific antibodies isolated 5-10 days post-vaccination have undergone significant somatic mutation, indicating a recall response in which memory B cell clones become activated and expand. We isolated polysaccharide-specific antibodies pre- and 7 days post-immunization with the pneumococcal polysaccharide vaccine. Our results demonstrate significant differences in variable gene usage and unassociated somatic mutations between B cells isolated pre-vaccination and those isolated 7 days post-vaccination. Our data suggest that the sequences isolated post-vaccination do not represent a clonal expansion of B cell precursors in the periphery prior to vaccination and alternative sites should be explored.

## Introduction

The incidence of pneumococcal disease has decreased in recent years, largely due to the advent of pneumococcal vaccines. Nonetheless, the 23-valent pneumococcal polysaccharide vaccine, although effective in young adults, demonstrates reduced efficacy in elderly populations [1,2]. In the past, vaccine evaluation has been limited to serological assessment, with anti-pneumococcal polysaccharide (PPS) antibody concentrations serving as the main surrogate marker of protection. Previous work has demonstrated that antibody avidity as measured by opsonophagocytic assays, not concentration, is a more reliable predictor of functional antibody activity [3,4]. Furthermore, variation in antibody avidity in response to polysaccharide (PS) antigens has been correlated with differences in antibody molecular structure [5]. Thus, understanding the molecular diversity of PPS-specific antibodies is becoming increasingly important in predicting the human immune response against pneumococcal disease. We [6,7] and others [8-11] have performed studies characterizing the immune response to *Streptococcus pneumoniae* PS antigens on a molecular level. Previous data have established that the anti-PPS gene repertoire is largely limited to the VH3 gene family [9,10]. In addition, significant differences have been observed between serotypes [10,11], age groups [6,7] and vaccine composition [12].

Polysaccharide antigens have long been classified as T-cell independent (TI); it has therefore been assumed that they elicit a *de novo* antibody response. However, several studies have demonstrated that PPS-specific antibodies isolated 5-10 days post-vaccination have undergone significant somatic mutation, suggesting that immunological

memory is induced by previous exposure to *S. pneumoniae* or cross-reactive antigen [8-11]. These extensive mutations, occurring one week post-vaccination, indicate a recall response in which memory B-cell clones become activated and expand. Despite these findings, little is known regarding the molecular structure of PPS-specific antibodies existing prior to vaccination. Thus, the goal of this study was to characterize the PPS-specific pre-vaccination antibody repertoire and investigate its potential role on the post-vaccination repertoire.

Our results demonstrate significant differences in variable gene usage and unrelated somatic mutations between PPS4-specific B cells isolated prior to vaccination and those isolated 7 days post-vaccination. Therefore, the variable gene sequences isolated post-vaccination do not represent a clonal expansion of PPS-specific B cell precursors in the periphery prior to vaccination. Rather, they indicate that the pre-vaccination memory B cells induced by colonization or cross-reactive antigen do not circulate in the peripheral blood but are likely present in the spleen.

**Materials and Methods**

*Human volunteers.* A total of six young adults (<30 years old) were used for this study. Each individual was screened for past illness and present health as described previously [7]. Volunteers were vaccinated with a single dose of the 23-valent pneumococcal polysaccharide vaccine (Pneumovax, Merck & Co., Inc., Whitehouse Station, NJ). Blood samples were collected pre-immunization and 7 days post-vaccination for immunological analysis and B cell selection. All protocols and literature were reviewed and approved by the Institutional Review Board at the University Medical Center.

*Identification of human anti-PPS4 antibodies by ELISA.* Sera obtained from human donors were subjected to ELISA to detect the presence of anti-PPS4 specific human antibodies as described previously [13]. Serum antibody concentrations (μg/ml) were calculated based on a standard curve generated with US reference pneumococcal antiserum 89SF (a kind gift of Dr. Carl Frasch, FDA, Bethesda MD).

*PPS-specific B cell isolation and cDNA amplification.* The selection of PPS4-specific B cells and cDNA amplification was performed as described in detail elsewhere [7]. Briefly, biotinylated PPS4 and PPS23F, were bound to streptavidin-coated immunomagnetic beads (Dynal Biotech, Oslo, Norway). Beads coated with PPS23F were added to isolated lymphocytes to remove non-specific binding B cells. Bound B cells were separated from the population using a magnet and discarded. The remaining cell population was used to select for PPS4 specific B cells and cells were co-incubated with free (unbound) cell wall polysaccharide to remove additional non-specific antibodies. Synthesis of cDNA was performed using Dynabeads Oligo$(dT)_{25}$ (Dynal Biotech, Oslo, Norway) according to the manufacture's instructions and samples were PCR-amplified two times using variable (V) gene family specific primers [6,7]. The specificity of this method was confirmed as previously described [7].

*Sequence analysis of VH and VL chain gene usage.* PCR products were ligated into the TOPO TA vector (Invitrogen, Carlsbad, CA), transformed into Top 10 *E. coli* and sequenced. Variable region sequences were compared to the database of human Ig by using VBASE DNA PLOT (http://vbase.mrc-cpe.cam.ac.uk). All sequences are available from GenBank under accession numbers: DQ840661 – DQ841117.

*Statistical analysis.* Statistical differences in anti-PPS4 antibody concentration were determined using Student's Paired T-test (SPSS 11.5.1 software). Statistical differences in gene usage between time points were determined using Poisson regression models (GENMOD procedure in SAS version 9.1). These models take account of the clustering of sequences within each donor. Statistical differences in gene usage within each donor were determined using Fisher's Exact test (SPSS 11.5.1 software). $p$ values $< 0.05$ were considered to be significant for all statistical analysis.

**Results**

Serum antibody concentration

To determine the amount of PPS4-specific antibodies prior to vaccination, we measured serum antibody concentration by ELISA pre- and 7 days post-vaccination. All donors demonstrated detectable serum PPS4-specific IgM and IgG antibody concentrations pre-vaccination and all responded to vaccination with increased levels of PPS4 antibody (data not shown). Anti-PPS4 antibody ranged from 0.1-9.6μg/ml pre-vaccination and significantly increased to 0.8-31.9μg/ml post-vaccination ($p < 0.05$). These results are in agreement with previous studies which show that PPS-specific antibody is present prior to vaccination [7,9,10,14].

PPS4-specific variable gene usage

*Day 0 vs. unselected peripheral B cells*

We isolated B cells from our donors on day 0 and day 7, and compared their heavy (VH) and light (VL) chain gene usage to the variable gene repertoire of IgM+ and CD19+ B cells previously described [15-17] (Figures 1 and 2). We analyzed a total of 88

heavy chain and 47 light chain sequences from B cells isolated on day 0. Heavy chain gene analysis was limited to the VH3 gene family based on previous studies [9,10] indicating predominance of this family in response to pneumococcal polysaccharides. PPS4-specific sequences isolated on day 0 were characterized by a predominance of VH3-30, VH3-21, VH3-11 and VH3-33 gene loci, which were dramatically over-expressed compared to CD19+ B cells (Figure 1) [17]. Light chain gene usage on day 0 consisted of 7 gene families total with the predominant expression of Vκ1, Vκ2 and Vλ1. VL gene expression of the PPS-specific B cells isolated at day 0 was distinct from that of IgM+ B cells [15,16] (Figure 2), indicative of a PPS-specific repertoire.

*Day 0 vs. day 7*

To determine whether the post-vaccination PS-specific antibody repertoire results from expansion of PPS-specific memory B cells present prior to vaccination, we compared the variable gene repertoires of PPS-specific B cells isolated pre-vaccination to that of B cells isolated 7 days post-vaccination (Figures 1 and 2). We analyzed a total of 78 heavy chain and 80 light chain sequences from B cells isolated on day 7. Heavy chain gene usage demonstrated a dramatic shift from day 0 to day 7. Sequences isolated on day 7 demonstrated a significantly increased representation of VH3-07, VH3-15, VH3-33, VH3-66, VH3-72 and VH3-73 gene loci compared to sequences isolated at day 0 (Figure 1). In contrast, post-immunization gene loci, VH3-11, VH3-21 and VH3-48, were represented significantly less frequently than on day 0. Light chain gene usage also demonstrated significant differences from pre- to post-vaccination. Post immunization significantly more sequences belonging to the Vκ3, Vκ4 and Vλ2 gene families were

isolated than pre-immunization. In addition, there was a concomitant and significant decrease in sequences belonging to the Vκ1, Vκ2 and Vλ3 gene families versus pre-immunization. VH and VL gene expression was also analyzed by individual (Table 1). Significant differences in gene usage pre- to post-immunization were observed within each individual for both heavy and light chains with the exception of donor 1. The number of distinct VH3 gene loci or VL gene families expressed by each donor at day 0 and day 7 ranged between 1 and 5 (Table 1), however, no significant difference in oligoclonality between time points was observed (data not shown). Variable heavy chain genes isolated at day 0 and day 7 from selected donors are shown in Table 2. Identical gene loci, but not CDR3 region, were used between donors. Unique CDR3 regions identified at day 0 were not identified at day 7.

*Percent identity to germ line and somatic hypermutation*

The percent sequence identity to germ line was determined for all sequences (Table 3). Extensive somatic mutation of VH and VL sequences was observed both pre- and post-immunization. Percent identity to germ line ranged from 89.7 to 100% and no significant differences were observed between sequences isolated on day 0 and day 7 for VH and VL (Table 3). The majority of mutations occurred in the CDR regions, although framework mutations were not uncommon. Table 2 shows the CDR residues and replacement mutations of all VH sequences isolated at day 0 and day 7 of selected donors. Within each donor, all sequences isolated on day 0 were unique and not clonally related even if they used the same gene locus. In contrast, sequences isolated on day 7 that contained identical CDR3 residues, often presented varying substitutions in the

CDR1 and CDR2 residues, and therefore likely arose from the same initial rearrangement event (Table 2). Donor 1, for example, utilized the VH3-11 locus at day 0 and day 7. Two of the three VH3-11 sequences isolated on day 7 contain identical CDR3 regions (EIDLDGDSSGGFDS) yet have distinctly mutated CDR1 and CDR2 regions, characteristic of sequences derived from the same precursor. These patterns in heavy chain gene usage were observed in all donors (data not shown). In addition, light chain sequences demonstrated similar results, although CDR3 sequences were more conserved within and between donors (data not shown). VL sequences isolated pre-vaccination within donors were often distinct from one another, however, sequences isolated on day 7 showed evidence of being derived from the same rearrangement event (data not shown).

In summary, significant differences in VH and VL gene usage were found between sequences isolated on day 0 and day 7. In addition, the types of somatic mutations observed suggest that the sequences isolated 7 days post-vaccination do not reflect a clonally expanded pre-immunization B cell repertoire present in the peripheral circulation.

**Discussion**

Advances in molecular biology have allowed for investigation of the genetic diversity involved in the humoral immune response. However, our understanding of the molecular mechanisms associated with the response to pneumococcal antigens is limited. Capsular polysaccharides are T cell independent and have long been considered incapable of generating long-lasting immunological memory. Recent studies have begun to characterize the molecular antibody response to polysaccharide antigens following

pneumococcal vaccination [6-11]. Several investigators have observed a high frequency of somatic mutations in PPS-specific V genes isolated 7 days post-vaccination [8-11] which likely reflects a recall response generated from pre-existing memory B cells. To explore this concept further, we investigated the PPS4-specific variable gene repertoire prior to pneumococcal vaccination and its potential influence on the post-vaccination repertoire. We postulated that if extensively mutated PPS-specific sequences are indeed a result of activation and expansion of memory B cell clones, these clones may be present in the periphery prior to vaccination. However, our results demonstrated populations of B cells expressing two distinct V gene repertoires pre- vs. post-vaccination. Significant differences in gene expression were observed on a population level, as well as within individual donors. In addition, the majority of sequences had undergone somatic hypermutation and there was no significant difference between those isolated pre- vs. post-vaccination. Although some VH and VL sequences isolated 7 days post-vaccination were clonally related, there was no evidence of clonal expansion or affinity maturation of the pre-immune response.

Our data suggest that although such B cells can be found in the periphery, the precursors to the heavily mutated PPS4-specific cells likely reside elsewhere. Previous studies have demonstrated that activation of B cells by TI antigens predominantly occurs in the marginal zone (MZ) of the spleen [18-20]. In addition, human MZ B cells often have a pre-diversified Ig repertoire during ontogeny and prior to their differentiation into TI-responsive B cells [21]. While some MZ memory B cells have been found in circulation [22], most are localized to the spleen [18]. It is currently unknown whether

the spleen is the site for the generation of memory B cells or acts mainly to support their survival. It may be advantageous to target marginal zone B cells in an attempt to delineate the variable gene repertoire which precedes that of the post-vaccination PPS-specific B cell repertoire.

The identification of extensively modified PPS4-specific variable genes prior to vaccination, and the high levels of anti-PPS antibody seen in most donors, suggests previous natural exposure to either intact pneumococci, possibly via pneumococcal colonization, or exposure to other cross-reactive antigens. Pneumococcal colonization frequently occurs early in childhood [23] although it often does not result in invasive disease [24]. A recent study by Malley et al. [25] showed that immunity to pneumococcal colonization may be induced without antibody present, and protection from subsequent challenge requires the help of CD4+ T cells. Therefore, a TD response brought on by pneumococcal colonization may be sufficient for the induction of immunological memory in an unvaccinated host. Additionally, exposure to potentially cross-reactive antigens, i.e., polysaccharides naturally conjugated or within close proximity to peptides on the surface of the cell wall, could result in a similar TD memory response and could help explain the PPS-specific pre-vaccination repertoire observed here. Furthermore, numerous studies have demonstrated the role of pre-existing natural antibody in providing innate protection against polysaccharide antigens [26-28]. B-1a cells responsible for the majority of this natural antibody secretion were previously associated with a strictly limited variable gene repertoire [29,30]. However, more recent studies have demonstrated that they express diverse repertoires [31,32] and together with

B-1b cells, play key roles in innate and adaptive immunity to *S. pneumoniae* [26]. Therefore, we cannot rule out the potential contribution of B1 cell subsets to the PPS4-specific repertoire observed prior to vaccination.

To our knowledge, this is the first study performed to characterize the polysaccharide-specific antibody variable gene repertoire prior to vaccination. We observed significant differences in variable gene usage and unassociated somatic mutations between PPS4-specific B cells isolated prior to vaccination and those isolated 7 days post-vaccination. Our data imply that the variable gene sequences isolated post-vaccination do not represent a clonal expansion of PPS-specific B cell precursors in the periphery prior to vaccination. On the contrary, analysis of the pre-vaccination PPS4-specific B cells suggests the involvement of pre-existing antibody induced by pneumococcal colonization, cross-reactive antigens or natural antibody. Based on the results presented here and those reported previously [18,22], full exploration of the pre-vaccination B cell repertoire will likely require the isolation of splenic, specifically marginal zone B cells; hence murine studies should be considered as a practical alternative. Such studies would allow us to further our understanding of the mechanisms involved in the humoral response pneumococcal polysaccharides.

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Table 1. Individual donors' variable heavy and light chain gene usage of PPS4-specific B cells isolated on day 0 and day 7.

| | Variable Heavy Chain | | | | | Variable Light Chain | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Day 0 | | Day 7 | | | Day 0 | | Day 7 | |
| Donor | VH | % Usage | VH | % Usage | Donor | VL | % Usage | VL | % Usage |
| 1 | 3-11 | 23 | 3-11 | 8 | 1 | L1 | 46 | L1 | 73 |
| | 3-30 | 54 | 3-30 | 38 | | L2 | | L2 | 7 |
| | 3-33 | 23 | 3-33 | 31 | | L3 | 31 | L3 | 20 |
| | | | 3-15 | 15 | | K3 | 8 | | |
| | | | 3-73 | 8 | | K4 | 15 | | |
| 2 | 3-11 | 42 * | 3-11 | 7 * | 2 | ND | | L1 | 38 |
| | 3-15 | 4 | 3-07 | 36 * | | | | L3 | 8 |
| | 3-33 | 19 | 3-33 | 21 | | | | K1 | 38 |
| | 3-30 | 27 | 3-66 | 29 * | | | | K3 | 16 |
| | 3-48 | 8 | 3-73 | 7 | | | | | |
| 3 | 3-30 | 7 * | 3-30 | 91 * | 3 | K1 | 22 | K3 | 85 * |
| | 3-21 | 93 * | 3-33 | 9 | | K2 | 78 * | K2 | 15 |
| 4 | 3-21 | 67 * | 3-07 | 100 * | 4 | K1 | 55 | K1 | 14 |
| | 3-30 | 33 * | | | | K2 | 36 * | K2 | 50 * |
| | | | | | | L1 | 9 | L1 | 36 |
| 5 | 3-30 | 100 * | 3-30 | 47 * | 5 | K1 | 100 * | K1 | 25 * |
| | | | 3-07 | 15 | | | | K3 | 8 |
| | | | 3-11 | 15 | | | | K4 | 25 |
| | | | 3-33 | 23 | | | | L1 | 17 |
| | | | | | | | | L2 | 25 |
| 6 | 3-30 | 76 * | 3-30 | 25 * | 6 | L1 | 91 * | L1 | 15 * |
| | 3-33 | 8 | 3-33 | 17 | | L2 | 9 | L2 | 23 |
| | 3-72 | 8 * | 3-72 | 58 * | | | | K1 | 31 |
| | 3-11 | 8 | | | | | | K3 | 8 |
| | | | | | | | | K4 | 23 |

(*) denotes significant differences between day 0 and day 7. Statistical analysis performed using Poisson regression models, p values < 0.05 were considered significant.

Table 2. Comparison of VH3 gene repertoires of selected donors at day 0 vs. day 7 post-vaccination.

| Donor | Day of isolation | *n* | % ID | Gene locus | Sequence of H chain CDR1 | CDR2 | CDR3 | Accession no. |
|---|---|---|---|---|---|---|---|---|
| | | | | **3-11** | DYYMS | YISSSGSTIYYADSVKG | | |
| 1 | Day 0 | 1 | 97.9 | | ----- | ---------- | VDYYDSRDYFDY | DQ840890 |
| | | 1 | 96.5 | | ----- | ---------- | EVDYSGDY | DQ840884 |
| | | 1 | 99.7 | | ----- | ---------- | DGATRDFDY | DQ840885 |
| | | | | **3-30** | SYGMH | VISYDGSNKYYADSVKG | | |
| | | 1 | 96.5 | | D---S | G-DWN-GSTG------ | TSDGDYVGY | DQ840880 |
| | | 1 | 98.6 | | DFA-- | ---------- | SRDYCSGGSCYLAPIDY | DQ840883 |
| | | 1 | 94.4 | | --A-- | LVY---TT-F-----R- | DWDVYYGSEAFGY | DQ840891 |
| | | 2 | 99.6 | | ----- | ---------- | ARDYGDYARPILDY | DQ840886 |
| | | 1 | 99.3 | | ----- | ---------- | EKEQQGFDY | DQ840889 |
| | | 1 | 99.3 | | ----- | ---------- | DRGIAVANPAFDI | DQ840892 |
| | | | | **3-33** | SYGMH | VIWYDGSNKYYADSVKG | | |
| | | 1 | 94.7 | | --A-- | -------RRE-V----- | GSMYGDYSEYFHY | DQ840888 |
| | | 1 | 99.3 | | ----- | ---------- | DYGDYSPRYY | DQ840881 |
| | | 1 | 98.6 | | ----- | ---------- | QIAEIDC | DQ840887 |
| | | | | **3-11** | DYYMS | YISSSGSTIYYADSVKG | | |
| | Day 7 | 1 | 98.2 | | ----T | ---G---------- | STGYCSGGSCPNWFDP | DQ840902 |
| | | 1 | 98.6 | | ----T | ---G---------- | EIDLDGDSSGGFDS | DQ840898 |
| | | 1 | 94.8 | | S-G-H | VMWPD--NHW------- | EIDLDGDSSGGFDS | DQ840897 |
| | | | | **3-15** | NAWMS | RIKSKTDGGTTDYAAPVKG | | |
| | | 2 | 94.8 | | ----- | -----AH-A---------D | TVGFLGKDRDN | DQ840896 |
| | | | | **3-30** | SYGMH | VISYDGSNKYYADSVKG | | |
| | | 1 | 92.3 | | --A-- | -V-SG-EY--------- | ALSSYCHTTSCYSSGLDY | DQ840901 |
| | | 1 | 93.7 | | H-P-- | ---H---D-------R- | KPPGGGGSFDY | DQ840900 |
| | | 2 | 97.9 | | N---- | --W---------- | SYDYIWGSADY | DQ840899 |
| | | 1 | 94.7 | | D-Y-T | --W---------- | SYDYIWGSADY | DQ840894 |
| | | 1 | 96.6 | | ----- | --W---------- | SYDYIWGSADY | DQ840903 |
| | | | | **3-30** | SYGMH | VISYDGSNKYYADSVKG | | |
| 2 | Day 0 | 2 | 99.6 | | ----- | ---------- | DLGFEQWLFLPYFDY | DQ840831 |
| | | 2 | 99.3 | | ----- | I--------- | DRFHSSGYYEDWY | DQ840843 |
| | | 1 | 89.7 | | --S-N | Y--SSS-TI-------- | SRLVATIRDY | DQ840836 |
| | | | | **3-33** | SYGMH | VIWYDGSNKYYADSVKG | | |
| | | 5 | 94.9 | | -NA-- | -------K--H----R- | EVKDSSWDH | DQ840834 |
| | | | | **3-48** | SYSMN | YISSSSSTIYYADSVKG | | |
| | | 1 | 100 | | ----- | ---------- | DGPSSWPVGY | DQ840841 |
| | | 1 | 98.2 | | ----- | ---------- | LSPLLDYFDY | DQ840839 |
| | | | | **3-15** | NAWMS | RIKSKTDGGTTDYAAPVKG | | |
| | | 1 | 99.3 | | ----- | ---------- | VYDSSGSQVYYFDY | DQ840842 |
| | | | | **3-66** | SNYMS | VIYSGGSTYYADSVKG | | |
| | Day 7 | 1 | 96.8 | | ----- | ---------- | DPGLPNGMVVR | DQ840856 |
| | | 1 | 97.2 | | --A-- | ---------- | GGGSSSWYAY | DQ840852 |
| | | | | **3-33** | SYGMH | VIWYDGSNKYYADSVKG | | |
| | | 1 | 97.8 | | --A-- | --------EN-S----- | GGGSSSWYAY | DQ840858 |
| | | 1 | 93.6 | | TCW-T | --------EN-S----- | EMKRHQVTI | DQ840859 |
| | | 1 | 99.6 | | ----- | ---------- | ESLDITGTLDYFDY | DQ840861 |
| | | | | **3-11** | DYYMS | YISSSGSTIYYADSVKG | | |
| | | 1 | 91.5 | | ----T | --PNR--D-S-T----- | VRHLGTRYFFDY | DQ840853 |
| | | | | **3-07** | SYWMS | NIKQDGSEKYYVDSVKG | | |
| | | 2 | 95.5 | | -H--- | ---E-----DL-H---- | LDGYSRSLAY | DQ840851 |
| | | 1 | 95.1 | | DF--- | --NE-----H---P--- | ARGYGRC | DQ840854 |
| | | 1 | 95.8 | | -H--- | --NE-----H---P--- | ARGYGRC | DQ840862 |
| | | 1 | 95.5 | | TC--T | ------G-E-------- | GGWSGWPETLLSI | DQ840860 |
| 4 | Day 0 | | | **3-30** | SYGMH | VISYDGSNKYYADSVKG | | |
| | | 1 | 96.4 | | T---- | L-RD-----L------- | FGPTGLDY | DQ840753 |
| | | 1 | 99.2 | | --A-- | ---------- | GPGIAVAGTIFDVVDY | DQ840745 |
| | | 1 | 96.8 | | ----- | ---------- | GLVAGTFDY | DQ840744 |
| | | | | **3-21** | SYSMN | SISSSSSYIYYADSVKG | | |
| | | 6 | 98.2 | | ----- | -----I---------- | GLVAGTFDY | DQ840743 |
| | Day 7 | | | **3-07** | SYWMS | NIKQDGSEKYYVDSVKG | | |
| | | 1 | 99.3 | | ----- | ---------- | GERWLQSVGY | DQ840755 |
| | | 1 | 100 | | ----- | ---------- | VYCSSTSCPGWFDP | DQ840754 |
| | | 1 | 100 | | ----- | ---------- | AYCSSTSCPGWFDP | DQ840768 |
| | | 1 | 97.6 | | ----- | ---------- | DLIIVPAP | DQ840757 |
| | | 7 | 94.8 | | -D--N | I--------- | DLIIVPAP | DQ840762 |
| | | 2 | 92.4 | | NT--N | ---------- | DLIIVPAP | DQ840766 |

Table 3. Average percent identity to germ line of PPS4-specific VH and VL sequences isolated at day 0 vs. day 7.

| | VH | | VL | |
|---|---|---|---|---|
| | Day 0 | Day 7 | Day 0 | Day 7 |
| % ID | 96.3% | 96.2% | 97.4% | 97.6% |
| Range | 90.9-100% | 89.7-100% | 92.4-100% | 92.2-100% |

Figure 1. Variable heavy chain gene usage of unselected CD19+ B cells [17] and PPS4-specific B cells isolated on day 0 and day 7. (*) denotes significant differences between day 0 and day 7. Statistical analysis performed using Poisson regression models, p values $< 0.05$ were considered significant.

Figure 2. Variable light chain gene usage of unselected IgM+ B cells [15,16] and PPS4-specific B cells isolated on day 0 and day 7. (*) denotes significant differences between day 0 and day 7. Statistical analysis performed using Poisson regression models, p values $< 0.05$ were considered significant.

Figure 1

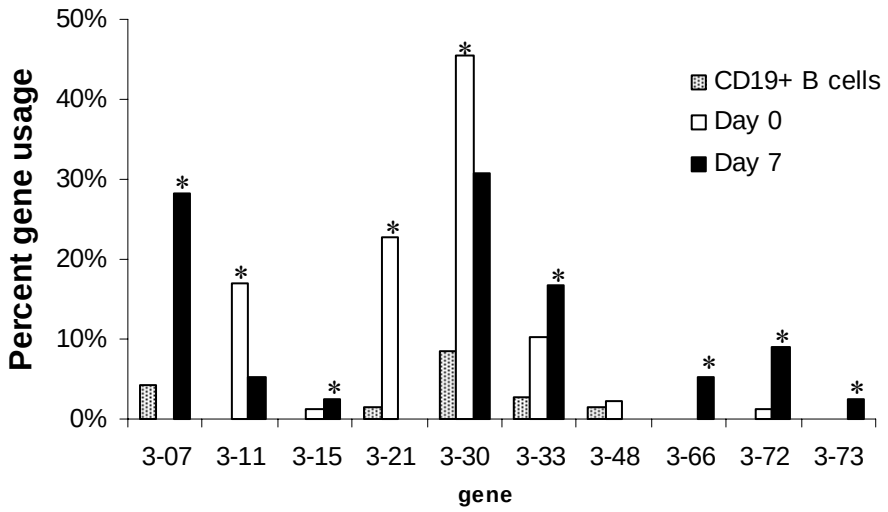
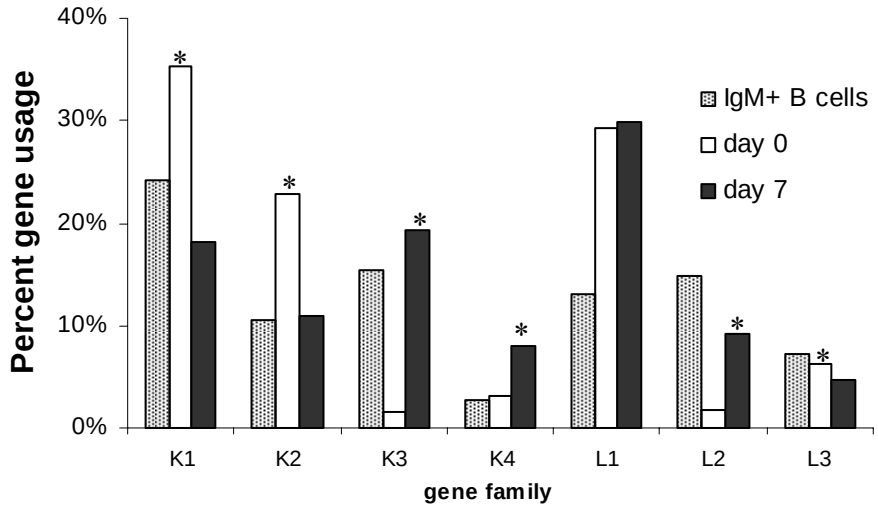

50%
40%
30%
20%
10%
0%
Percent gene usage
CD19+ B cells
Day 0
Day 7
3-07
3-11
3-15
3-21
3-30
3-33
3-48
3-66
3-72
3-73
gene

Figure 2


40%
30%
20%
10%
0%
Percent gene usage
IgM+ B cells
day 0
day 7
K1
K2
K3
K4
L1
L2
L3
gene family

# DISCUSSION

The incidence of pneumococcal disease has declined over the past few decades, largely due to the advent of the pneumococcal polysaccharide and conjugate vaccines. Nevertheless, they have failed to elicit a highly immunogenic response in elderly adults (Hirschmann and Lipsky, 1994; Shapiro et al., 1991). While PPS-specific antibody concentrations in elderly adults are similar to those of young adults, antibody functional activity is markedly decreased (Romero-Steiner et al., 1999). Studies have shown that the reduction in functional antibody activity is likely related to a decrease in antibody avidity (Lucas et al., 1998; Nicoletti et al., 1991) and therefore, may correlate with differences in antibody molecular structure. Furthermore, several studies have shown that the response to PS antigens utilizes a limited gene repertoire (Abadi et al., 1998; Baxendale, 2000; Lucas et al., 1994, 1998) and undergoes significant changes with increasing age (Kolibab et al., 2005a; Nicoletti et al., 1991; Smithson et al., 2005). Therefore the differences in variable gene usage observed between young and elderly adults may account for the differences observed in vaccine efficacy between age groups. However, our understanding of the molecular mechanisms associated with the response to pneumococcal antigens is limited.

The purpose of these studies was to investigate possible factors that play a role in determining antibody variable gene usage. Studies have demonstrated that PPS-specific antibodies isolated 5-10 days post-vaccination have undergone significant somatic mutation, indicative of a memory response induced by previous exposure to *S. pneumoniae* or cross-reactive antigen (Baxendale, 2000; Zhou et al., 2002, 2004). Such

previous exposure is likely to take part in the PPS-specific antibody variable gene usage following vaccination. In addition, other factors such as T cells, accessory cells and cytokine environment undergo significant changes associated with increasing age (Aydar et al., 2004; Chelvarajan et al., 2005; McNerlan et al., 2002; Sandmand et al., 2002; Saurwein-Teissl et al., 2002). We wished to investigate whether or not factors extrinsic to B cells are capable of influencing variable gene usage in response to the pneumococcal vaccination.

First we investigated the role of cytokines on the age-related changes in anti-PPS VH and VL gene expression (Shriner et al., 2006b). We used a reconstituted SCID mouse model to analyze the V gene repertoire of PPS-specific young and elderly B cells in the presence of a more controlled cytokine environment. We hypothesized that elderly B cells would express a gene repertoire resembling that of young B cells when stimulated in the presence of a similar cytokine environment. However, overall, the gene repertoire used by B cells isolated from E PBL SCID mice was significantly different than that observed in Y PBL SCID mice. Thus age-related differences in gene expression persisted in hu-PBL-SCID mice despite their stimulation in similar cytokine environments. The generation of antigen-specific human antibody in the hu-PBL-SCID mouse model used in this study requires the transfer of the donors' accessory cells as well as B cells. Previous work has demonstrated that defects in accessory cell function, specifically cytokine secretion, occur with age (Bondada et al., 2001). It is therefore plausible that, based on the presence of age-specific accessory cells, differences in cytokine environment in Y PBL SCID and E PBL SCID remained despite manipulation

with IL-12 and TRF. These differences in cytokine environment may have been sufficient to result in age-related differences in gene expression.

Furthermore, to examine the influence of a modified cytokine environment on variable gene usage, we compared the gene expression of PPS-specific B cells recovered from donors to those recovered from SCID mice (Shriner et al., 2006b). Our results showed that significant differences in V gene usage arose between donors and their respective hu-PBL-SCID mice. This may, in part, be due to the altered cytokine environment encountered by B cells in the SCID mice. It has been previously demonstrated that cytokines are capable of influencing gene expression, (Hikida et al., 1998) and work in our laboratory has revealed similar results using the same model in response to MCPS (Smithson et al., 1999). In addition, comparison of the antibody response in donors to that of SCID mice was hampered by differences in B cell populations used for the stimulation and recovery of PPS-specific B cells. The SCID mice were reconstituted with circulating PBL of human donors and consequently, lymphocytes that resided in the bone marrow or spleen did not contribute to the population of cells available for stimulation within the mice. Moreover, in the donors, the majority of lymphocytes retreat to the bone marrow following antigen stimulation (Kruetzmann et al., 2003; Vinuesa et al., 2003), and therefore were not circulating at the time of B cell isolation. Therefore, it cannot be definitively assumed that the changes in V gene usage observed between donors and SCID mice reflect changes brought on by a modified cytokine environment. However, we did observe similar trends in gene usage among young donor/SCID pairs and elderly donor/SCID pairs in support of the notion

that a modified cytokine environment is capable of altering V gene expression, and thus potentially affects antibody functional activity.

Our second study was designed to investigate the role immunizing agent has on variable gene usage (Shriner et al., 2006a). We used the hu-PBL-SCID mouse model to analyze a single individual's B cell response to two different forms of antigen, TI-2 and TD, and defined differences in gene usage elicited by these vaccines. The results of this study demonstrated that significant differences in heavy and light chain variable gene usage occurred between B cells stimulated with the PPV compared to those stimulated with CV. Interestingly, there was a common trend in light chain usage observed between the PPV and CV. There was a notable shift in light chain variable gene usage from a predominance of VLλ gene families used in response to PPV to a predominance of VLκ gene families used in response to CV. This trend was observed regardless of the age of the donors. The significance of VL gene expression on antibody function and specificity has been demonstrated previously in response to *S. pneumoniae* (Nicoletti et al., 1991) and Hib-PS antigens (Lucas et al., 1998).

This study was unique in that it allowed us to analyze variable gene usage from the same population of B cells in response to two different vaccines. As the SCID mice were reconstituted with PBL from the same donor, the accessory cells, cytokine environment and lymphocyte populations remained constant between groups of PPV and CV SCID mice. Thus, the only difference between groups was the nature of antigen presentation (TI versus TD). Therefore, our results suggest that the significant shift in VL gene expression was directly related to vaccine composition and that these changes

may be related to the additional T cell and accessory cell involvement in the immune response to the conjugate vaccine. As a result, the distinct mechanisms of protection elicited by these two vaccines likely results in an altered cytokine environment. The nature of the immunizing agent and the microenvironment it creates may influence gene usage and could account for differences in antibody functional activity.

The third study was performed to further investigate the anti-pneumococcal antibody response by characterizing the anti-PPS B cell repertoire prior to vaccination with PPV (Shriner et al., 2006c). As mentioned previously, the extensively mutated PPS-specific antibody response observed 5-10 days post-vaccination suggests previous exposure to *S. pneumoniae* or cross-reactive antigens (Baxendale, 2000; Zhou et al., 2002, 2004). Therefore the antibodies isolated following immunization may reflect a recall response and clonal expansion of PPS-specific B cells present prior to vaccination. To explore these concepts further, we characterized the PPS4-specific circulating B cells of young adults prior to vaccination and compared it to the PPS4-specific antibody response 7 days post-vaccination. We hypothesized that if the extensively mutated PPS-specific sequences were indeed a result of activation and expansion of memory B cell clones, these clones may be present in the peripheral circulation prior to vaccination. Our results, however, demonstrated populations of B cells expressing two distinct V gene repertoires pre- versus post-vaccination. The majority of sequences had undergone somatic hypermutation and there was no significant difference in mutational frequency between those isolated pre- versus post-vaccination. Although some VH and VL

sequences isolated 7 days post-vaccination were clonally related, there was no evidence of clonal expansion or affinity maturation of the pre-immune response.

The results from this study suggest that the precursors to the heavily mutated PPS4-specific cells are not present in the peripheral circulation, but likely reside elsewhere such as the marginal zone of the spleen. Previous studies have demonstrated that the immune response to TI antigens occurs in the marginal zone (Kruetzmann et al., 2003). Furthermore, the PPS4-specific mutated B cells isolated from the periphery prior to vaccination support the hypothesis that previous exposure to pneumococcal polysaccharides, or other cross-reactive antigen, has stimulated the immune system. The PPS-specific cells present pre-vaccination may reflect the involvement of natural antibody produced by B1 cells, previously shown to play a role in the response to *S. pneumoniae* (Haas et al., 2005).

Taken together, these studies have demonstrated that a variety of external factors are capable of altering antibody variable gene usage and molecular structure in response to pneumococcal vaccination. Changes in external stimuli such as cytokine production, T cells, accessory cells and pre-existing memory cells likely contribute substantially to variations in antibody molecular structure and therefore may significantly impact antibody functional activity. However, the exploration of the exact mechanisms by which these changes takes place remains to be elucidated.

Understanding the molecular diversity of polysaccharide-specific antibodies is becoming increasingly important in predicting the human immune response against pneumococcal disease. However, the human immune system is complex and highly

variable and investigating the precise mechanisms involved in the generation of antibody diversity can be difficult. The establishment of the hu-PBL-SCID mouse model has shown potential as a useful tool to study the human immune system; however, it is not without limitation. Therefore, alternative models such as NOD/SCID mice have been developed which will likely be of great value in investigating the human immune response to a variety of antigens. Further investigation of the factors involved in determining antibody molecular structure and functional activity will be of great value to future vaccine design.

## SUMMARY

The studies presented describe the investigation of factors that may influence antibody variable gene usage in response to pneumococcal vaccination.

We investigated the role of cytokines on the age-related changes in anti-PPS VH and VL gene expression using a reconstituted SCID mouse model. This allowed us to analyze the V gene repertoire of PPS-specific young and elderly B cells in the presence of a more controlled cytokine environment. We hypothesized that elderly B cells could express a gene repertoire resembling that of young B cells when stimulated in the presence of a similar cytokine environment. However, the gene repertoire used by B cells isolated from E PBL SCID mice was significantly different than that observed in Y PBL SCID mice. Thus age-related differences in gene expression persisted in hu-PBL-SCID mice despite their stimulation in similar cytokine environments. Furthermore, to examine the influence of a modified cytokine environment on variable gene usage, we compared the gene expression of PPS-specific B cells recovered from donors to those recovered from SCID mice. Our results showed that significant differences in V gene usage arose between donors and their respective hu-PBL-SCID mice. However, despite these persistent differences, we did observe similar trends in gene usage among young donor/SCID pairs and elderly donor/SCID pairs in support of the notion that a modified cytokine environment is capable of altering V gene expression and thus potentially affects antibody functional activity.

Our second study was designed to investigate the role nature immunizing agent has on variable gene usage. We used the hu-PBL-SCID mouse model to analyze a single

individual's B cell response to two different forms of antigen and defined differences in gene usage elicited by these vaccines. The results of this study demonstrated that significant differences in heavy and light chain variable gene usage occurred between B cells stimulated with the PPV compared to those stimulated with CV. Comparing PPS-specific variable gene usage between groups, there was a notable shift in light chain gene expression from a predominance of VLλ gene families used in response to PPV to a predominance of VLκ gene families used in response to CV. Our results imply that the significant shift in VL gene expression was directly related to vaccine composition and suggests that these changes may be related to the additional T cell and accessory cell involvement in the immune response to the conjugate vaccine.

The third study was performed to further investigate the anti-pneumococcal antibody response by characterizing the anti-PPS B cell repertoire prior to vaccination with PPV. We characterized the PPS4-specific antibody response of young adults prior to vaccination and compared it to the PPS4-specific antibody response 7 days post-vaccination. We hypothesized that if the extensively mutated PPS-specific sequences are indeed a result of activation and expansion of memory B cell clones, these clones may be present in the peripheral circulation prior to vaccination. However, our results demonstrated populations of B cells expressing two distinct V gene repertoires pre- versus post-vaccination. The majority of sequences had undergone somatic hypermutation and there was no significant difference in mutational frequency between those isolated pre- versus post-vaccination. Although some VH and VL sequences

isolated 7 days post-vaccination were clonally related, there was no evidence of clonal expansion or affinity maturation of the pre-immune response.

Taken together, these studies have demonstrated that a variety of external factors are capable of altering antibody variable gene usage and molecular structure in response to pneumococcal vaccination. Changes in external stimuli such as cytokine production, T cells, accessory cells and pre-existing memory cells likely make a substantial contribution to variations in antibody molecular structure, and therefore may significantly impact antibody functional activity.

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## ABSTRACT

*Streptococcus pneumoniae* is a major human pathogen and leading cause of morbidity and mortality worldwide. Pneumococcal disease has a characteristic age distribution with the highest incidence occurring at the extremes of age. The incidence of pneumococcal disease has decreased in recent years, largely due to the advent of pneumococcal vaccines. Nonetheless, the 23-valent pneumococcal polysaccharide vaccine (PPV), although effective in young adults, demonstrates reduced efficacy in elderly populations. The pneumococcal conjugate vaccine (CV), although highly immunogenic in children under 2, offers no advantage over the PPV in elderly adults. In the past, vaccine evaluation has been limited to serological assessment, with anti-pneumococcal polysaccharide (PPS) antibody concentrations serving as the main surrogate marker of protection. Previous work has demonstrated that antibody avidity as measured by opsonophagocytic assays, not concentration, is a more reliable predictor of functional antibody activity. Furthermore, variation in antibody avidity in response to polysaccharide (PS) antigens has correlated with differences in antibody molecular structure. Thus, understanding the molecular diversity of PPS-specific antibodies is becoming increasingly important in predicting the human immune response against pneumococcal disease. Numerous factors may influence the expressed B cell repertoire in response to pneumococcal vaccination, including pre-existing antibody, T cells, accessory cells and cytokine environment. We hypothesized that differences in cytokine environment, antigen presentation and the presence of pre-existing antibody significantly influence variable gene usage. We used a severe combined immunodeficient (SCID)

mouse model to study the influence of cytokines on young and elderly variable gene usage. We observed numerous significant differences in gene usage between young and elderly B cells despite their stimulation in a more controlled cytokine environment. We used the same SCID model to study the influence of antigen presentation on variable gene usage. We observed significant differences in variable gene usage when lymphocytes were stimulated with two different forms of antigen (PPV versus CV), regardless of the donors' age. Lastly, we characterized the PPS-specific pre-vaccination antibody repertoire and investigated its potential influence on the post-vaccination repertoire. We observed two distinctly different PPS-specific antibody repertoires pre- versus post-vaccination.