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Polyreactive and antigen-specific B-cell response to Streptococcus pneumoniae

Rebecca S. Thompson

The University of Toledo

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A Dissertation

entitled

Polyreactive and Antigen-specific B-cell Response to *Streptococcus pneumoniae*

by

Rebecca S. Thompson

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Sciences

Dr. MA Julie Westerink, Committee Chair

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June 2012
An Abstract of

Polyreactive and Antigen-specific B-cell Response to *Streptococcus pneumoniae*

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Rebecca S. Thompson

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The University of Toledo
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*Streptococcus pneumoniae* is a prevalent cause of disease and mortality worldwide. Pneumococcal disease includes pneumonia, otitis media, bacteremia and meningitis. Antibodies against the pneumococcal capsular polysaccharide are protective and aid in clearance of the bacteria.

The phenotype of human anti-pneumococcal polysaccharide (PPS) B cells has not been well characterized. Immune-compromised individuals often respond poorly to the 23-valent purified pneumococcal polysaccharide vaccine (PPV) and are often deficient in IgM memory and switched memory IgM\(^+\) cells. We hypothesized that the anti-PPS B cells which respond to PPV are IgM memory cells. To directly characterize anti-PPS B cells, fluorescently labeled PPS14 and PPS23F in conjunction with a FACS Aria flow cytometer were used to detect and characterize PPS-specific B cells from healthy young adult volunteers pre- and post-vaccination with PPV. Post-vaccination PPS-specific B cells expressed a dominant IgM memory phenotype, CD27\(^+\)IgM\(^+\), suggesting a key role of IgM memory cells in the immune response to PPS. These results help explain the poor
PPS-specific antibody response post-vaccination in individuals deficient in IgM+/CD27+ B cells.

When PPS-specific antibodies are not present, natural antibodies serve as the body’s first line of defense against pneumococcal challenge. These low avidity, polyreactive anti-PPS antibodies have not been extensively studied in humans. We hypothesized that while these antibodies are low avidity, they elicit protection against pneumococcal challenge. In this project, PPS binding B cells were isolated using fluorescently labeled PPS and expanded in tissue culture. Although these antibodies were isolated from individual B cells and possessed unique VH/VL, sequence analysis and avidity studies revealed similar characteristics. The VL CDR3 was restricted in length and VH CDR3s expressed a statistically higher number of flexible amino acids when compared to PPS-specific antibodies. To investigate the role of the constant region in antibody avidity, these polyreactive variable regions were expressed as IgG1 or IgG2. Kinetic analysis demonstrated that IgG1 antibodies were more avid when compared to IgG2. The IgG1 isotype is more flexible than IgG2 allowing unrestricted movement to bind PPS antigens more avidly. Our results suggest IgM+ memory cells respond to PPV and polyreactive antibodies possess specific characteristics that enable recognition of multiple PPS.
First, I would like to thank my mentor Julie for her guidance and support over the last five years. Without her my work would not have been possible. I would also like to thank the other members of the lab. After countless hours of brainstorming and collaboration you are more than co-workers, you have become an extended family.

My dissertation is also dedicated to my loving husband Ken and family, especially my parents Jerry and Kim. At a young age I developed a love for science and my parents did everything to nurture and inspire my curiosity. Without their support and encouragement throughout the years I would not be the person I am today.
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# Contents

Abstract ..................................................................................................................... iii

Acknowledgments .................................................................................................... vi

Contents .................................................................................................................. vii

List of Figures .......................................................................................................... ix

List of Abbreviations ............................................................................................... x

Preface ..................................................................................................................... xii

1 Literature ............................................................................................................. 1

1.1 Epidemiology .................................................................................................... 3

1.2 Pathogenesis ..................................................................................................... 6

1.3 Virulence factors ............................................................................................... 8

1.4 Innate immune response .................................................................................. 11

1.5 Specific immune response ............................................................................... 14

1.6 Therapeutic intervention ................................................................................ 17

1.7 Prevention ....................................................................................................... 18

1.8 Antibody structure ......................................................................................... 21

1.9 Anti-pneumococcal antibodies ....................................................................... 26

1.10 Human B cell response .................................................................................. 29
1.11 Natural antibodies ........................................................................................................ 31

2 Phenotypic analysis of pneumococcal polysaccharide-specific B cells .............. 34
   2.1 Abstract .................................................................................................................. 35
   2.2 Introduction ............................................................................................................ 37
   2.3 Materials and Methods ......................................................................................... 39
   2.4 Results .................................................................................................................. 43
   2.5 Discussion ............................................................................................................. 49

3 Isolation and characterization of human polyreactive pneumococcal polysaccharide
   antibodies .................................................................................................................... 72
   3.1 Abstract .................................................................................................................. 73
   3.2 Introduction ............................................................................................................ 75
   3.3 Material and Methods ......................................................................................... 77
   3.4 Results .................................................................................................................. 82
   3.5 Discussion ............................................................................................................. 87

4 Discussion .................................................................................................................. 114

References .................................................................................................................... 117
List of Figures

1-1 *Streptococcus pneumoniae* in sputum smear ................................................................. 1
1-2 Surface components of *Streptococcus pneumoniae* ......................................................... 2
1-3 Incidence of Invasive Pneumococcal Disease .................................................................. 4
1-4 Main virulence factors of *Streptococcus pneumoniae* ..................................................... 8
1-5 Complement pathways involved in clearance of *Streptococcus pneumoniae* ................. 14
1-6 Characteristics of T-cell dependent and independent antigens .................................. 16
1-7 General antibody structure ............................................................................................... 21
1-8 Antibody assembly ............................................................................................................ 23
1-9 Surface plasmon resonance model .................................................................................. 29
List of Abbreviations

AA........................................Amino acid
AI .........................................Avidity index
APC .......................................Antigen-presenting cell
ASC .......................................Antibody secreting cell

BAFF ........................................B-cell activating factor
BCR .........................................B cell receptor
BSA ..........................................Bovine serum albumin

CB ...........................................Cascade Blue Ethylenediamine
CBC ..........................................Complete blood count
CDR3 .......................................Complimentary determining region 3
CPS ..........................................Cell wall polysaccharide
CVID .......................................Common variable immune deficiency
CWPS ......................................Cell wall polysaccharide

DTAF ........................................5 - (4,6 - Dichlorotriazinyl)aminofluorescein

FcR ..........................................Fc receptor

HAART .....................................Highly active anti-retroviral therapy
Hib ...........................................Haemophilus influenzae type b
Hyl ...........................................Hyaluronate lyase

Ig .............................................Immunoglobulin
IPD ..........................................Invasive pneumococcal disease

LPS ..........................................Lipopolysaccharide

MAb .........................................Monoclonal antibody
MAC .........................................Membrane attack complex
MBL ..........................................Mannose-binding lectin
MHC ..........................................Major histocompatibility complex
MZ ...........................................Marginal zone

NaSCN .....................................Sodium thiocyanate
OI = Opsonophagocytic index
OPI = Opsonophagocytic index
OPSA = Opsonophagocytic assay

PAMP = Pathogen-associated molecular patterns
PBMC = Peripheral blood mononuclear cell
PBST = PBS + 0.1% Tween 20
PRR = Pattern recognition receptor
PCR = Polymerase chain reaction
PCV = Pneumococcal conjugate vaccine
pIgR = Polymeric immunoglobulin receptor
Ply = Pneumolysin
PPS = Pneumococcal polysaccharide
PPV = Pneumococcal polysaccharide vaccine
PPV23 = 23-valent pneumococcal polysaccharide vaccine
PspA = Pneumococcal surface protein A
PspC = Pneumococcal surface protein C

SHM = Somatic hypermutation
*S. pneumoniae* = *Streptococcus pneumoniae*
SPR = Surface plasmon resonance

T<sub>H1</sub> = T helper type 1
T<sub>H2</sub> = T helper type 2
TD = T cell dependent
TI = T cell independent
TLR = Toll-like receptor

VH = Variable heavy
VL = Variable light
Preface

*Streptococcus pneumoniae*, also referred to as pneumococcus, is a pathogen that asymptotically colonizes the nasopharynx. Despite use of modern medicine and vaccines, pneumococcus is still a major cause of morbidity and mortality worldwide. Invasive pneumococcal disease occurs when pneumococci move from the nasopharynx to normally sterile organs and blood. Groups at high risk, namely the very young, elderly and HIV-positive are most susceptible to pneumococcal diseases such as bacteremia, meningitis, otitis media and pneumonia. These populations lack an effective immune response against *S. pneumoniae*. There are two licensed vaccine types to elicit an anti-pneumococcal antibody response. In 1983, a 23-valent purified pneumococcal vaccine was introduced. The 23 serotypes included in this vaccine accounted for 90% of the disease causing serotypes. This vaccine is recommended for the elderly over the age of 65, asplenic and immune compromised. Children under the age of two do not respond well to T-cell independent type 2 antigens such as purified PPS. After the success of the conjugate *Haemophilus influenzae* type b vaccine, a conjugate vaccine was developed for PPS. Conjugation of PPS to non-toxic diphtheria CRM197 protein, a T-cell dependent antigen, is recognized by children’s immune systems. The pneumococcal conjugate vaccine protects children from invasive pneumococcal disease. However four doses are recommended and each dose is expensive. This hinders widespread administration in
developing countries and areas of low socioeconomic status. Creation of these vaccines significantly decreased the incidence of disease however invasive pneumococcal disease continues to be a major cause of death in developing countries and results in abundant health care costs. In order to develop more efficacious treatments and vaccines, the human B cells response to PPS needs to be better characterized.

Previous studies have suggested the role of IgM memory cells in response to polysaccharide antigens however the phenotype of B cells that respond to PPV have not been well defined. Analysis of the B cell response to PPV in healthy young adults will serve as a control for the standard response to vaccination. Comparison of the cellular response to vaccination in the elderly and HIV-positive could help identify dysfunction in cellular immunity.

When specific PPS antibodies are not present, natural antibodies serve as the first line of defense against pneumococcal challenge. Human, polyreactive PPS-binding antibodies have not been characterized. Isolation and expansion of PPS binding B cells allowed for sequence analysis of the variable gene regions. Cloning of the variable regions into IgG1 and IgG2 expression cassettes allowed for the calculation of the binding constant and comparison of the effects of different constant regions. Exploring the variable region sequence and binding constant of polyreactive antibodies will help to predict the quality of the natural antibody response to *Streptococcus pneumoniae*.

We conducted these studies to test the following hypotheses:

1. The anti-pneumococcal polysaccharide B cells which respond to PPV are IgM memory cells.
   a. Populations that poorly respond to PPV are deficient in IgM memory cells.
b. Prior studies have implicated CD27+IgM+ memory cells as the phenotype of B-lymphocytes responsible for the immune response following vaccination with PPV.

2. Human polyreactive anti-pneumococcal antibodies are low avidity but elicit protection from pneumococcal challenge.
   a. Murine studies have identified B1 cells that spontaneously secrete polyreactive antibodies.
   b. These studies have demonstrated functional activity of polyreactive antibodies.
1 Literature

*Streptococcus pneumoniae* is a member of the genus *Streptococcus*. Members of this genus are coci shaped cells, usually growing in pairs or chains and tend to remain attached to host tissues (Ryan, Ray, & Sherris, 2010). Clinically pertinent streptococci are nonmotile, not acid fast and do not form spores (Ryan et al., 2010).

![Streptococcus pneumoniae in sputum smear](image)

Figure 1-1 *Streptococcus pneumoniae* in sputum smear

*Streptococcus pneumoniae* is an alpha hemolytic bacteria meaning the pneumococcus partially lyses red blood cells. This is used as a diagnostic tool for identification of bacteria from patient samples. Positive identification results in a green coloration under the bacterial colonies when grown on blood agar. Hydrogen peroxide
produced by the pneumococcus oxidizes hemoglobin to methemoglobin which is green in color. Another diagnostic tool is Gram staining. Pneumococci stain Gram-positive due to the thick layer of peptidoglycan surrounding the cell. The peptidoglycan retains the crystal violet and iodine complex within the bacterial cell. Retention of the dye results in purple pneumococci when the bacteria are observed under a microscope.

Figure 1-2 Surface components of *Streptococcus pneumoniae*

*Streptococcus pneumoniae*’s capsular polysaccharide is the main virulence factor for the initiation of the host immune response. Different strains of pneumococci are distinguished by variances in the structure of their capsular polysaccharide. To date more than 90 different serotypes have been identified. Host antibodies directed against the
pneumococcal polysaccharide (PPS) provide immunological protection specific for each serotype.

1.1 Epidemiology

*Streptococcus pneumoniae* is responsible for considerable morbidity and mortality worldwide. Each year in the U.S., pneumococcus is responsible for an estimated 3,000 cases of meningitis, 50,000 cases of bacteremia and 500,000 cases of pneumonia (Abbas, Lichtman, & Pillai, 2011). In 2000, there were approximately 14.5 million documented cases of invasive pneumococcal disease (IPD) in children under the age of 5. Pneumococcal colonization may result in invasive disease, including bacterial meningitis and bacteremia, or noninvasive mucosal disease (Block, 1997; Schuchat et al., 1997; Wald, 1992). Invasive pneumococcal disease exhibits a characteristic age distribution with the majority of cases occurring in the very young (<2 years old) and the elderly (>65 years old) (Robinson et al., 2001).

Pneumococcal disease has had the greatest impact on young children resulting in approximately 0.7-1 million deaths from pneumococcal infections per year due to a high incidence of bacteremia and meningitis in this age population. *Streptococcus pneumoniae* is now the most common bacteria isolated from children with bacterial meningitis after the success of the *Haemophilus influenzae* type b conjugate vaccine ("Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP)," 1997). Since the widespread administration of PCV, the death rate for children under the age of two in the U.S. has dropped from 6.4% in 2000 to 0.44% in 2010 (Figure 3) ("CDC - ABCs: 2010 Strep pneumoniae Surveillance Report - Active Bacterial Core surveillance,"). Prior to administration of the pneumococcal conjugate
vaccine (PCV), it was the most common organism isolated from middle ear fluid of
cchildren with otitis media.

![Incidence of Invasive Pneumococcal Disease in U.S.](image)

**Figure 1-3 Incidence of Invasive Pneumococcal Disease**

In the elderly, pneumococcal infection most often results in pneumonia
(Robinson et al., 2001). Pneumococcus is the most common bacteria isolated in patients
with community-acquired pneumonia (Marston et al., 1997). To date more than two-
thirds of the elderly population has received a pneumococcal vaccination (Jackson &
Janoff, 2008). The death rate in the elderly over the age of 65 has also dropped from
10.7% in 2000 to 5.61% in 2010 (Figure 3). Additionally, herd immunity has decreased
invasive pneumococcal infections in the elderly by 38% (Jackson & Janoff, 2008). In
adults there are additional risk factors that contribute to the risk of pneumococcal
infection. Diabetes, chronic renal disease, asplenia, alcoholism, lower socioeconomic
status and ethnicity are all associated with a higher incidence of pneumococcal disease.
A recent respiratory viral infection also increases the risk of pneumococcal infection due to decreased mucosal clearance (McCullers & Bartmess, 2003; O'Brien et al., 2000). In 1918, pneumococcal pneumonia often followed influenza infection and likely contributed to many of the deaths in the historic influenza epidemic (Morens, Taubenberger, & Fauci, 2008).

Another group highly susceptible to pneumococcal infection is the immune compromised. This population includes persons who cannot produce antibodies against PPSs, lack complement components or those with defective phagocytosis (Sanders et al., 1993). Hypogammaglobulinemia, IgG deficiency, IgA deficiency and impaired polysaccharide responsiveness are examples of these conditions (Cunningham-Rundles & Bodian, 1999; D. T. Johnston et al., 2006; Schroeder, Schroeder, & Sheikh, 2004; Stiehm, 2008). The functionally or surgically asplenic are also at a significantly increased risk for developing IPD as the spleen plays an important role in the clearance of pneumococci (Styrt, 1990; Wara, 1981).

A large population of the immune compromised group is HIV-positive individuals. Pneumococcal infection is the most common bacterial respiratory pathogen in the HIV-positive population (Boschini et al., 1996; Janoff, Breiman, Daley, & Hopewell, 1992; Redd et al., 1990; Schuchat, Broome, Hightower, Costa, & Parkin, 1991; Selwyn et al., 1992). Before the availability of highly active anti-retroviral therapy (HAART), the incidence of IPD in HIV-positive individuals was 100-fold higher than in age-matched HIV-negative individuals (Redd et al., 1990). However even with the widespread use of
HAART, HIV-positive patients remain at a 35-fold increased risk of invasive pneumococcal infection (Heffernan et al., 2005). The immune systems of children, elderly and immune compromised are defective in effectively clearing *S. pneumoniae* resulting in increased colonization and pathogenesis of disease.

1.2 Pathogenesis

The human nasopharynx is the only reservoir of *S. pneumoniae*. The organism evades phagocytosis as its capsular polysaccharide prevents complement deposition. Other pneumococcal components cause damage to epithelial tissues resulting in better adherence of pneumococci. Colonization progresses to invasive disease when pneumococci move from the nasopharynx and invade a normally sterile site. From there, pneumococci can spread to adjacent mucosal tissues and cause infections such as otitis media, sinusitis and pneumonia. Occasionally, *S. pneumoniae* can invade the mucosa and reach the bloodstream causing invasive infections such as bacteremia, meningitis, septic arthritis and osteomyelitis (Myers & Gervaix, 2007).

Inhalation of pneumococci into the lungs is a common route of infection (Tuomanen, Austrian, & Masure, 1995). Usually the host’s immune system is able to clear pneumococci through phagocytosis by aveolar macrophages, coughing and a functional ciliary epithelium (Ryan et al., 2010). However, when pneumococci escape these host defenses, they reach the aveoli and begin dividing. Once pneumococci have adhered to aveoli, virulence factors trigger local inflammation. Leukocyte recruitment and edema may result in migration of pneumococci into the bloodstream. If this occurs, the infection becomes systemic with high levels of bacteremia possibly resulting in
bacterial meningitis (Daum, Scheifele, Syriopoulou, Averill, & Smith, 1978). Diagnosis of bacteremia in children is difficult because fever is often the only symptom (Myers & Gervaix, 2007).

In children, pneumococci can adhere to the Eustachian tubes causing otitis media (Linder, Daniels, Lim, & DeMaria, 1994; Tong, McIver, Fisher, & DeMaria, 1999). Eustachian tubes vent, protect and clear pathogens from the middle ear. However in children under the age of 7 the Eustachian tube is horizontal and smaller in diameter hindering clearance of bacteria. A recent viral infection also increases the risk of acute otitis media due to impairment of the mucociliary clearance and underdeveloped anatomy of the Eustachian tubes (Bluestone, 1996; Wald, 2011). Children exposed to tobacco smoke are at greater risk for IPD (DiFranza, Aligne, & Weitzman, 2004; T. F. Murphy, 2006; Uhari, Mantysaari, & Niemela, 1996). Cigarette smoke impairs the clearance of bacteria by slowing the mucociliary action of the respiratory epithelium and inducing inflammation (T. F. Murphy, 2006; Stanley, Wilson, Greenstone, MacWilliam, & Cole, 1986; Willemse, ten Hacken, Rutgers, Postma, & Timens, 2005).

The pneumococcus exhibits several virulence factors that enable the colonization of the nasopharynx (Ryan et al., 2010). A major factor in adherence is the result of pneumococcal surface proteins binding to host epithelial cells. Adherence is enhanced by additional virulence factors including those which expose more receptors on the surface of the host cell (Ryan et al., 2010).
1.3 Virulence factors

*Streptococcus pneumoniae* express many virulence factors that help evade the host immune system and contribute to host colonization (Figure 4). The pneumococcal capsule and surface proteins prevent entrapment in the nasal mucous and opsonization. In pneumonia and other respiratory tract infections, pneumococcus evades complement activation through enzymes and inhibitory proteins.

Figure 1-4 Main virulence factors of *Streptococcus pneumoniae*
The primary virulence factor is the pneumococcal capsular polysaccharide (PPS) (Henrichsen, 1995). Pneumococcal strains without PPS are unable to colonize the nasopharynx (Magee & Yother, 2001). This surface polysaccharide interferes with phagocytosis by blocking access to complement receptors and antibodies. The capsule masks C3b and subsequent deposition of complement effectively preventing recognition by phagocytes.

Another main virulence factor is pneumolysin (Ply) (Rossjohn et al., 1998). This protein’s sequence is well conserved and found in almost all pneumococcal isolates (Kadioglu, Weiser, Paton, & Andrew, 2008; Lock, Zhang, Berry, & Paton, 1996). Pneumolysin is a pore-forming toxin involved in acute inflammation. It is able to increase pneumococcal adherence, damaging host cells (Ashida et al., 2011). Pneumolysin is a cytoplasmic enzyme that is released as pneumococcal autolysin slowly degrades the bacterial cell wall. Upon release, Ply is cytotoxic to ciliated epithelium in the lungs, which interferes with clearance of pneumococcus from respiratory mucosa (Jedrzejas, 2001; Rayner et al., 1995; Steinfort et al., 1989). Additionally, Ply disrupts alveolar tight junctions allowing for penetration of pneumococci into the pulmonary interstitium (Rubins & Janoff, 1998). The multiple effects of Ply are critical for the early establishment of infection by pneumococci (Rubins et al., 1998). Another protein necessary for virulence is hyaluronate lysase (Hyl). This enzyme facilitates the degradation of extracellular matrix components to increase tissue permeability and expose host cells to other pneumococcal toxins such as pneumolysin (Kelly, Taylor, Li, & Jedrzejas, 2001).
Other important pneumococcal virulence factors include the pneumococcal cell-surface proteins. Pneumococcal surface protein A (PspA) is negatively charged and interferes with the binding of complement factor C3, inhibiting complement-mediated opsonization (D. E. Briles et al., 1997; Jedrzejas, 2001; Jedrzejas et al., 2000). Additionally, PspA is a lactoferrin-binding protein which protects the bacteria from the bactericidal enzyme apolactoferrin (D. Briles, 2006; Johnston, Briles, Myers, & Hollingshead, 2006; Shaper, Hollingshead, Benjamin, & Briles, 2004). Pneumococcal surface protein C (PspC) increases cellular adherence by binding to the polymeric immunoglobulin receptor (pIgR). After binding to pIgR, the pneumococci are able to penetrate mucosal epithelial cells (Zhang et al., 2000).

Originally pneumococcal surface antigen A (PsaA) was defined as an adhesin. In pneumococci that lacked PsaA, binding to mammalian cells was deficient (Berry & Paton, 1996; D. E. Briles et al., 2000). Furthermore, the sequence of PsaA closely resembled that of other identified pneumococcal adhesins (Sampson, O'Connor, Stinson, Tharpe, & Russell, 1994). Pneumococcal surface antigen A is actually part of an ATP-binding cassette (ABC) that utilizes the energy from ATP to carry out biological functions such as gene expression (Davidson, Dassa, Orelle, & Chen, 2008; Kadioglu et al., 2008; Lennarz et al., 2004). This ABC has specificity for manganese and without PsaA, a divalent metal-ion binding protein, pneumococci do not obtain the manganese needed for normal growth (Dintilhac, Alloing, Granadel, & Claverys, 1997; McAllister et al., 2004). Even though PsaA is not an adhesin, without PsaA pneumococci lack the nutrients to grow and produce proteins including adhesins (Kadioglu et al., 2008).
Virulence factors allow for the colonization of the host. Usually pneumococci maintain an asymptomatic relationship in the nasopharynx. However breakdown of the mucosal barrier enables migration of pneumococci to other tissues causing invasive disease. These same factors that allow for colonization are also used to detect the presence of pneumococci by the host immune system to initiate pneumococcal clearance.

1.4 Innate immune response

The initial recognition of *S. pneumoniae* by the host is mediated by binding of pathogen-associated molecular patterns (PAMP) on pneumococci. Members of the complement pathway and pattern recognition receptors (PRR) family recognize PAMP and launch downstream events that result in bacterial clearance. These receptors bind bacterial virulence factors, microbe-associated molecular patterns and molecules released by the host (Koppe, Suttrop, & Opitz, 2011).

There are several PRR families including the transmembrane toll-like receptors (TLR). Toll-like receptors recognize extracellular or endosomal PAMP (Beutler et al., 2006). Components of the pneumococcal cell wall, i.e. peptidoglycan, lipoteichoic acid and lipoproteins, are bound by TLR2 (Akira, Uematsu, & Takeuchi, 2006; Koppe et al., 2011; Yoshimura et al., 1999). After activation of TLR2, pro-inflammatory cytokines IL-1, IL-6 and TNF-α are released from immune cells, including macrophages (Schwandner, Dziarski, Wesche, Rothe, & Kirschning, 1999). Toll-like receptor 2 stimulation may be detrimental to the host during early colonization as activation of TLR2 decreases tight epithelial junctions allowing for pneumococcal relocation (Clarke, Francell, Huegel, & Weiser, 2011). Toll-like receptor 4 may play a role in recognition of pneumococci when
combined with TLR2. Toll-like receptor 4 knockout mice were more susceptible to infection with *S. pneumoniae* (Koppe et al., 2011). There is also evidence that TLR4 recognizes pneumolysin (Dessing, Florquin, Paton, & van der Poll, 2008). Toll-like receptor 9 recognizes unmethylated CpG motifs within endosomes. Bacterial DNA contains more unmethylated CpGs than human DNA therefore exposure to unmethylated CpGs alerts the immune system. Upon CpG binding this intracellular receptor signals for the production of proinflammatory cytokines IL-12 and type 1 interferons.

The dominant complement pathway in response to *S. pneumoniae* infection is the classical pathway (Brown et al., 2002). The classical pathway of complement is initiated by recruitment of C1 proteins to either IgG or IgM bound to a pneumococcal surface antigen or C1 binding directly to the surface of the bacteria (Figure 5) (J A Winkelstein, 1981). Different IgG subclasses bind C1 with different strengths, with IgG1 binding more strongly than IgG2 (Flanagan & Rabbitts, 1982; van Loghem, 1986). Binding of C1 to the Fc region of antibodies or a bacterial surface activates the C1 protease subunit, C1r. Activated C1 cleaves C4 into C4a and C4b, and C4b binds to the surface of the pneumococcus. The C1 complex also cleaves C2 into C2a that is released and C2b that binds to the C4b deposited on the cell surface. Combined C2b and C4b form C3 convertase that cleaves C3 into C3a and C3b. C3b binds to C3 convertase or the surface of the bacteria where it acts as an opsonin. The C5 complex forms when C2b, C4b and C3b bind to each other. The C5 complex cleaves the C5 complement protein into C5a that is released and C5b that recruits the formation of the membrane attack complex (MAC). This complex is a membrane bound pore that mediates cell lysis. Recognition of IgG Fc regions and C3b on the cell surface promotes bacterial clearance by phagocytosis.
Additionally, pneumolysin may stimulate the classical pathway by binding to the Fc region of IgG (Paton, 1996).

The other two complement pathways, the mannose-binding lectin (MBL) pathway and alternative pathway, are involved to a lesser extent. Mannose-binding lectin binds poorly to the sugar residues on pneumococci and seems to have little effect on complement deposition (Brown et al., 2002; Hyams, Camberlein, Cohen, Bax, & Brown, 2010; Neth et al., 2000). Protein C3 is spontaneously cleaved in the plasma and deposition of C3b fragments to the pneumococcal cell surface recruits binding of Factor B (Figure 5). Nascent C3b may bind cell wall polysaccharide, teichoic acid and peptidoglycan activating the alternative pathway of complement (Quinn, Crosson, Winkelstein, & Moxon, 1977; Tauber, Polley, & Zabriskie, 1976). This pathway is often spontaneous and independent of antibody recognition (Forsgren & Forsum, 1972; Root, Ellman, & Frank, 1972; J. A. Winkelstein, Shin, & Wood, 1972). Factor D cleaves Factor B and Factor Bb remains bound to C3b on the cell surface. Properdin may bind to stabilize the Bb/C3b complex. This complex acts as a C3 convertase and continues the same cascade as the classical pathway.
1.5 Specific immune response

While innate immunity is activated following recognition of *S. pneumoniae*, adaptive immunity is only activated if the innate response is unable to clear the pathogen. After recognition of *S. pneumoniae* by TLR, cytokine production initiates the migration of APCs such as dendritic cells, macrophages and B cells to the site of infection. Antigen presenting cells encounter antigens in the blood or tissue, process the antigen and display
fragments of the antigen on its surface via the major histocompatibility complex II (MHC II). Here dendritic cells act as APCs to B and T cells initiating the adaptive immune response (Majcherczyk, 2004).

Depending on the *S. pneumoniae* antigen presented, dendritic cells may direct an anti-protein or anti-polysaccharide specific antibody response. The immunoglobulin response to pneumococcal protein PspA is dependent on CD4+ T cells through co-stimulation of B cell protein CD80 and T cell receptor CD28 (Khan, Lees, & Snapper, 2004; Lee, Sen, & Snapper, 2005; Wu et al., 2000). After antigen recognition, CD4+ T cells proliferate and differentiate into either T helper type 1 (T\(_{H1}\)) or T helper type 2 (T\(_{H2}\)) cells. The main function of T\(_{H1}\) cells is cell-mediated immunity after stimulation from activated macrophages while the primary function of T\(_{H2}\) cells is activation of B cells to stimulate antibody production (Curtis, 2005). Antigens that provoke T cell help are classified as T cell dependent (TD) antigens (Figure 6). Pneumococcal surface protein A is a TD antigen eliciting priming of T cells after recognition.
Figure 1-6 Characteristics of T-cell dependent and independent antigens

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>T cell dependent antigen</th>
<th>T cell independent type 1 antigen</th>
<th>T cell independent type 2 antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody production in athymic individual</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Antibody response in absence of all T cells</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Antibody response in infants</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Primes T cells</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Polyclonal B cell activation</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Requires repeating epitopes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Examples</td>
<td>Diphtheria toxin</td>
<td>Bacterial lipopolysaccharide</td>
<td>Pneumococcal polysaccharide</td>
</tr>
</tbody>
</table>

In contrast, PPS and CWPS are T-cell independent (TI) antigens as polysaccharides do not associate with MHC II (K. Murphy, 2011). The complete specific immune response to PPS is unknown. T-cell independent antigens stimulate antibody response in the absence of T-cells and do not induce immunological memory (Lesinski & Westerink, 2001). However CD4+ T-cells are necessary for stimulation of CD80 (Wu et al., 2000; Wu et al., 2002). The anti-PPS IgG response is more dependent on CD4+ T-cells and CD80 stimulation compared to the anti-CWPS (Khan et al., 2004).

There are two classes of TI antigens. Type 1 possesses an inherent activity that directly induces B cell division. These antigens are also referred to as B-cell mitogens as they induce cells to undergo mitosis. (K. Murphy, 2011). At high concentrations, type 1 antigens, regardless of their specificity, can induce B cell proliferation and differentiation. Antigens that stimulate this polyclonal activation include bacterial
lipopolysaccharide (LPS). Type 2 antigens do not have this intrinsic ability to stimulate B cells. Type 2 antigens can only stimulate specific mature B cells by crosslinking multiple B cell receptors (BCR). Type 2 antigens are made of highly repetitive structures that include bacterial capsular polysaccharides, such as PPS. Cross-linking of multiple BCR on mature B cells leads to IgM antibody production (K. Murphy, 2011). Activated dendritic cells provide a co-stimulatory cytokine, B-cell activating factor (BAFF) that augments antibody production against type 2 antigens.

An alternative possibility is binding of PPS cross-linked on B cells by a surface molecule present on all helper T cells. Activation of B cells by T cell independent antigens occur primarily in the marginal zone of the spleen (Carsetti et al., 2005; Kruetzmann et al., 2003). Comparison of peripheral blood subsets from infants, asplenic and healthy volunteers revealed an absence of IgM memory B cells in populations more susceptible to pneumococcal disease (Kruetzmann et al., 2003). Recognition by T cells causes cytokine production that stimulates the B cells to undergo isotype switching. Although the exact mechanism is unknown, CD1, a nonclassical MHC I-like molecule, expressed on APC and B cells presents PPS to CD8+ T cells (Kobrynski, Sousa, Nahmias, & Lee, 2005).

1.6 Therapeutic intervention

In 1891, Klemperer and Klemperer were able to demonstrate the protective properties of antiserum. Serum from a patient with a pneumococcal infection administered to a uninfected individual was sufficient to protect the individual from future infection (Klemperer, 1891). The next major progress in therapy was two decades
later when Neufeld demonstrated opsonization and serotype specific immunity to pneumococcus with immune sera (Neufeld & Rimpau, 2009).

In the mid-20th century, a major scientific advancement in the field of medicine was discovered with the use of penicillin to treat gram-positive bacterial infections (Hansman, Glasgow, Sturt, Devitt, & Douglas, 1971). Penicillin is a beta-lactam antibiotic and inhibits the synthesis of the peptidoglycan layer of a bacterial cell (Fisher, Meroueh, & Mobashery, 2005). However mutations in pneumococcal penicillin-binding proteins soon altered binding to penicillin, failing to fully disrupt cell wall synthesis. By the 1960s, penicillin-resistant pneumococci were on the rise and other therapeutic/preventative options needed to be explored (Doern, 2000).

1.7 Prevention

Increased bacterial resistance led to the reexamination of potential vaccine candidates. Of all pneumococcal virulence factors, purified capsular polysaccharide is most protective. The first vaccine contained several different purified PPS serotypes pooled together. In the early 1970s, 6-, 12- and 13-valent PPS vaccines were tested on a group of young South African gold miners (Austrian et al., 1976; Smit, Oberholzer, Hayden-Smith, Koornhof, & Hilleman, 1977). Within this population, the 13-valent vaccine was most effective, preventing 78.5% of pneumococcal pneumonia and 82.3% of bacteremia (Austrian et al., 1976). After the success of this vaccine trial, a 14-valent vaccine containing 50ug of each purified polysaccharide was developed and licensed for use in 1977. The 14 serotypes in this vaccine accounted for almost 68% of IPD (Broome & Facklam, 1981). In 1983, a 23-valent vaccine containing 25ug of each purified
polysaccharide was introduced to include additional clinically relevant serotypes (Robbins et al., 1983). The 23-valent vaccine contains serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F. These purified polysaccharides are dissolved in saline with phenol or thimerosal and contains no adjuvant. This vaccine is recommended for all individuals over the age of 65, the asplenic and those of an ethnicity or socioeconomic status associated with a higher risk of disease ("Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP)," 1997).

Despite high efficacy in healthy young adults, prevention of IPD is greatly diminished in elderly vaccinated with PPV (Melegaro & Edmunds, 2004). Pneumococcal polysaccharide-specific antibody concentrations are similar to healthy young adults however antibody functional activity is largely reduced (Romero-Steiner et al., 1999b). Due to the increased incidence and cost associated with IPD in the elderly, cost-effectiveness studies were conducted on PPV. Despite the vaccine’s reduced efficacy, several groups have found PPV to be cost effective in preventing bacteremia, pneumonia and improving overall health in the elderly (Ament, Fedson, & Christie, 2001; Sisk et al., 1997).

Pneumococcal polysaccharide is a T-cell independent antigen and therefore poorly immunogenic in infants due to their underdeveloped immune system. Consequently the 23-valent purified PPS vaccine was not efficacious in children under the age of 2. After the success of the Haemophilus influenzae type b (Hib) conjugate vaccine, a PCV was developed coupling PPS to the same diphtheria toxoid, CRM197, a T-cell dependent antigen (Kayhty & Eskola, 1996). This method however requires the
conjugation of each polysaccharide to CRM197 limiting the number of serotypes that could be included. In 2000, the first 7-valent conjugate vaccine was licensed containing the most common disease causing serotypes in children, 4, 6B, 9V, 14, 18C, 19F and 23F. Each dose contains approximately 2ug of each polysaccharide, except 4ug of serotype 6B, 20ug CRM197 and 0.125mg of aluminum as an aluminum phosphate adjuvant. Four doses at 2, 4, 6, and 12-15 months of age are administered to boost immunity (Bridy-Pappas, Margolis, Center, & Isaacman, 2005; Makela, 2003). These seven serotypes represented 76% of otitis media and 80-90% of IPD in children (Block et al., 2004).

In addition to the dramatic reduction of vaccine-serotype associated disease in children, administration of the 7-valent conjugate vaccine also resulted in a decrease in IPD amongst non-vaccinated adults (Pilishvili et al., 2010; Whitney et al., 2003). This phenomenon, known as ‘herd immunity’, is explained by the frequent exposure of adults to pneumococci spread by young. However after the introduction of the 7-valent PCV there was a shift in the prevalence of infectious serotypes. Invasive pneumococcal disease of serotypes included in the vaccine decreased significantly while non-vaccine serotypes increased. To compensate for the observed serotype shift, a new 13-valent conjugate vaccine, adding 1, 3, 5, 6A, 7F and 19A, was licensed in the US in 2010 (Nuorti & Whitney, 2010). Each dose contains 2.2ug of each serotype, 4.4ug of 6B, 34ug of CRM197, 100ug polysorbate 80, 295ug succinate buffer and 125ug aluminum as an aluminum phosphate adjuvant.

Invasive pneumococcal disease in children is a worldwide problem with the greatest number of deaths occurring in developing countries. While vaccination with
PCV is effective at preventing disease, the PCV is costly and requires multiple doses. A single dose of Prevnar®, the 13-valent PCV licensed in the US, costs $120.95 per dose (CDC, 2012). Organizations such as the Global Alliance for Vaccines and Immunizations provide subsidies in developing countries however the financial burden of this vaccine often limits widespread use (Miller, 2000; Shepard, Walsh, Kleinau, Stansfield, & Bhalotra, 1995; Sinha, Levine, Knoll, Muhib, & Lieu, 2007).

1.8 Antibody structure

The basic antibody structure is illustrated in Figure 7 below. Antibodies are composed of two identical heavy and light chains joined together by disulfide bonds. Each chain has a variable and constant region. The variable region recognizes and binds different molecules while the constant region is responsible for effector functions, i.e. recognition by phagocytes, and antibody fine specificity.

In an immunoglobulin, the variable regions produce two paratopes that bind and recognize different epitopes of the antigen. Variable region diversity is the result of gene rearrangements. There are seven different variable heavy gene families with 51 gene loci,
27 diversity segments and 6 joining segments. Additionally, the variable light chain offers even more diversity as there are two types kappa (κ) and lambda (λ). In total, the variable light chain repertoire consists of 71 variable genes and 9 joining segments (K. Murphy, 2011).

Each gene segment undergoes somatic rearrangement to create a functional V, D and J segment (Figure 8). This segment forms a complete and unique variable region. The large number of possible gene segment combinations results in the expression of 3 million different variable regions. Additionally nucleotide insertions and deletions in the CDR3, variable heavy and light gene pairing and somatic hypermutation increase the diversity. Somatic hypermutation occurs after the assembly of the whole B cell receptor and antigen stimulation. This process introduces mutations into the variable region often increasing the variable region’s affinity for an antigen.
Figure 1-8 Antibody assembly

Sequence analysis using single cell polymerase chain reaction (PCR), combinatorial libraries and human hybridomas have shown the genetic diversity of the human antibody response to PPS (Baxendale et al., 2000; Lucas, Moulton, Tang, & Reason, 2001a; X. Wang & Stollar, 2000; Zhou, Lottenbach, Barenkamp, Lucas, & Reason, 2002). Even though there are 7 variable heavy gene families, the response to PPS is frequently restricted to VH3 (Abadi et al., 1998). Within the VH3 gene family there is also bias for expression of gene loci 3-23, 3-30, 3-53, 3-49 and 3-07 (Brezinschek, Brezinschek, & Lipsky, 1995; Brezinschek et al., 1997). Gene restriction has also been observed for other polysaccharide antigens namely *Haemophilus influenzae* type b (Hib) and *Cryptococcus neoformans* (Lucas & Reason, 1999; Pinchuk, Nottenburg, & Milner,
This bias in gene usage is thought to be the result of immunoglobulin structural restraints for polysaccharide antigens (Dorner et al., 1998).

The constant region of the antibody determines the antibody isotype and consequently the role the antibody plays in the immune system. There are five different antibody isotypes, IgA, IgD, IgE, IgM and IgG. Immunoglobulin A is secreted primarily by mucous epithelium in the respiratory and intestinal tract. It is a poor activator of complement and functions primarily as a neutralizing antibody (K. Murphy, 2011). Immunoglobulins A and M have a J chain which enables secretion in the mucosa thru binding to pIgR (Paul, 2008). While its exact functions are unknown, IgD is believed to enhance mucosal immunity and stimulate basophils (K. Chen & Cerutti, 2011).

Immunoglobulin E is bound mainly to the surface of mast cells. Binding of an antigen to IgE results in an allergic response generated by the release of histamine and other chemical mediators.

Immunoglobulin M is the first antibody secreted in humoral immunity as IgM is positioned first on the constant region gene segment and produced without isotype switching (K. Murphy, 2011). The early IgM response occurs before B cells have undergone somatic hypermutation and tend to be low affinity. Immunoglobulin M is secreted as a pentamer with ten antigen-binding sites in total. Although the individual variable regions are of low affinity when multiple binding sites are attached there is an additive effect resulting an overall higher avidity. This pentameric structure makes IgM a good complement activator. Immunoglobulin M is able to bind in two conformations, star and staple (Feinstein & Munn, 1969; Svehag, Bloth, & Seligmann, 1969). The staple
conformation has thought to be important in binding multivalent surfaces such as bacterial surfaces.

Immunoglobulin G is the predominate antibody in human serum. There are four classes of IgG that are differentiated by the number of disulfide bonds and amino acids in the hinge region. The hinge region determines the flexibility of the antibody and is located between the Fab arms and the CH2 and CH3 regions (Burton, Gregory, & Jefferis, 1986; Pumphrey, 1986). The two main isotypes produced in response to S. pneumoniae are IgG1 and IgG2. The IgG1 isotype has 15 amino acids in the hinge region and two disulfide bonds. Immunoglobulin G2 is made of 12 amino acids in the hinge region and 4 disulfide bonds. The increased number of amino acids in the hinge region and decreased number of disulfide bonds enable IgG1 to be more flexible than IgG2. As a result IgG1, is unimpeded by limitations in Fab arm movement allowing for recognition of larger antigens (Morelock et al., 1994; Torres, Fernandez-Fuentes, Fiser, & Casadevall, 2007).

Phagocytosis is initiated by the interaction of the Fc region of the antibody and Fc receptors (FcRs) on macrophages, monocytes and dendritic cells. There are three classes of FcRs, FcαR bind IgA, FcεR bind IgE and FcγR bind IgG. There are no FcRs that bind IgM rendering IgM ineffective in facilitating phagocytosis. Furthermore, there are three types of FcγR that recognize IgG, FcγRI, FcγRII and FcγRIII. Immunoglobulin G1 induces phagocytosis better than IgG2 when bound to FcRs on effector cells. Additionally IgG1 is recognized by all three receptors while IgG2 is only recognized by FcγRII.
1.9 Anti-pneumococcal antibodies

Specific anti-PPS antibodies provide protection from pneumococcal disease. These antibodies bind to the surface of *S. pneumoniae* and after antibody Fc recognition induce opsonophagocytosis. Analysis of anti-PPS antibodies can act as a correlate of protection from IPD. However several factors need to be included to fully understand the anti-PPS antibody repertoire.

Initially the concentration of circulating anti-PPS antibodies was detected using the Farr assay which measured antibody binding to radioactively labeled PPS (Schiffman, Douglas, Bonner, Robbins, & Austrian, 1980). This method was not ideal, as it required large amounts of serum and radioactive reagents. For these reasons a more sensitive, safer assay was developed (Musher, Johnson, & Watson, 1990; M. H. Nahm, Siber, & Olander, 1996). The PPS-specific enzyme-linked immunosorbent assay (ELISA) is more specific, requires less serum and does not use radiolabeled PPS. However, this method detected both PPS-specific and CWPS-specific antibodies. Cell wall polysaccharide-specific antibodies are not protective and inclusion of cross-reactive CWPS-specific antibodies resulted in an overestimation of the specific antibody response (Frasch & Concepcion, 2000; Watson, 1993).

In order to eliminate inclusion of CWPS-specific antibodies, the ELISA protocol was amended to include an absorption step with CWPS to eliminate cross-reactive CWPS-specific antibodies (Musher et al., 1986). This step improved the correlation between antibody concentration and functional activity. Recently other immunogenic contaminants have been detected in purified PPS even after absorption with CWPS. It has been shown that addition of PPS22F to the absorption step decreases contaminant binding
to cross-reacting antibodies (Concepcion & Frasch, 2001). Therefore, absorption with CWPS and PPS22F was included in the PPS-specific ELISA protocol. The World Health Organization has adopted this method for detecting and quantifying PPS-specific antibodies (Wernette et al., 2003).

Pneumococcal polysaccharide-specific antibodies elicit protection by initiating complement or opsoninization resulting in phagocytosis. The affinity of the variable region for PPS controls whether the bacteria are detected by the immune system. Thus PPS-specific antibody functional activity is just as important as antibody concentration. An opsonophagocytic assay (OSPA) is used to test functional activity. Pneumococci are incubated with human serum and newborn rabbit serum is added as a source of complement. Phagocytic cells are added and functional activity is measured by calculating the number of phagocytosed vs. non-phagocytosed pneumococci. The higher percentage of phagocytosed pneumococci the more efficient the antibody is at facilitating opsonophagocytosis of pneumococci.

Another component of functional activity is avidity or how strongly an antibody binds to its antigen. This is not to be confused with affinity. Affinity is the strength of binding for one variable region segment. Avidity is defined as the strength of binding of the whole antibody. Avidity measurement enables analysis of the quality of the humoral immune response. There are several methods by which to measure antibody avidity. A common method is by disruption ELISA. Antibody samples are incubated with varying molar concentrations of a chaotropic agent, usually sodium thiocyanate. The agent disrupts binding to the antigen (PPS) by interrupting molecular forces. The higher the molar concentration needed to disrupt binding, the more avid the antibody is for its
antigen. Disruption of binding by 50% determines the avidity index. This value can be used to compare the avidity of different antibodies.

Calculation of the avidity index is an adequate tool for general comparison. To more specifically compare antibodies a binding constant is measured. The binding constant is calculated from the rate of association and dissociation of an antibody from its antigen. Surface plasmon resonance (SPR) is a technology used to measure the rates of these molecular interactions.

The 1950s began the pioneering of SPR technology (Ritchie, 1985). Surface plasmons are light waves trapped on the surface of a conductor by interacting with free electrons (Willander & Al-Hilli, 2009). The free electrons on the gold surface oscillate in resonance with the light wave. Concentrating light from a laser enhances the electric field used to manipulate light-mass interactions (Willander & Al-Hilli, 2009). Aqueous interactions occur over a gold chip on top of a prism (Figure 9). A laser shines light through the prism and when there are changes on the surface of the chip the angles by which light refracts through the prism changes. A computer records these angle changes and plots them on a graph. Interactions are measured in mRIU, which is equivalent to approximately 1ng/mm².
The use of SPR is an invaluable tool to directly quantify an antibody’s avidity for its antigen on the molecular level. Comparison of binding constants for different anti-PPS antibodies in combination with OPI measurements enables analysis of the quality of the human B cell response to *S. pneumoniae*.

### 1.10 Human B cell response

The specific subset(s) of B lymphocytes responsible for immune response to polysaccharide antigens remains to be elucidated. Studies have implicated the role of IgM memory cells, by some postulated to be splenic marginal zone B cells, in response to polysaccharide antigens (Kruetzmann et al., 2003; Park & Nahm, 2011; Pillai, Cariappa,
& Moran, 2005; Weller et al., 2004). The nature of the B cell phenotype responsible for the production of anti-polysaccharide antibodies remains to be defined.

There is some controversy considering the surface receptors expressed on anti-PPS B cells. Two populations, CD27\(^+\)IgM\(^+\) and CD27\(^+\)IgM\(^-\) have been implicated as possible phenotypes of B cells responsible for anti-PPS antibody production (Takizawa, Sugane, & Agematsu, 2006; Wardemann, Boehm, Dear, & Carsetti, 2002b; Weller et al., 2004). There are several arguments supporting the role of IgM memory (CD27\(^+\)IgM\(^+\)) B cells in the response to PPS.

Asplenic individuals and young children have severely reduced number of IgM memory cells increasing their risk of pneumococcal infection (Kruetzmann et al., 2003; Shi et al., 2005; Weller et al., 2004). These populations also respond poorly to polysaccharide vaccines (Hart et al., 2007; Timens, Boes, Rozeboom-Uiterwijk, & Poppema, 1989; Zandvoort & Timens, 2002). Additionally there is a correlation between the number of IgM memory cells and incidence of IPD in these populations (Kruetzmann et al., 2003). A decreased percentage of IgM memory cells have also been reported in HIV-positive individuals.

There is also evidence that IgM memory cells are not solely responsible for the immune response to PPS. In groups at high risk for IPD namely the asplenic, common variable immunodeficient and elderly also have a decreased number of switched memory (CD27\(^+\)IgM\(^-\)) B cells (Kruetzmann et al., 2003; Shi et al., 2005). Additionally, antibodies sequenced 5 days post-vaccination are predominately IgG and IgA (Kolibab, Smithson, Rabquer, Khuder, & Westerink, 2005; Lucas et al., 2001a; Zhou et al., 2002; Zhou, Lottenbach, Barenkamp, & Reason, 2004). After stimulation \textit{in vitro} switched memory
cells produce higher levels of anti-PPS antibodies than IgM memory B cells (Takizawa et al., 2006). Recent studies in SCID mice with human lymphocyte subsets suggested switch memory cells produced anti-PPS IgG following PPV (Moens, Wuyts, Meyts, De Boeck, & Bossuyt, 2008).

Immunoglobulin M+ IgD+ B cells represent antigen naïve cells expressing unmutated variable regions. Recently, Klein et al described two populations within this classically naïve IgM+IgD+ group (Klein, Rajewsky, & Kuppers, 1998). These two populations are distinguished by expression of CD27, a member of the TNF receptor family. B cells that are CD27− represent the antigen naïve, unmutated population. However, CD27+ is a marker for human memory B cells and is expressed on activated B cells (Tangye & Good, 2007). CD27+ B cells are localized to the human spleen and secrete 5- to 100-fold more immunoglobulin that CD27− B cells when stimulated in vitro (Maurer et al., 1992; Maurer, Holter, Majdic, Fischer, & Knapp, 1990). CD27+IgM+ memory B cells have undergone somatic hypermutation (SHM) but not class-switching. These cells are believed to begin SHM in the pre-immune repertoire and mediate responses to T-cell independent antigens.

1.11 Natural antibodies

Natural antibodies are able to recognize and bind a variety of foreign and self-antigens including protein and polysaccharide antigens however the characteristics of these antibodies remains to be elucidated. Polyreactive antibodies although low avidity are believed to be the body’s first line of defense against pathogens and can initiate complement deposition in the absence of specific anti-PPS antibodies. Natural,
Polyreactive antibodies include IgM, IgG and IgA isotypes, mainly use VH3, VH4 or VH5 genes and classically contain few mutations, i.e. resemble germline sequences (Casali & Notkins, 1989; Ochsenbein & Zinkernagel, 2000). Highly mutated, specific anti-PPS antibodies are derived from conventional antibody producing B2 cells that are generated through antigen-driven somatic hypermutation. Another subset of B cells, B1 cells, play a role in the innate immunity to encapsulated organisms. In mice, these cells are phenotypically defined by CD5 expression. B1 cells are unique in several aspects including phenotype and production of natural Abs (Berland & Wortis, 2002; Hardy & Hayakawa, 2001). B1 cell antibodies bind autoantigens and are broadly cross-reactive to a variety of bacterial antigens i.e. PPS and LPS (Mackenzie et al., 1991). As stated in the previous section, murine studies have demonstrated that these CD5⁺IgM⁺ B cells spontaneously secrete natural antibodies that appear to play a crucial role as the first line of defense against bacterial and viral pathogens including the pneumococcus (Baumgarth et al., 2000; Boes, Prodeus, Schmidt, Carroll, & Chen, 1998; D. E. Briles et al., 1981; Ochsenbein et al., 1999). Thus CD5⁺ B cells, in particular the CD5⁺ B1 cells, are thought to be responsible for the production of natural antibodies in humans.

By definition, protective antibodies must have functional activity. Natural antibodies with a high opsonophagocytic activity and low avidity have previously been reported (Baxendale & Goldblatt, 2006; Romero-Steiner et al., 1999a). Additionally Casali et al. demonstrated that B1 cells could generate hypermutated antibodies contributing to antibody avidity and in turn functional activity (P. Casali & E. W. Schettino, 1996).
Currently, vaccines for pneumococcus are lacking. The PCV is expensive to manufacture and very limited in the number of serotypes covered. The PPV does not stimulate an adequate immune response in groups at greatest risk for IPD. The human B cell response to pneumococcus is not well understood. In the following studies we have analyzed the B-cell response to PPS in two ways. In the first manuscript, we developed a method to directly labeled anti-PPS cells with fluorescent PPS14 and 23F. Identification of anti-PPS cells enabled characterization of the phenotype of B-cells that respond to PPV. The second manuscript explored the properties of polyreactive PPS antibodies. Sequence analysis of polyreactive anti-PPS antibodies enables the estimation of the quality of the innate immune response to PPS. The results of these studies will help to better understand the human B cell response to *S. pneumoniae*. Once the human B cell response to pneumococcus has been analyzed, vaccines and treatment can specifically be designed to stimulate the appropriate immune response.
2 Phenotypic analysis of pneumococcal polysaccharide-specific B cells

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Running title: Pneumococcal PPS B cell phenotype
2.1 Abstract

The phenotype of B cells responsible for the production of anti-pneumococcal polysaccharide antibody has been unclear. Although individuals that respond poorly to the 23-valent pneumococcal polysaccharide(s) (PPS) vaccine, Pneumovax®, such as children <2 years, the asplenic and a subset of Common Variable Immunodeficiency (CVID) patients are profoundly deficient or lack IgM memory cells (CD27⁺IgM⁺), they are also deficient in the switched memory (CD27⁺ IgM⁻) compartment. Direct characterization of PPS-specific B cells has not been performed. In this study we labeled PPS14 and PPS23F with fluorescent markers. Fluorescent labeled PPSs were used in FACS Aria flow cytometry to characterize the phenotype of PPS-specific B cells obtained from 18 young adults pre- and post-immunization with Pneumovax®. The labeled PPS were capable of inhibiting binding of antibody to the native PPS. Similarly, the native PPS were able to inhibit binding of PPS-specific B cells in a flow cytometric assay demonstrating specificity and functionality. Phenotypic analysis of unselected B cells, pre- and post-immunization demonstrated a predominance of naïve CD27⁻IgM⁺ cells accounting for 61.4% of B cells. Likewise, the PPS-specific B cells obtained pre-immunization consisted primarily of naïve, CD27⁻ B cells, 55.4-63.8%. In contrast, the PPS-specific B cells obtained post-immunization were predominantly IgM memory cells displaying the CD27⁺IgM⁻, 54.2% for PPS14 and 66% for PPS23F, significantly higher than both unselected B cells and PPS-specific B cells. There was no significant difference in switched memory B cell populations (CD27⁺IgM⁻) between groups. These results
suggest a dominant role of IgM memory cells in the immune response to pneumococcal polysaccharides.
2.2 Introduction

*Streptococcus pneumoniae* is a major cause of morbidity and mortality in young children, elderly adults, and immune compromised hosts. There are currently two types of vaccines that offer protection against pneumococcal disease: conjugate vaccines for children under 2 years of age and a 23-valent pneumococcal polysaccharide vaccine (PPV23) for protection in adults (1). Both vaccines elicit serotype specific opsonic antibodies, which are necessary for protection (2, 3). The phenotype of the B lymphocyte population responsible for the immune response to the purified pneumococcal vaccine (Pneumovax®) has been controversial. The debate centers primarily on the surface antigens expressed by the responding B lymphocytes. Recently, it has been suggested that peripheral blood CD27+ IgM+ or IgM memory B lymphocytes are recirculating splenic marginal zone (MZ) B lymphocytes (4, 5). These lymphocytes are believed to recognize TI-2 antigens such as pneumococcal polysaccharide by virtue of a pre-diversified sIgM and respond immediately without T cell help (6, 7). This view treats CD27+ IgM+ B lymphocytes as innate immune cells in the first line of defense (8-10).

In support of this concept, it has been shown that persons with decreased or absent IgM memory B lymphocytes such as the splenectomized, infants under 2 years of age, elderly, HIV infected, and a subgroup of common variable immunodeficiency patients, all respond poorly to polysaccharide vaccines and are highly susceptible to infections with encapsulated organisms (5-7, 11-13). It is however unlikely that IgM memory B lymphocytes are exclusively responsible for anti-polysaccharide antibody production as
switched memory B lymphocytes (IgM\(^{+}\)CD27\(^{+}\)) secrete anti-PPS antibody following \textit{in vitro} stimulation (14). Furthermore, sequence analysis of anti-PPS antibodies, 5 days post-vaccination, demonstrate a predominance of IgG and IgA antibodies, derived from switched memory cells that have undergone somatic hypermutation (15-17).

Moreover, IgM and switched memory B cells likely play important roles in the immune response to PPV. Although several studies have demonstrated that loss of IgM and/or switched memory B cells in the HIV-negative and HIV-infected populations, they did not focus on the PPS-specific cells (7, 13, 18). We have established a technique to identify PPS-specific B lymphocytes, enabling us to characterize the phenotype of PPS-specific B lymphocytes. In this study we have identified PPS specific B lymphocytes using fluorescently labeled polysaccharides and analyzed the phenotype of these polysaccharide-specific B cells by flow cytometry. The results of our study demonstrates a significant increased representation of IgM memory B cells in the polysaccharide-specific B cell fraction compared to the unselected B cell fraction, providing direct evidence of the importance of IgM memory cells in the response to pneumococcal polysaccharides.
2.3 Materials and Methods

*Human volunteers*

Twenty two pneumococcal polysaccharide vaccine-naïve healthy volunteers between the ages of 18-30 years (mean=24) participated in the University of Toledo IRB committee approved study (IRB # 105137). Each individual was questioned about medications, previous illness and present health. In addition, Hepatitis B, Hepatitis C, HTLV 1 &2, and HIV screening was performed. Each individual was explained about study design and protocol in detail. Informed consent was obtained from all participants. Volunteers were immunized with the 23-valent pneumococcal polysaccharide vaccine (Pneumovax® 23, Merck). Blood samples were collected pre-vaccination, day six, and four – six weeks post-vaccination. Lymphocytes were obtained for flow cytometric analysis at day 0 and day 6 post-vaccination. Serum samples obtained at day 0 and between 4-6 weeks were used to measure serum antibody responses and opsonophagocytic activity.

*Labeling of polysaccharide 14 and 23F with fluorescent dye*

Conjugation of PPS-14 to Cascade Blue Ethylenediamine (CB) (Invitogen Cat # C-621); or PPS-23F to 5 - (4,6 - Dichlorotriazinyl)aminofluorescein (DTAF) (Sigma Fluka Cat # 36565) was carried out as follows. Ten mg of PPS-14 or 23F (10 mg/ml in 0.1 M Borate Buffer, pH 9.0) was incubated with 1.0 mg of Cascade Blue or 1.0 mg of 5-DTAF respectively for 2.5 hours at 4°C. The mixture was dialyzed against PBS for 24 hours at 4°C with 4 changes of PBS (MW cutoff =
Approximately 10µl of 5M Sodium cyanoborohydride was added to the dialysate and the samples were mixed for another 30 minutes at 4°C in the dark. The samples were again dialyzed against PBS for 24 hours at 4°C with 4 changes of PBS. Finally, samples were subjected to chromatography on a Sephadex G-25 column (1cm Diameter x 17cm height) at 4°C in the dark. Fractions (150µl each) containing PPS-14 or PPS-23F complexed with Cascade Blue or DTAF respectively were pooled and stored at -20°C.

**Fluorescent labeling of polysaccharide-specific hybridoma cells and human B lymphocytes**

Mouse hybridoma cells with specificity for pneumococcal polysaccharides 14 and 23F, used as positive control were a gift from Pfizer (Pearl River, N.Y.). Hybridoma cells with specificity for PPS14 and/or PPS23 were stained by incubation with 10µg/ml fluorescently labeled pneumococcal polysaccharides 14-Cascade Blue and 23F-DTAF. PPS14 and PPS23-positive B lymphocytes were identified using the following fluorescently labeled antibodies: anti-CD19-PE, anti-CD27-PerCP-Cy5.5, anti-IgM-APC, anti-IgD (Alexa Fluor 700) (BD Pharmingen), and fluorescently labeled PPS 14-Cascade Blue and 23F-DTAF (BioCentra LLC, Sugar Land, TX).

PBMC were collected from immunized volunteers at day zero and 6 days post-vaccination. Buffy coats were harvested and mixed 1:1 with PBS 2mM EDTA and layered onto lymphocyte separation medium (Cellgro®) followed by centrifugation. Cells were then resuspended in RBC lysis buffer followed by addition of 10ml PBS 2mM EDTA 0.1% BSA. Cells were counted, centrifuged, and resuspended to 1x10⁸ cells/ml. Before staining, cells were absorbed with 10µg/ml of cell wall polysaccharide (CPS).
(Statens Serum Institut, 3459; MiraVista Diagnostics, Indianapolis, IN) and PPS 22F (ATCC) for 20 minutes; this step has been shown to reduce non-specific binding in ELISA (19). For inhibition flow experiments listed concentrations of homologous pneumococcal polysaccharide were included during the absorption step. Cells were then labeled with 10µg/ml of labeled polysaccharide, either 14-CB or 23F-DTAF, and previously mentioned markers per manufacturer’s instructions. Cells were washed and resuspended in PBS and analyzed with three laser FACSaria using FACSDiva software (BD Biosciences). FCS files were further analyzed using FlowJo software (TreeStar, Ashland, OR).

**Pneumococcal Polysaccharide ELISA**

ELISA was performed to examine the anti-PPS specific human antibodies in all volunteers. The pneumococcal polysaccharide ELISA is a modification of the WHO assay (20). Briefly, 5 µg/ml of pneumococcal polysaccharide, either 14 or 23F, were absorbed onto Nunc Maxisorp microtiter plates (Nunc Roskilde, Denmark) at 37°C overnight. Plates were then washed with PBS + 0.1%Tween-20 (PBST). Sera was diluted 1/200 in PBST then adsorbed with CPS (10 mg/ml) and 22F (10 mg/ml) for 30min at room temperature. After absorption sera were serially diluted onto the plates and incubated at 37°C for 2hrs; the standard serum 89-SF was used as a positive control. Plates were washed and bound antibody was detected using HRP-conjugated anti-human Ig(H+L) monoclonal antibody (Southern Biotech) diluted 1/3000 in 1% BSA PBST and
incubated at 37°C for 1hr. After washing, plates were developed by using an OPD substrate and the O.D. was read at a wavelength of 490nm.

Inhibition ELISA

The inhibition ELISA is similar to the 22F absorption ELISA as reported by Concepcion and Frasch (19). Briefly, 10μg/ml of purified pneumococcal polysaccharides, either 14 or 23F, were adsorbed onto Nunc Maxisorp (Nunc Roskilde, Denmark) high-binding microtiter plates at 37°C overnight. The plates were blocked with 1% bovine serum albumin (BSA) in PBS/0.1% Tween-20 (PBST) for 2hrs at 37°C. Supernatants were absorbed with 10ug/ml CPS (Statens Serum Institut, 3459; MiraVista Diagnostics, Indianapolis, IN) and 22F (ATCC) for 30 min at room temperature. Furthermore, supernatants were blocked for 30 min at room temperature with increasing concentrations of homologous fluorescently labeled polysaccharide, either 14-cascade blue or 23F-DTAF, to show inhibition. For negative controls the previously described CPS and 22F absorbed samples were blocked with heterologous polysaccharide 23F-CB for polysaccharide 14 and 14-CB for 23F. Supernatants were added to the ELISA plates and incubated at 37°C for 1hr. The standard serum 89-SF was used as a positive control. Bound antibody was detected using HRP-conjugated anti-human Ig(H+L) monoclonal antibody (Southern Biotech) diluted 1/3000 in 1% BSA PBST and incubated at 37°C for 1hr. Plates were developed by using OPD substrate, stopped with H₂SO₄ and O.D. was read at a wavelength of 490nm.
Opsonophagocytic assay

Opsonophagocytic assay was performed as previously described (21, 22) to determine functional vaccine response to pneumococcal polysaccharides 14 and 23F. Briefly, *S. pneumoniae*, serotypes 14 and 23F were incubated with serial diluted heat inactivated pre-vaccination and post-vaccination sera. Newborn rabbit serum (Pel-Freez, Brown Deer, WI) was added as a source of complement. Differentiated HL-60 cells were added at an effector/target ratio of 400:1. All sera were tested in duplicate. The opsonophagocytic titer was determined as the reciprocal of the dilution with 50% killing when compared to serum free controls and analyzed using the Opsititer1 software program from the University of Alabama at Birmingham.

Statistical analysis

Geometric mean concentration of IgG, IgM and IgA and Flow cell numbers, specific to PPS14 and 23F were calculated for each group. Correlation between two groups was examined using Pearson’s correlation coefficient. Comparison between two group values was performed using unpaired t test. P values less than 0.05 were considered to be significant.

2.4 Results

*Donor antibody response to vaccination*
To confirm pneumococcal polysaccharide specific immune response to vaccination we obtained pre-vaccination sera, day zero, and post-vaccination sera, day 28-42, from healthy young adults and measured antibody responses to serotypes 14 and 23F. Antibody concentrations were determined following absorption with PS22F and cell wall polysaccharide (CPS) as previous studies have demonstrated that absence of absorption with 22F and CPS results in an over estimation of polysaccharide-specific antibody concentration (19, 21). Post-vaccination, donors had a significant increase in concentration of PPS14 specific IgG from 2.96±2.5 µg/ml to 29.78±17.07 µg/ml (p<0.0001) and IgM from 1.37±0.49 µg/ml to 28.17±9.26 µg/ml (p<0.0001). Although post-vaccination PPS14 specific IgA titers were higher than pre-vaccination titers, but no significant difference was found (p=0.08). Similarly, post-vaccination responses to PPS23F were significantly increased compared to pre-vaccination sera, IgG from 1.54±0.71 µg/ml to 21.54±13.09 µg/ml (p<0.0001) and IgM from 0.17±0.22 µg/ml to 8.86±10.57 µg/ml (p<0.0001). In our sample population the greatest increase in pre- to post-vaccination concentration was for the isotype IgG followed by IgM for both PPS14 and PPS23F. There was a positive correlation between pre- and post-vaccination PPS14-specific IgM ($r^2 = 0.88; p < 0.0001$) and pre- and post-vaccination PPS23F-specific IgM ($r^2 = 0.98, p < 0.0001$) and IgG ($r^2 = 0.79, p < 0.0001$) and IgA ($r^2 = 0.81; p < 0.001$). In summary all donors displayed an increase in serotype specific antibody response in immunoglobulin isotypes IgG, IgM, and IgA with the exception of donor 4 whose IgA levels remained undetectable after vaccination. This data confirms that the young healthy
donors used in this study responded to Pneumovax23®. Donor antibody responses are summarized in Figure 1A.

**Functional antibody response**

The functional or opsonophagocytic response of serum antibody obtained pre-vaccination (day 0) and 28-42 days post-vaccination against both serotype 14 and 23F PPS was determined for all donors (Figure 1B). Data is reported as the opsonophagocytic index (OPI) or reciprocal of the antibody dilution required to obtain 50% opsonophagocytic killing by differentiated HL-60 cells. For all donors, post-vaccination sera had a significant increase in OPI against serotype 14 and serotype 23F when compared to pre-vaccination sera. Positive correlations were found between post-vaccination IgG and post-vaccination OPI for both PPS14 and PPS23F ($r^2 = 0.8$, $p<0.0001$; $r^2 = 0.86$, $p<0.0001$ respectively). Moreover, the sample population displayed a functional immune response after vaccination with Pneumovax23®.

**Labeled polysaccharide maintains native epitope(s)**

We evaluated the ability of fluorescently labeled pneumococcal polysaccharide to maintain the native epitope(s) of unlabeled polysaccharide. To this purpose, we performed an ELISA using increasing concentrations of fluorescently labeled PPS to inhibit binding of the polyclonal control serum 89-SF to wells coated with native unlabeled PPS14 or 23F. Unlabeled homologous polysaccharide was used as a positive control and unlabeled heterologous PPS was used as a negative control. The ability of
native pneumococcal polysaccharide and fluorescently labeled pneumococcal polysaccharide to inhibit 89-SF binding to homologous polysaccharides were similar as shown in Figure 2. At 1µg/ml both cascade blue labeled PPS14 and unlabeled PPS14 were able to inhibit 89-SF binding by greater than 50%. Incremental addition of inhibitory PPS, up to 25µg/ml, further increased inhibition of 89-SF binding for both native and cascade blue labeled PPS14. Likewise, DTAF labeled PPS23F was able to inhibit 89-SF binding comparable to the native unlabeled PPS23F. Both labeled PPS14 and PPS23F displayed the same trend and similar magnitude of inhibitory affect as their unlabeled homologous counterparts, while minimal inhibition was seen in control wells where heterologous polysaccharide was used.

Labeled polysaccharides bind homologous and not heterologous anti-PPS hybridoma cells

To further confirm functionality and specificity of the labeled polysaccharides, hybridoma cells with specificity for PPS14 (α14g2b) and for PPS23F (α23F) were incubated with PPS14-CB and with PPS23F-DTAF and subjected to flow cytometry. The results of these studies demonstrated that hybridoma cells α23F with specificity for PPS23F, uniquely bound PPS23F-DTAF and failed to bind PPS14-CB in a concentration dependent manner as shown in Figure 3. A sub-population of small likely non-secreting cells consistently failed to bind PPS23F-DTAF. Conversely, hybridoma cells α14g2b with specificity for PPS14, uniquely bound PPS14-CB and failed to bind PPS23F-DTAF, demonstrating both specificity and functionality of the labeled PPSs.

Fluorescently labeled polysaccharides recognize PPS-specific B lymphocytes
To demonstrate the specificity of binding of the fluorescently labeled PPS, an inhibition assay was performed. This was accomplished by pre-treating lymphocytes isolated 6 days post-vaccination, with increasing concentrations of homologous unlabeled polysaccharide before addition of fluorescently labeled polysaccharide. Inhibition of fluorescent polysaccharide binding was 68% at 250 ug/ml inhibitory PPS for PPS14-CB and greater than 80% for PPS23F-DTAF. Inhibition occurred in a concentration dependent manner as shown in Figure 4.

**Phenotypic analysis of polysaccharide specific B lymphocytes**

To determine the phenotype of B lymphocytes that respond to vaccination with Pneumovax23®, 18 healthy young donors were immunized. Pre-vaccination (n=9) and six days post-vaccination (n=18) circulating PBMC were isolated, labeled and subjected to Flow cytometry analyses by using the following fluorescently labeled antibodies/antigen: CD19, CD27, IgM, IgD, PPS14 and PPS23F. The phenotype of the pre- and post-vaccination specific B cells was compared to the phenotype of unselected B cells. CD19⁺ B lymphocytes were subdivided into four categories: naïve (CD27⁻ IgM⁺), CD27⁻ IgM⁻ class switched, IgM memory (CD27⁺ IgM⁺), and class switched memory (CD27⁺ IgM⁻) B cells.

Analysis of the unselected B cell populations obtained pre-vaccination showed that a large proportion, 71.2% (41.8-97.2%), of these B cell were naïve or CD27⁻. The majority (61.4%) of the CD27⁻ B cells expressed the CD27IgM⁺ phenotype, while a minority, (9.8%) of total B cells were CD27IgM⁻. The memory or CD27⁺ B cell population
represented 28.7% of the B cells with 14.3% expressing the IgM memory phenotype (CD27^+IgM^+) and 14.4% were classic switched memory B cells (CD27^+ IgM^-). Analysis of the post-immunization unselected B cell population did not differ significantly from pre-immunization values (Figure 5A and Fig 5B).

In the pre-immunization samples, a small percentage of B cells, 0.56±0.34%, stained with fluorescently labeled PPS (Table 1). The PPS14 and PPS23F-specific B cells in this population demonstrated a predominance of CD27^+ B cells with a total of 55.4% (14-80.3%) for PPS14 and 63.8% (55.8-81.5%) for PPS23F. The CD27^+IgM^+ phenotype represented the majority of these cells with 47.4% for PPS14 and 55.8% for PPS23F of the total B cell population. The CD27^+ population represented a total of 44.5% (8.1-86.6) and 36.2% (10.3-48.2) of the PPS14 and PPS23F-specific B cells with the IgM memory component (CD27^+IgM^+) representing 21.8% of the total PPS14-specific B cells and 25.6% of the PPS23F-specific B cells. The remainder of the B cell population consisted of switched memory B cells (CD27^+IgM^-), accounting for 22.8% of the PPS14-specific B cells and 10.6% of the PPS23F-specific B cells (Figure 5A).

In the 6 day post-immunization samples, the percentage of PPS fluorescently labeled B cells increased significantly to 2.75±1.48% for PPS14 and 2.08±1.17% for PPS23F (Table 1). In sharp contrast to both the unselected and pre-immunization PPS-selected B cell populations, the minority of post-immunization PPS-specific B cell populations consisted of naïve CD27^- B cells, 27.6% for PPS14 and 20.5% for PPS23F. The naïve B cell population consisted primarily of CD27^+IgM^- B cells, 23.8% and 17.5% for PPS14 and PPS23F respectively. A small percentage, 3.8% and 3% of B cells were naïve, class switched CD27^+IgM^- B cells. The majority of the PPS-selected B cells were memory B
cells (CD27+) accounting for 72-79% of the total B cell population (Figures 5B and 6). Moreover, the IgM memory population, CD27+IgM+, was significantly overrepresented compared to both unselected and pre-immunization PPS-specific B cells, representing 54.2% of the PPS14-specific B cells and 66% of the PPS23F-specific B cells (Fig. 5B and 5C). In contrast, there was no significant difference in switched memory (CD27+IgM-) population between post-immunization PPS14 and PPS23F-specific B cells and the unselected or pre-immunization PPS-selected populations, accounting for 18.2% and 13.5% respectively. Furthermore, there was a strong correlation between post-immunization IgM antibody concentration and post-immunization IgM memory B cell percentage for both PPS14 (r²=0.87) and PPS23F (r²=0.88). In contrast, the correlation between post-immunization IgG antibody concentration and post-immunization switched memory B cell percentage was much lower, r²=0.56 for PPS14 and r²=0.51 for PPS23F.

2.5 Discussion

The goal of this study was to characterize the phenotype of B cells responding to Pneumovax23® vaccination. We specifically chose pneumococcal polysaccharides 14 and 23F as they are the most common disease causing serotypes found in adult high risk groups such as the elderly and HIV infected (23-25). To assess the immune competency of our volunteers we studied pre- and post-vaccination polysaccharide specific immunoglobulin concentration and opsonophagocytic assays. All individuals responded to vaccination displaying a significant increase in anti-polysaccharide antibody concentration, specifically IgG, with a concomitant significant increase in opsonophagocytic activity. Within our test group there was variability in pre-
and post-vaccination PPS specific antibody concentration. Variability in antibody concentrations occurred between individuals and between serotypes within individuals. Therefore, a high response to one PPS present in the multivalent vaccine did not necessarily correlate with a high response to other PPS included in the vaccine. This data is consistent with previous studies investigating the pre- and post-vaccination anti-PPS antibody response in young healthy individuals to all serotypes included in Pneumovax23® (26). Moreover, all volunteers demonstrated a two-fold or higher increase in pre- versus post-immunization antibody concentrations. A positive correlation between pre- and post-immunization titers was noted and between post-immunization IgG and opsonophagocytic index. Although not the main focus of our study these basic immunological assays demonstrate the immune competency of our study group.

The specific subset(s) of B lymphocytes responsible for immune response to polysaccharide antigens remains to be elucidated. Studies have implicated the role of IgM memory cells, by some postulated to be splenic marginal zone B cells, in response to polysaccharide antigens (5, 7, 9, 27). The nature of the B cell phenotype responsible for the production of anti-polysaccharide antibodies, remains to be defined.

We used fluorescently labeled PPS in conjunction with flow cytometry for identification and analysis of PPS-specific B lymphocytes. Identification of antigen specific B lymphocytes using fluorescently labeled antigen has several advantages over previously used methods. Direct labeling of the polysaccharides allows for polysaccharide-specific B cell phenotype analysis while minimizing potential cross reactivity to linking agents used by indirect labeling methods. Overall this results in lower background binding and
more accurate phenotype analysis. We have demonstrated the ability to identify pneumococcal polysaccharide specific B lymphocytes using fluorescently labeled polysaccharide in conjunction with flow cytometry. The specificity of our labeled PPS is supported by their ability to inhibit binding of the control sera 89-SF to homologous unlabeled polysaccharide in ELISA and the ability to bind to PPS14 or PPS23F-specific monoclonal cell lines. Likewise, binding of the labeled PPS to post-vaccination peripheral blood B lymphocytes in flow cytometry can be inhibited with addition of homologous unlabeled polysaccharide.

Flow cytometric analysis of unselected CD19^+ B cells showed a predominance of naïve CD27^-IgM^+ B cells, as previously described in the peripheral blood of healthy adults (28-30). The CD27^+ memory B cell population constituted about 30% of total B cells with an equal distribution between IgM memory and switched memory B cells. We found no significant difference in phenotype distribution between pre- and post-immunization unselected samples. This is not surprising as the PPS-specific B cells represented a small fraction, between 2-2.75%, of the total post-immunization B cell population insufficient to cause a significant shift in overall phenotype distribution. Analysis of the pre-immunization polysaccharide-specific B cells, 0.5% of the total B cell population, resulted in a phenotypic pattern very similar to that of unselected B cells with a predominance of naïve CD27^+IgM^+ B cells. There was however an increased percentage of CD27^+IgM^+ cells in the PPS-specific B cells although this was only significant for PPS23F. In contrast, in the 6 day post-immunization PPS-specific B cells, there was a highly significant shift in cell surface expression. The majority of post-immunization
PPS14 and PPS23F-specific B cells expressed the IgM memory, CD27⁺IgM⁺, phenotype with a concomitant decrease in naïve, CD27⁻IgM⁺, expression when compared to the unselected B cells and to the pre-immunization PPS-specific B cells. It should be mentioned that we found a strong correlation between the post-immunization PPS-specific IgM and the number of PPS-specific IgM memory B cells. Notably, there was no significant change in the CD27⁺IgM⁻ or switched memory population post-immunization and it correlated poorly with the IgG antibody concentration.

Isolation of antigen-specific B cells is known to be a challenging enterprise due to their limited presence in the B cell population. Overall, antigen selected B cells represent <1-2% (31-33) of total B cells, depending on technique and timing of isolation, and in line with our findings. Moreover, although a variety of techniques have been used, all have limitations in yield and purity. Both yield and purity can affect phenotype analysis performed in our studies. We employed direct fluorescent labeling of PPS in conjunction with flow cytometry/sorting. The PPS-selected population was inhibited 68-80% by unlabeled PPS, suggesting that two-thirds to 80%, depending on serotype, of our PPS-labeled B cells were indeed PPS-specific. In accordance, the remaining 20-32% of our PPS-labeled cells were not PPS-specific, false-positive, thus likely expressing the unselected cell phenotype, i.e. 14.3-15.6% CD27⁺IgM⁺, a much lower percentage than the PPS-positive B cells. Moreover, the presence of the false-positive PPS-labeled population therefore likely diminished, not increased, virtual expression of the CD27⁺IgM⁺ phenotype in our PPS-specific population. As both unselected and PPS-selected populations expressed a similar percentage of CD27⁺IgM⁻, it is unlikely that the
contaminating or false-positive PPS-labeled population changed the percentage of switched memory B cells in the PPS-specific population.

These data suggest that a large portion of the PPS-responding B cells are IgM memory cells and this concept is supported by several experimental and clinical findings. First, both asplenic individuals and children younger than two years of age have an undetectable or severely reduced number of IgM memory cells, are at increased risk of pneumococcal infection and respond poorly to polysaccharide vaccines (5-7, 11-13). Second, the percentage of IgM memory cells in CVID patients correlates with incidence of encapsulated bacterial infection (7). In further support of these findings, Kruetzmann et al established a significant correlation between serum anti-polysaccharide serotype 3, 9 and 22 IgM antibody concentration and the number of IgM memory B cells in the peripheral blood of children. Several recent studies have focused on memory B lymphocyte sub-populations and immune response to pneumococcal polysaccharide vaccination particularly in HIV-positive individuals (13, 18). These studies analyzed the peripheral blood B lymphocyte population in HIV-positive individuals and correlated the results with either quantitative IgG or IgM antibody response to PPV. Moreover, both studies demonstrated a significant decrease in the switched memory B cell population in the HIV-infected. However, there was no correlation found between the number of switched memory B cells and anti-PPS IgG antibody concentration. In another study, Hart et al (13) described a highly significant loss of IgM memory B cells in HIV-positive individuals. The loss of B memory cells correlated with the decrease in the anti-PPS IgM antibody response. It should be mentioned that in these studies, as well as others, B
lymphocyte populations were not selected for PPS-specificity complicating the interpretation of the data. The results of the present study however, support the hypothesis that IgM memory B cells play an important, if not crucial role in the immune response to pneumococcal polysaccharides. As stated previously, one could argue that IgM memory B cells are not solely responsible for the antibody response to pneumococcal polysaccharide antigens. First, the elderly, asplenic and CVID patients not only have reduced or absent IgM memory cells but also significantly reduced numbers of switched memory B cells (6, 7). Second, switched memory (CD27⁺IgM⁻) B cells secrete higher levels of anti-pneumococcal polysaccharide antibody than IgM memory B cells (CD27⁺IgM⁺) following in vitro stimulation (14). Third, sequence analysis of anti-pneumococcal polysaccharide antibodies, obtained 5 days post-immunization, are predominately IgG and IgA isotypes (15-17, 34). Fourth, recent studies performed in SCID mice transplanted with human lymphocyte subsets demonstrated that switched memory B cells produced an IgG anti-polysaccharide response following vaccination with PPS (35). In support of this finding, Wardemann et al reported that both switched memory B cells (CD27⁺ IgG⁺) and naïve B cells expressed Ig with specificity for T-independent antigens (36, 37). In the present study, we did not find a significant quantitative difference in the switched memory B cell populations in the unselected versus selected pre- and post-immunization samples. All samples consisted of approximately 10-20% CD27⁺IgM⁻ B cells. It should be mentioned however that the peripheral blood samples were obtained early, i.e. 6 days post-vaccination, at which time the highest number of antibody secreting cells (ASC) are found in the peripheral circulation (32, 38) while serum antibody response was evaluated at 4-6 weeks. The
number of ASC diminish rapidly thereafter, significantly compromising analysis of PPS-specific B cells at later time points. Moens et al (35) demonstrated that mice reconstituted with purified CD27^+IgM^+ B cells produced both an anti-PPS IgM and IgG response following immunization. They hypothesized that immunization with PPS induced an isotype switch from IgM memory cells to IgG producing plasma cells. We examined the B cell phenotype in a small (n=4) number of individuals 4 to 6 weeks post-vaccination. These preliminary studies indicated that the number of PPS-specific B cells was similar to pre-immunization values, at a mere 0.5% of total B cells. Moreover, the phenotype of the PPS-specific B cells isolated at 4-6 weeks was not significantly different than the pre-immunization PPS-specific B cells. These data strongly suggest that the antibody secreting B cells are no longer present in the peripheral circulation at 4 to 6 weeks post-immunization. Extensive analysis of the phenotype of the PPS-specific B cells present in the peripheral blood at 4 to 6 weeks would probably result in an invalid analysis of the antibody secreting PPS-specific B cell population, likely present elsewhere in the B cell compartment, such as the spleen or bone marrow, at more distant time points. Thus a large number of questions remain to be elucidated.

We are presently expanding our studies to include a 2, 3 and 4 week time point post-vaccination for analysis of PPS-specific B cell phenotype. In addition, immune response and B cell phenotype following Pneumovax® will be compared to those generated following conjugate vaccination. Finally, although beyond the scope of the present studies, it will be interesting to determine cytokine profiles at various time points post-vaccination. Recent studies have demonstrated that immunization with PPS induced secretion of IL-6 and TNF-α from macrophages attributed to the presence of TLR2 and
TLR4 ligands in Pneumovax® and thought to be responsible for the IgG component of the immune response to this T-independent type 2 antigen (39).

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My contribution to this paper included development of methodology for fluorescent labeling of pneumococcal polysaccharides. Without this reagent, the flow experiments could not have been conducted.

Disclosures

The authors have no financial conflicts of interest.
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Footnote

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Abbreviations used in this article: PPS, pneumococcal polysaccharide, CVID, Common Variable Immunodeficiency, PPV, 23-valent pneumococcal polysaccharide vaccine, MZ, marginal zone, DTAF, 5 - (4,6 - Dichlorotriazinyl)aminofluorescein, CB, cascade blue, HIV, human immunodeficiency virus.
Figure 1. Serum antibody response and opsonophagocytic activity. Healthy young volunteers (n=18) were immunized with Pneumovax®. Serum samples were obtained pre- and 4-6 weeks post-immunization. Serum samples were tested for PPS14 and PPS23F specific IgG, IgA, IgM (A) and opsonophagocytic activity (B). Serum antibody levels are expressed as µg/mL and opsonophagocytic activity is expressed as opsonophagocytic index.
Figure 2. Inhibition ELISA with fluorescent-labeled PPS14 and PPS23F. Various amounts of labeled PPS14 or PPS23F were pre-incubated with a 1:1000 dilution of standard serum 89SF. Percent inhibition was calculated compared to uninhibited samples.
Figure 3. Specific staining of hybridoma cells. Hybridoma cells with specificity for PPS14 (α14g2b) (A) or PPS23F (α23F) (B) were incubated with various amounts of PPS23F-DTAF and PPS14-CB, and subjected to flow cytometry. PPS14 hybridoma cells specifically bound PPS14-CB in a dose dependent manner and failed to stain with PPS23F-DTAF. PPS23F hybridoma cells specifically bound PPS23F-DTAF in a dose dependent manner and failed to stain with PPS14-CB. Fifty thousand events were recorded.
sisted primarily of CD27+ IgM+B cells, 23.8% and 17.5% for PPS14 and PPS23F, respectively. A small percentage, 3.8 and 3%, of B cells were naive, class-switched CD27+ IgM+B cells. The majority of the PPS-selected B cells were memory B cells (CD27+), accounting for 72–79% of the total B cell population (Figs. 5B, 6). Moreover, the IgM memory population, CD27+ IgM+, was significantly overrepresented compared with both unselected and preimmunization PPS-specific B cells, representing 54.2% of the PPS14-specific B cells and 66% of the PPS23F-specific B cells (Fig. 5B, 5C). In contrast, there was no significant difference in switched memory (CD27+ IgM+) population between postimmunization PPS14- and PPS23F-specific B cells and the unselected or preimmunization PPS-selected populations, accounting for 18.2 and 13.5%, respectively. Furthermore, there was a strong correlation between postimmunization IgM Ab concentration and postimmunization IgM memory B cell percentage for both PPS14 ($r^2 = 0.87$) and PPS23F ($r^2 = 0.88$). In contrast, the correlation between postimmunization IgG Ab concentration and postimmunization switched memory B cell percentage was much lower, $r^2 = 0.56$ for PPS14 and $r^2 = 0.51$ for PPS23F.

**Discussion**

The goal of this study was to characterize the phenotype of B cells responding to Pneumovax 23 vaccination. We specifically chose pneumococcal polysaccharides 14 and 23F as they are the most common disease-causing serotypes found in adult high-risk groups such as the elderly and HIV infected (23–25).

To assess the immune competency of our volunteers, we studied pre- and postvaccination polysaccharide-specific Ig concentration and opsonophagocytic assays. All individuals responded to vaccination with PPS14 (Cascade Blue) and PPS23F (5-DTAF).

**Figure 3.** Specific staining of hybridoma cells. Hybridoma cells with specificity for PPS14 (a14g2b) (A) or PPS23F (a23F) (B) were incubated with various amounts of PPS23F-DTAF and PPS14-CB and subjected to flow cytometry. PPS14 hybridoma cells specifically bound PPS14-CB in a dose-dependent manner and failed to stain with PPS23F-DTAF. PPS23F hybridoma cells specifically bound PPS23F-DTAF in a dose-dependent manner and failed to stain with PPS14-CB. Fifty thousand events were recorded.

**Figure 4.** Inhibition of binding fluorescently labeled PPS. Lymphocytes isolated 7 days post-vaccination were pre-treated with increasing concentrations of homologous unlabeled polysaccharide before addition of fluorescently labeled polysaccharide A=PPS14 and B=PPS23F. Fifty thousand events were recorded. Percent inhibition of binding to fluorescently labeled PPS was determined by comparison to the uninhibited cells.
A

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<td>59.8 (41.2-78.5)</td>
<td>23.8 (6.5-38.8)</td>
<td>17.5 (0.1-35.1)</td>
</tr>
<tr>
<td>CD27 IgM</td>
<td>08.8 (2.5-15.6)</td>
<td>03.8 (0.2-9.4)</td>
<td>03.6 (0.6-4.7)</td>
</tr>
<tr>
<td>CD27 IgM</td>
<td>15.6 (4.8-30.6)</td>
<td>54.2 (21.2-59.2)</td>
<td>66.0 (53.6-83.2)</td>
</tr>
<tr>
<td>CD27 IgM</td>
<td>15.8 (6.6-27.6)</td>
<td>18.2 (7.2-31.9)</td>
<td>13.5 (1.0-21.8)</td>
</tr>
</tbody>
</table>
Figure 5. B cell phenotypes. The phenotype of B lymphocytes that respond to vaccination with Pneumovax23® was determined by flow cytometry. Pre-vaccination (n=6) and seven days post-vaccination (n=18) circulating PBMC were isolated and labeled for analysis using the following fluorescently labeled antibodies/antigen: CD19, CD27, IgM, IgD, PPS14 and PPS23F. The phenotype of pre-vaccination unselected B cells were compared to PPS-specific B cells (A), the phenotype of post-vaccination unselected B cells were compared to PPS-specific B cells (B) and pre-immunization PPS-specific B cells were compared to post-immunization PPS-specific B cells (C). In each sample 75,000 events were recorded. *p<0.0001 **p=0.0002 #p=0.037 ##p=0.006
Figure 6. Phenotype analyses of B cells in the human peripheral blood. Healthy donor PBMC sample was stained with Abs to CD19, CD27, IgM, and fluorescent labeled PPS14 (A and B) and 23F (C and D). CD19⁺ B cells (shown in histogram, dotted line = isotype control) were gated on PPS23F or PPS14. PPS14 or 23F specific B cells (CD19⁺ PPS14⁺, CD19⁺PPS23F⁺) and PPS14 or 23F negative B cells (CD19⁺PPS14⁻, CD19⁺ PPS23F⁻) cells were separated into CD27⁺IgM⁺, CD27⁺IgM⁻, CD27⁻IgM⁺ and CD27⁻IgM⁻. In each sample 75,000 events were recorded. Representative data of FACS analyses; (A and C) pre-vaccination, (B and D) post-vaccination of PPS14 (A and B) or PPS23F (C and D)
3 Isolation and characterization of human polyreactive pneumococcal polysaccharide antibodies

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Running title: Characteristics of human polyreactive pneumococcal polysaccharide antibodies
3.1 Abstract

Natural antibodies serve as the body’s first line of defense against pneumococcal challenge. Nonspecific human pneumococcal polysaccharide IgG antibodies have not been extensively studied. We analyzed human polyreactive antibodies that bind multiple pneumococcal polysaccharides, including PPS14 and PPS23F. These antibodies were isolated from single pneumococcal polysaccharide specific B cells allowing for the analysis of human immunoglobulins with natively paired variable regions. Although isolated individually, these antibodies demonstrated similar characteristics. Most antibodies possessed a variable light chain with a CDR3 length made up of nine amino acids and relatively high number of flexible amino acids in combined VH/VL. While these antibodies were polyreactive and structurally alike, kinetic analysis revealed unique $K_D$ values. Variable chains are responsible for antigen recognition whereas antibody fine specificity is affected by isotype structure. To investigate the contribution of the constant region of these isotypes and their effect on antibody avidity to pneumococcal polysaccharide, the nonspecific variable regions were expressed as IgG1 or IgG2 and subjected to kinetic analysis. The IgG1 antibodies uniformly had a stronger avidity to PPS14 and PPS23F compared to IgG2. To further document the importance of the constant region in antibody avidity and fine specificity, analysis of antibody F(ab)’2 fragment binding to PPS14 and PPS23F resulted in similar $K_D$ values. These studies suggest that antigen recognition by polyreactive antibodies is determined by a conserved variable light chain CDR3 length and longer, more flexible variable heavy CDR3s when
compared to pneumococcal polysaccharide-specific sequences while differences in specific avidities are modulated by antibody isotype.
3.2 Introduction

_Streptococcus pneumoniae_ is a human bacterial pathogen which colonizes the nasopharynx and is a major cause of pneumonia, meningitis and acute otitis media (1, 50). The main virulence factor of _S. pneumoniae_ is the capsular polysaccharide (4). Antibodies against pneumococcal polysaccharide (PPS) provide protection against disease. Natural or nonspecific antibodies are believed to provide a first line of defense against pneumococcal invasion. These antibodies are part of the early phase of the immune reaction and protect against infection and/or decrease the bacterial load if infection is already present (3). Little is known about inherent human nonspecific PPS antibodies. It is thought that human polyreactive antibodies are selected into the marginal zone B cell compartment, as demonstrated in immunoglobulin (Ig) transgenic mice (17, 22, 27, 51).

Murine studies have identified a B cell subset responsible for the production of these antibodies. B1a cells are a distinct population characterized by the CD5 surface marker (3). B1a cells spontaneously secrete low avidity, polyreactive antibodies against PPSs and other antigens. Experimental evidence has suggested that the B1 cell population decreases with age possibly contributing to disease susceptibility (19).

We isolated PPS-binding single B cells and several B cell clones were identified that bound multiple PPSs. The goal of this study was to explore the structural and kinetic characteristics of isolated polyreactive human PPS antibodies. Furthermore, we analyzed
the role of the antibody constant region by comparing avidity values for variable heavy and variable light (VH/VL) pairs expressed as IgG1, IgG2 and F(ab’)2.
3.3 Material and Methods

Human volunteers and vaccination. Healthy young adult volunteers (18 to 30 years old) participated in this study. Volunteers were recruited from the student population at the University of Toledo Health Science Campus (Toledo, OH). Each volunteer was questioned concerning previous pneumococcal vaccination, medications, previous illness, and present health. In addition, we obtained complete blood count (CBC); a comprehensive chemistry profile; total B cells; T-cell subsets; and total immunoglobulin G (IgG), IgM, and IgA levels. Individuals previously immunized with the pneumococcal vaccine and any individual considered to be immunocompromised did not qualify. Informed consent was obtained from all participants using protocols reviewed and approved by the Institutional Review Board at the University of Toledo.

Pneumococcal Polysaccharide ELISA. The pneumococcal polysaccharide ELISA is a modification of the WHO assay (32). Briefly, 5 µg/ml of pneumococcal polysaccharide, either 4, 6B, 14 or 23F, were absorbed onto Nunc Maxisorp microtiter plates (Nunc Roskilde, Denmark) at 37°C overnight. Plates were washed with PBS + 0.1%Tween-20 (PBST). Immunoglobulins were adsorbed with cell wall polysaccharide (CWPS) (10 µg/ml) and PPS22F (10 µg/ml) for 30 minutes at room temperature. After absorption immunoglobulins were serially diluted onto the plates and incubated at 37°C for 2hrs; the standard serum 89SF was used as a positive control. Plates were washed and bound antibody was detected using HRP-conjugated anti-human Ig(H+L) monoclonal antibody (Southern Biotech) diluted 1/3000 in 1% BSA PBST and incubated
at 37°C for 1hr. After washing, plates were developed by using an OPD substrate and the O.D. was read at a wavelength of 490nm.

Disruption ELISA. Disruption ELISA is a technique used to analyze the general avidity of an antibody to PPS. Briefly, 5 µg/ml of pneumococcal polysaccharide, either 14 or 23F, were absorbed onto Nunc Maxisorp microtiter plates (Nunc Roskilde, Denmark) at 37°C overnight. Plates were washed with PBS + 0.1%Tween-20 (PBST). Immunoglobulins were mixed with a disrupting agent, sodium thiocyanate (NaSCN), ranging in concentration from 4M to 0.0625M. Immunoglobulins with NaSCN were serially diluted onto the plates and incubated at 37°C for 2 hours with the standard 89SF serum as a positive control. Plates were washed and bound antibody was detected using HRP-conjugated anti-human Ig(H+L) monoclonal antibody (Southern Biotech) diluted 1/3000 in 1% BSA PBST and incubated at 37°C for 1hr. After washing, plates were developed by using an OPD substrate and the O.D. was read at a wavelength of 490nm.

Opsonophagocytic assay. To determine functional activity against PPS14 and PPS23F opsonophagocytic assay was performed as previously described (36). Briefly, S. pneumoniae serotypes 14 and 23F were incubated with serially diluted immunoglobulins. Newborn rabbit serum (Pel-Freez, Brown Deer, WI) was added as a source of complement. Differentiated HL-60 cells were added at an effector/target ratio of 400:1. All immunoglobulins were tested in duplicate. The opsonophagocytic index was determined as the reciprocal of the dilution with 50% killing when compared to serum
free controls and analyzed using the Opsotiter1 software program from the University of Alabama at Birmingham.

Purification of PPS-specific B cells. Pneumococcal polysaccharide 14 and 23F were fluorescently labeled using Alexa 488 as previously described (40). Peripheral blood lymphocytes collected day 7 post-vaccination were obtained using Lymphocyte Separation Medium (Mediatech, Inc., Manassas, VA). Cross-reactive B cells were depleted from the population by incubation with beads coated with PPS22F and CWPS and discarded. Isolation of PPS-specific B cells was achieved by incubation with fluorescently labeled PPS14 or PPS23F. Flow cytometric sorting and cloning of single PPS-specific B cells was performed as described previously (47). PPS-specific B cells were cultured and expanded for 14 days. The supernatants were tested for immunoglobulin secretion and anti-PPS activity via ELISA. PPS-positive B cell cultures were harvested and lysed for preparation of cDNA.

Production of variable chain plasmids. The cDNA obtained from single PPS-specific B cells was used as a template in PCR for the amplification of the variable chains. Primer sets described previously (48) and Taq polymerase were used to generate variable chain fragments. Heavy and light chain fragments were individually ligated into the TA cloning vector system and transformed into Top 10 Escherichia coli cells. Positive clones were sequenced and re-PCRed to add restriction sites for cloning into the IgG1, pHC-huCg1, or IgG2, huCg2, and kappa, pLC-huCk, expression cassettes (29).
The CH1 domain of the antibody is conserved in both IgG1 and IgG2 expression cassettes. Correct sequence insertion was confirmed by sequence analysis.

Human recombinant immunoglobulin expression. The paired pHc and pLC plasmids were co-transfected into HEK293 cells using FuGene HD (Roche Basel, Switzerland). After 48 hours, supernatants were tested for immunoglobulin secretion and PPS binding by ELISA as described previously (49).

Generation of F(ab’)2 fragments. Antibodies were digested using a F(ab’)2 preparation kit (Pierce, Rockford, IL). Briefly antibodies were desalted and added to spin columns containing resin with immobilized pepsin. The tubes were incubated at 37ºC for 2 hours while rotating on a end-over-end mixer. Columns were centrifuged and flow-through was collected. F(ab’)2 fragments were analyzed by SDS-PAGE.

Antibody Avidity Surface Plasmon Resonance. Surface plasmon resonance (SPR) avidity analysis of the recombinant human antibodies was performed using a Reichert SR7000DC instrument with a computer interface for system control and data acquisition (Reichert, Inc., Depew, NY). All experiments were conducted at 25ºC with a flow rate of 10 µl/min with HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20) as the running buffer. Anti-human IgG or anti-human F(ab’)2 (SouthernBiotech, Birmingham, AL) was diluted to 10ug/mL in HBS-EP and covalently immobilized to the surface of the sensor chip using amine coupling chemistry. The recombinant human antibodies or fragments were passed over the chip and captured.
on the chip’s surface. Varying concentrations of PPS14 or PPS23F were then passed over the chip. Avidity measurements for each recombinant antibody were determined by differences in mRIU values. Regeneration was achieved using 3M MgCl₂. Data was analyzed using Scrubber2 software (Campbell, Australia).
3.4 Results

ELISA studies of IgG1 and IgG2 expressed VH/VL. All VH/VL pairs were cloned into both vectors containing human IgG1 Fc and human IgG2 Fc. The expressed immunoglobulins were tested in PPS-specific ELISA. All immunoglobulins were absorbed with CWPS and PPS22F as described in Materials and Methods. The results of these studies demonstrated that all monoclonal antibodies expressed as IgG1 and IgG2 isotype bound multiple PPSs as shown in Figure 1. Although there were differences in PPS binding between the IgG1 isotype versus IgG2 isotype with identical VH/VL as measured by ELISA, these differences were not significant.

Disruption ELISA. Binding to PPS was disrupted by adding different dilutions of NaSCN to the ELISA well. Overall avidity was measured by the concentration of NaSCN required to disrupt 50% of binding measured at 0M NaSCN. These studies demonstrated relatively weak binding to both PPS14 and PPS23F. On average, 0.083M NaSCN was needed to disrupt monoclonal binding to both PPS14 and PPS23F (Figure 2A). In many cases, a higher concentration of NaSCN was needed to disrupt the IgG1 isotype binding compared to the IgG2 isotype although the difference was not significant.

Opsonophagocytic studies. To ascertain the functional activity of the expressed immunoglobulins, an opsonophagocytic assay was performed. As shown in Figure 2B, monoclonal antibodies expressed as IgG1 had a high opsonophagocytic index (OI) against PPS14. Moreover, all monoclonal antibodies expressed as IgG2 also killed type
14 pneumococcus, however the OI was considerably lower than for the IgG1 antibodies despite identical VH/VL. Similarly, all IgG1 monoclonal antibodies also killed S. pneumonia serotype 23F with greater opsonophagocytic index than their VH/VL identical IgG2 counterparts.

Variable region analysis. We performed sequence analysis of VL and VH regions of all monoclonal antibodies as shown in Table 1. The eight VL regions belonged to a total of 5 different VL gene families, namely A20, O12, L5, A27 and B3. The predominant VL gene families represented were A20 and O12. The CDR3 region, thought to be crucial in antigen binding, of all monoclonal antibodies (MAbs) with the exception of 33E2, expressed a kappa VL gene with a CDR3 length of 9 amino acids (AA) (Figure 3A). In addition there was a striking absence of overall positive or negatively charged AA in the CDR3 region (Figure 3B and 3C). The number of mutations in the VL varied greatly between monoclonal antibodies from 10 mutations in MAb 33G8 to 37 in the heavily mutated MAb 21B2. Monoclonal antibodies 21B2 and 32E8 displayed identical VL however they were paired with different VH.

Analysis of the VH regions demonstrated that 7 of the 8 VH region expressed gene products belonged to the VH3 gene family. The VH3-23 gene family was predominantly expressed although none of the VH3-23 sequences were identical. In contrast to the VL CDR3s, the VH chains of these antibodies showed varying CDR3 lengths ranging from 12 AA in MAb 33E2 to 24 AA in MAb 24F5. The number of charged molecules in the VH CDR3s ranged from a total of 3 to 6 positively and/or
negatively charged amino acids per CDR3 (Figure 3C). Amino acids arginine, tryptophan and tyrosine have been reported to be responsible for increasing the plasticity of the antigen binding region, allowing the antibody to recognize multiple antigens (30). All VH CDR3 regions possessed varying numbers of these three amino acids ranging from 2 to 9. Monoclonal antibody 33G8 has the highest number of arginine residues in the VH CDR3. Monoclonal antibody 24F5 has the highest number of tryptophan residues. The number of mutations in the VH CDR3 varied greatly from 4 mutations in MAb 24F5 and 33E2 to 36 mutations in MAb 31E2. The VH regions with longer CDR3 regions had a tendency towards lower number of mutations (Table 1).

There was no correlation between the length of the CDR3 of the VL and the VH regions. Thus, MAbs with varying VH CDR3 lengths ranging from 12 to 24 amino acids were all paired with a VL with a CDR3 length of 9 amino acids. Some heavily mutated VL regions, for example VL 21B2 and VL 24F5 were paired with minimally mutated VH regions that closely resembled germline sequence. Monoclonal antibody 32E8 was the only clone that expressed a heavily mutated VH and VL.

The monoclonals analyzed in this study were compared to previously published polyreactive and PPS-specific antibodies (8, 24, 35, 38, 52). Variable heavy and light CDR3 percent homology to germline sequences were not significantly different between PPS-specific and polyreactive populations (Figure 4A). In contrast, the average CDR3 length was notably longer in polyreactive VH CDR3 compared to PPS-specific antibodies (Figure 4B). Overall, the polyreactive antibodies possessed a significantly
higher number of flexible amino acids in the VH CDR3 than the PPS-specific VH CDR3 (Figure 4C). There was no significant difference in the number of flexible amino acids in the VL CDR3 between the polyreactive and PPS-specific antibodies (Figure 4D).

Avidity analysis of IgG antibodies. In addition to the disruption avidity ELISA studies, both MAb IgG1 and IgG2 clones were tested for antibody avidity using SPR. A chip was coated with anti-human IgG by amine coupling. Cell culture supernatant containing monoclonal antibody preparation was passed over the chip, capturing the monoclonal antibody. This method allows for uniform binding of the recombinant antibody to the chip. Varying concentrations of PPS14 or PPS23F were passed over the surface of the chip and antigen-antibody binding was measured by SPR. As expected, these polyreactive antibodies exhibited low avidity for pneumococcal polysaccharide (Figure 5). Binding rates were detectable in the micromolar range, from 5.3 to 0.12, for all clones. In contrast, a mouse monoclonal antibody specific for PPS14 was calculated to have an avidity of 70nM which is significantly stronger than the polyreactive antibodies analyzed.

As shown in Figure 5A, all MAbs expressed as IgG1 molecules, with the exception of MAb 33G8, bound PPS14 more avidly than their homologous MAb expressed as IgG2. There was a notable difference in antibody avidity between IgG1 and IgG2 forms of the same VH/VL pair in all cases. A similar trend was detected in MAb binding to PPS23F. Most Mabs expressed in the IgG1 form bound more avidly than their IgG2 counterpart with the exception of Mabs 33G8 and 32E8. Differences in binding
between the IgG1 and IgG2 isoforms were observed for only 3 of the 5 MAbs tested, namely 21B2, 24F5 and 33G8. There was no correlation between the antibody avidity and length of the VH CDR3 or between avidity and number of mutations in either VH or VL or combined VH/VL. Overall for both polysaccharides the IgG1 isoform bound more avidly than its IgG2 counterpart although these differences were not statistically significant.

Avidity analysis of F(ab)'2 fragments. Recombinant antibodies were digested with pepsin to produce F(ab)'2 fragments. F(ab)'2 fragment purity was analyzed using SDS-PAGE as demonstrated in Figure 6B. Pepsin digestion removes the CH2 and CH3 domains keeping the hinge region intact. Surface plasmon resonance analysis of the pepsin digested fragments resulted in the elimination of the differences in binding kinetics observed between the IgG1 and IgG2 isotypes as shown in Figure 6A. The binding constants for these fragments were significantly lower than the values of their undigested IgG1 and IgG2 forms.
3.5 Discussion

Natural antibodies are able to recognize and bind a variety of foreign and self-antigens including protein and polysaccharide antigens. Natural antibodies have been reported for several bacterial polysaccharides however the characteristics of these antibodies remain to be elucidated. Low avidity polyreactive antibodies are believed to be the body’s first line of defense against pathogens and can initiate complement deposition. Polyreactive antibodies, referred to as 'natural antibodies', include IgM, IgG and IgA isotypes (34), mainly use VH3, VH4 or VH5 genes and classically contain few mutations, i.e. resemble germline sequences (14). It is generally accepted that highly mutated Abs are derived from conventional antibody producing cells, referred to as B2 cells, and generated through antigen-driven somatic hypermutation. Another subset of B cells, B1 cells, may play a significant role in the innate immunity to encapsulated organisms. In mice, these cells are phenotypically defined by CD5 expression. Compared to conventional B2 cells, they are unique in several aspects including phenotype and production of natural Abs (9, 20). The antibodies produced by B1 cells bind autoantigens and are broadly cross-reactive to a variety of bacterial antigens i.e. PPS and LPS (26). Murine studies demonstrate that these CD5⁺IgM⁺ B cells spontaneously secrete natural antibodies that appear to play a crucial role as the first line of defense against bacterial and viral pathogens including the pneumococcus (5, 10, 13, 33). Thus CD5⁺ B cells, in particular the CD5⁺ B1 cells, are thought to be responsible for the production of natural antibodies in humans.
In effort to better characterize human polyreactive pneumococcal immunoglobulins, we initially tested the specificity of our MAbs using a PPS ELISA. The monoclonal antibodies studied here bound multiple pneumococcal polysaccharides and therefore were considered polyreactive antibodies. Despite low antibody avidity, all polyreactive antibodies tested had high opsonophagocytic activity and induced opsonization of both serotypes, PPS14 and PPS23F (Figure 2B) suggesting they are not only polyreactive but are indeed functional. Antibodies with a high opsonophagocytic activity and low avidity have previously been reported by Baxendale et al. and Romero-Steiner et al. (7, 37). This observation is in sharp contrast to studies that have shown that reduced or absent functional antibody activity, as determined by opsonophagocytic or mouse protection assays, is directly related to low antibody avidity (41, 44). Although some studies directly correlate antibody avidity with functional activity, this appears not to be true for all antigen-specific antibodies. Several factors are likely to influence antibody avidity. These include factors that affect intrinsic affinity, such as VH/VL molecular structure, but also by other factors, potentially constant region structure, that may affect the number of binding sites and antibody flexibility (18). The criterion governing antibody avidity and functional activity of both antigen-specific and polyreactive antibodies however, remains to be elucidated.

Monoclonal antibody avidity was tested by both disruption ELISA and directly by surface plasmon resonance. While these antibodies bound multiple polysaccharides in ELISA studies, their binding was relatively weak as demonstrated by the low concentrations of NaSCN required to disrupt binding to PPS. The avidity constant, as
measured by SPR corresponded directly to the NaSCN ELISA studies and demonstrated weak antigen binding. The results of these studies are in accordance with previous findings concerning antibody avidity of natural antibodies (9, 37).

Antibody avidity for pneumococcal polysaccharides is determined not only by the sequence of the variable region but also by the way the variable region interacts with the surface of the polysaccharide. The pneumococcal polysaccharide antigen consists of repeating saccharide units. Anti-PPS antibody variable regions are directed against two different molecular patterns. They recognize either one or two monosaccharides at the terminal ends of the polysaccharide or alternatively, recognize extended sugar epitopes of 6 or 7 monosaccharides (2). Moreover, structural constraints of the PPS epitopes may limit the interaction with the antibody variable region (53). It has been postulated that a uniformed CDR3 length may allow for the binding of similarly spaced epitopes. The polyreactive monoclonals analyzed have VL CDR3s of similar length. Preserved VL CDR3 length has been reported with other polyreactive antibodies (11, 12). In contrast, the length of the VL chain CDR3 in PPS-specific antibodies has been reported to be variable ranging from 4 to 11 amino acids (6, 52, 53). Variable light CDR3 length reportedly influences the loop configuration of the antibody/antigen-binding site. In fact, Wang et al. has suggested that a change in length of the VL CDR3 may have a more significant effect than amino acid substitutions (46). These findings suggest that the homogeneous VL CDR3 length may be a feature of polyreactive antibodies while PPS-specific antibodies do not have this restriction. Alternatively, this range of VL CDR3 length in PPS-specific antibodies may be related to the restricted use of the A23 and L6
gene families, at least in the case of PPS23F-specific antibodies (52). These gene families were absent in our eight polyreactive PPS23F-binding monoclonal antibodies. Moreover, the polyreactive VL CDR3s lacked positive or negatively charged amino acids. Charged amino acids in the CDR3 may result in repulsion of the antigen preventing antibody binding. However, it should be mentioned that some PPS-specific VL CDR3s also lack highly charged amino acids.

In contrast to the uniform VL CDR3 length, the VH CDR3 varied greatly in length ranging from 12 to 24 amino acids. Similarly, the VH CDR3 of PPS-specific antibodies is reported to vary greatly from 4 to 20 amino acids, although the median VH CDR3 length of polyreactive antibodies is notably longer, some overlap is evident (8, 25, 35, 38, 52, 53). Thus the overall longer length of the VH CDR3 is more commonly observed with polyreactivity. Moreover, germline sequences tend to possess a longer CDR3 than heavily mutated Abs. The longer CDR3 of these polyreactive antibodies may facilitate binding to larger epitopes (16, 21).

The amino acid arginine has been associated with antibody polyreactivity (45). Polyreactivity may be the result of electrostatic interactions mediated by arginine in the VH chain CDR3. Among our polyreactive clones, all but one possess at least one arginine in the VH CDR3. Other amino acids which contribute to polyreactivity are tryptophan and tyrosine. These residues interact with a diverse array of antigens due to their ability to form hydrogen bonds, hydrophobic interactions, electrostatic interactions and aromatic rings (45). Amino acids with flexible side rings may also make the binding
region more plastic allowing for interaction with different antigens. Monoclonals 21B2 and 32E8 both possess the same VL chain. However, 21B2 VH chain exhibits more of the flexible amino acids and bound both PPS14 and PPS23F more avidly than 32E8 in SPR studies. Pneumococcal polysaccharide-specific antibodies tend to express fewer flexible amino acids, on average 3.6 amino acids in combined VL/VH CDR3s, in contrast to our polyreactive clones which contained 6.4 flexible molecules. This lack of flexible amino acids theoretically decreases antibody elasticity and may therefore result in a more rigid and specific antibody molecule (52).

Natural antibody variable regions classically possess few mutations and are closely related to germ-line. Among the clones analyzed in our study the number of variable region mutations varied greatly. The number of VL mutations varied from 10 to 37 while the number of VH mutations varied from 4 to 36. Monoclonals 33E2, 21B2, 24F5 and 33G8 conform to the classic idea of natural antibodies with few mutations in their VH. There was no association between the number of VL mutations and the number of VH mutations found in these clones. Similarly, there was no correlation between the number of mutations and the CDR3 length. In summary, some of our polyreactive antibodies conformed to the classic image of polyreactive antibodies while others were highly mutated in either the VH or VL or both. Casali et al. demonstrated that B1 cells could generate hypermutated antibodies. Moreover, using a variety of techniques including gene shuffling and site-directed mutagenesis, they showed that the immunoglobulin VH region provides the major structural predictor for antibody polyreactivity, mainly provided by the heavy chain CDR3 (15). The ability of B1 cells to
generate hypermutated antibodies may explain the high number of mutations in clones 32E8, 31E2 and 31B5.

The polyreactive antibodies we analyzed were expressed in IgG1 and IgG2 form. It has been reported that antibody isotype, thus antibody constant region, may influence antibody fine specificity and avidity. Schreiber et al. expressed a VL/VH pair with specificity for *Haemophilus influenzae* type b as various isotypes. These investigators noted that different isotypes with identical VL/VH resulted in variable protective immunity likely attributable to variable binding avidity (39). Similarly, Baxendale et al. found differences in antibody avidity to PPS between IgA1 and IgG2 clones with identical VL/VH, suggesting the Fc region may influence antigen binding (15). Torres et al. analyzed IgG murine antibodies with identical variable regions demonstrated diverse binding affinity and fine specificity suggesting the constant region affects the variable region binding to *Cryplococcus neoformans* (42). This discrepancy in antigen binding despite identical variable regions has also been documented for protein antigens (31). Consistent with previous reports, we also found a significant difference in binding avidity between IgG1 and IgG2 isotypes with identical VH/VL regions. The IgG1 isoform of most of our polyreactive monoclonal antibodies demonstrated a greater avidity and bactericidal activity than IgG2 isoforms expressing identical VL/VH pairs. Morelock et al. attributes this increased avidity of IgG1 isotype to unimpeded Fab arm movement and increased hinge flexibility. Thus the constant region appears to affect the conformation of the variable region (43). Moreover, changes in conformation imposed by the constant region may allow for different antibody isotypes with identical variable regions to
recognize different epitopes. However when these antibodies were digested to F(ab)’2 fragments these differences were abolished. This phenomenon was previously reported with a protein antigen (28). This suggests the CH2 and CH3 regions of the constant region act as a supportive structure to maintain the conformation of the antibody and result in significant differences in binding avidity between isotypes (23). Loss of the CH2 and CH3 removes supportive scaffolding causing loss of a structured hinge which may explain the differences in avidity between IgG1, IgG2 and F(ab)’2 fragments. Additionally, there is evidence that polyreactivity may be influenced by the surrounding constant region structures. Thus, structures that make the antigen-binding site more flexible, may allow the antibody to bind multiple antigens (42). The IgG1 isoform possesses fewer disulfide bonds and more amino acids in the constant region resulting in greater flexibility. This increase in molecular flexibility may, in part, explain the overall increased avidity of the IgG1 isotype for these polyreactive monoclonal antibodies. Although the variable region is responsible for antigen recognition, the constant region of the antibody is necessary for stability, fine specificity and optimal avidity.

Polyreactive antibodies to PPS in our study despite low avidity by disruption ELISA and SPR were bactericidal against multiple serotypes of pneumococcus suggesting clinical significance. The VH regions expressed were mainly VH3, namely VH 3-23. Although VL conserved CDR3 length, VH CDR3 length varied greatly as did the number of mutations. Moreover, the VH CDR3 was characteristically longer than PPS-specific antibodies. Additionally the CDR3 possessed more arginine, tyrosine and tryptophan amino acids allowing for a greater flexibility compared to PPS-specific
antibodies. Each VH/VL pair was expressed as IgG1 and IgG2 isotypes. Overall for both PPS14 and PPS23F, the IgG1 isotype bound more avidity to PPS and was more functional corresponding to higher avidity suggesting a role of the constant region in antibody specificity and function. The common characteristics discovered in these human polyreactive antibodies may aid in establishing guidelines for the detection of the natural human anti-pneumococcal antibody in the repertoire.
Figure 1. Monoclonal antibodies, 33G8 (A), 32E8 (B), 31E2 (C), 31B5 (D), 21B2 (E) and 24F5 (F), bound multiple pneumococcal polysaccharides as assessed by ELISA.
Figure 2. A) Disruption ELISA. Monoclonal antibodies were incubated with varying concentrations of NaSCN. Avidity index (AI) equals the molar concentration of NaSCN needed to disrupt monoclonal binding to PPS by 50%. B) Opsonophagocytic assay of clones against serotype 14 and 23F pneumococcus. Bactericidal ability is associated with functional activity.
### Table 1. Sequence analysis of polyreactive clones isolated from single B cell culture.

Shown are VH (A) and VL (B) CDR3 sequence, number of mutations, homology to germline, V and J gene usage.
Figure 3. A) The length of the CDR3 in number of amino acids. While the VH chain CDR3s exhibit varying length, the VL chain CDR3s share the same number of amino acids, 9, with the exception of 33E2 which has 8 amino acids. Another shared
characteristic of these nonspecific clones is the absence of negative amino acids in their Vk CDR3 (B) and an increase in the number of flexible amino acids in the VH CDR3 (C).
Figure 4. A) Analysis of homology of the CDR3 to germline. There was no significant difference in germline homology between PPS-specific and polyreactive CDR3. B) The average VH CDR3 length was notably longer for polyreactive antibodies when compared to the PPS-specific sequences. Comprehensive VH (C) and Vk (D) CDR3 amino acid analysis of published anti-pneumococcal polysaccharide antibodies. Amino acid abbreviations: Arginine - R, tryptophan - W, tyrosine - Y, histidine - H, lysine - K, aspartic acid - D and glutamic acid - E. Number of sequences analyzed for each serotype in parentheses. Difference between RWY VH composition is statistically significant *** p <0.0001.
Figure 5. Kinetic analysis of MAbs avidity to PPS14 (A) and PPS23F (B) using SPR.

Antigen binding is expressed as Kd mol$^{-1}$. 
Figure 6. A) Kinetic analysis of F(ab’)2 fragment avidity to PPS14 and PPS23F. Recombinant antibodies were digested with pepsin. B) Analysis of antibody digestion with pepsin to produce F(ab’)2 fragments. Lane: 1. 24F5 IgG1 F(ab’)2 digested, 2. 24F5 IgG1 F(ab’)2 digested + protein A column, 3. 24F5 IgG2 F(ab’)2 digested, 4. 24F5 IgG2 F(ab’)2 digested + protein A column
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4 Discussion

*Streptococcus pneumoniae* is a significant pathogen in the U.S. and worldwide. Despite advances in treatment and prevention, pneumococcus is still a main cause of morbidity and mortality. Therefore it is important to improve the understanding of the human immune response to this bacterium. The main correlate of protection is the presence of functional, high avidity antibodies that bind PPS and promote clearance of pneumococci through lysis and phagocytosis. The purpose of these studies was to define the phenotype of B cells that respond to PPV and identify unique characteristics of polyreactive PPS antibodies.

Murine studies have explored the B cell response to pneumococcal polysaccharides to define the phenotype of B cells that respond to PPS. Previous studies have identified B1 cells as anti-PPS SC. These cells are identified by B220^CD5^CD20^CD5^IgD^-IgM^+(Wardemann et al., 2002b). In humans, the phenotype of PPS-specific B cells has not been characterized. Our development of fluorescently labeled PPS14 and 23F enabled us to identify B cells that respond to PPV. Using flow cytometry and fluorescently labeled polysaccharide, PPS-specific cells were identified and sorted by surface markers CD19, CD27, IgD and IgM. Pre- and post-vaccination unselected B cells were mainly naïve CD27^- cells. Post-vaccination, health young donors had a significant increase in anti-PPS14 and anti-PPS23F IgG and IgM antibodies. This increase in specific PPS antibody
titers after vaccination confirmed immune responsiveness of this control population. Functional activity was measured by OPSA and the opsonophagocytic index (OPI) increased significantly after vaccination. These results support the typical immune response to PPV by producing higher concentrations of functional anti-PPS antibodies. Analysis of PPS-specific peripheral blood mononuclear cells (PBMC) from healthy young volunteers 6 days post-vaccination revealed an increase in the CD$27^+$IgM$^+$ memory cells population. Additionally there was a substantial correlation between CD$27^+$IgM$^+$ cell percentage and anti-PPS IgM antibody concentration. Defining the phenotype of B-cells that respond to vaccination with the purified pneumococcal polysaccharide is an essential first step in improving prevention and treatment of adults in high-risk populations, namely the elderly and HIV that lack these B cell subsets.

When specific-antibodies are not in circulation, natural antibodies are the first line of defense for protection against pneumococcal infection. Many studies have been conducted to define the B-cell type and antibody sequence of polyreactive antibodies, as there is little cross-reactivity between PPS-specific antibodies. We isolated and characterized several B cells that produced antibody that bound multiple polysaccharides. Analysis of these polyreactive antibodies revealed similar characteristics. All but one monoclonal antibody expressed a VL CDR3 length of nine amino acids. Previous research has implicated the importance of restricted VL CDR3 length in antigen recognition, suggesting VL CDR3 length is more important than amino acid composition (Bridges et al., 1995). The characteristics of CDR3 amino acids of polyreactive antibodies were compared with PPS-specific sequences. The charge and rigidity of the anti-PPS antibodies effects the interaction with PPS. Three amino acids categories were
calculated, flexible, positive and negative. Polyreactive VH CDR3 expressed a significantly higher number of flexible amino acids than PPS-specific VH CDR3. Flexible amino acids are thought to increase the plasticity of the antigen binding groove allowing for recognition of multiple, large antigen such as bacterial polysaccharides (van Esch et al., 2002).

Antibody isotype also has an effect on antibody avidity. The two main isotype subclasses secreted in response to PPS are IgG1 and IgG2. The polyreactive VH/VL chains were expressed as both of these subclasses. For both PPS14 and 23F, IgG1 had an overall higher functional activity. Immunoglobulin G1 is more flexible allowing for more unrestricted movement of the antibody.

Knowledge of the human antibody response to is key to developing future vaccines and therapies. Without a sufficient understanding of how B cells respond to pneumococcal challenge, it is difficult to augment the immune response in high-risk groups. The results of these studies in healthy young volunteers have identified the phenotype of PPV responding B cells and clarified certain characteristics of natural polyreactive antibodies. These results taken together enable analysis of the overall quality of the immune response in groups at high risk for IPD. By studying the immune response to PPS, prediction of risk for IPD can be correlated. Future studies will explore the immune response to PPV in the elderly and HIV-positive volunteers. By understanding the impaired immune response in these populations guidelines can be established to improve the immune response to vaccination.
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