A Dissertation

entitled

Pneumococcal Vaccination in Aging HIV-Infected Individuals

by

Jennifer A. Ohtola

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

Doctor of Philosophy Degree in Biomedical Sciences

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An Abstract of

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Advanced age and human immunodeficiency virus (HIV) infection are both risk factors for *Streptococcus pneumoniae* infections due to immunological dysfunction. The aging HIV-infected (HIV+) population may be at higher risk for pneumococcal disease due to the combination of these factors on humoral immunity. Current recommendations for pneumococcal vaccination in HIV+ adults include a priming dose of the 13-valent pneumococcal conjugate vaccine followed by one dose of the 23-valent pneumococcal polysaccharide vaccine 8 weeks later (PCV/PPV). We compared quantitative and qualitative antibody responses to PCV/PPV versus a single dose of PPV in HIV+ adults aged 50-65 years with CD4+ T cells/µl (CD4) >200 on antiretroviral therapy ≥1 year. We found that PCV/PPV did not demonstrate a clear immunological advantage to PPV alone, as serotype-specific IgG levels and functional titers postvaccination were similar between groups. In addition, these antibody responses were significantly reduced in HIV+ subjects vaccinated with PCV/PPV compared to age-matched, uninfected (HIV−) controls who received PCV/PPV. We also characterized the phenotype and surface

expression of several receptors on serotype-specific B cells that may influence vaccine responses. HIV+ subjects vaccinated with PCV/PPV generated significantly reduced frequencies of circulating serotype-specific B cells postvaccination compared to those who received PPV only. However, phenotypic distributions of serotype-specific memory B cell subsets were similar between groups. Transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI)$^+$ serotype-specific B cell percentages were significantly decreased in HIV+ PCV/PPV compared to PPV groups, indicating that prior PCV altered TACI expression. It remains unclear if this impact provides any benefit to vaccine responses. CD21$^+$ serotype-specific B cells were also significantly reduced in HIV+ compared to HIV− PCV/PPV groups which may contribute to diminished antibody responses. Collectively, our findings suggest that continued efforts aimed at developing more effective vaccination strategies in susceptible adult populations are warranted, and further investigation into the immunological mechanisms that increase the risk of pneumococcal disease and induce potent vaccine responses are necessary.

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List of Abbreviations

ACIP ....Advisory Committee on Immunization Practices
AIDS ....Acquired immunodeficiency syndrome
APRIL ....A proliferation-inducing ligand
ANCOVA ....Analysis of covariance
ANOVA ....Analysis of variance
ART............................Antiretroviral therapy

BAFF..........................B cell activating factor
BAFF-R......................BAFF receptor
BCMA........................B cell maturation antigen
BCR............................B cell receptor

CAP............................Community-acquired pneumonia
CBC............................Complete blood count
CD4............................$CD4^+$ T cells/µl
CDC ....Centers for Disease Control and Prevention
CI................................Confidence interval
CVID..........................Common variable immunodeficiency

ELISA ....Enzyme-linked immunosorbent assay

FDA............................Food and Drug Administration

GC ....Germinal center

HIV ....Human immunodeficiency virus
HIV+ ....HIV-infected
HIV− ....HIV-uninfected

Ig ....Immunoglobulin
IPD ....Invasive pneumococcal disease
IRB..............................Institutional Review Board

MHC ....Major histocompatibility complex
MZ..............................Marginal zone

OPA............................Opsonophagocytic killing assay

PBMCs......................Peripheral blood mononuclear cells
Pcp............................Pneumococcal choline-binding protein
PCV...........................13-valent pneumococcal conjugate vaccine
PPS............................Pneumococcal polysaccharide
PPV ....23-valent pneumococcal polysaccharide vaccine
Psp.............................Pneumococcal surface protein

SCID ....Severe combined immunodeficiency

TACI ....Transmembrane activator and calcium-modulating cyclophilin
ligand modulator
TD ....T cell-dependent
TI................................T cell-independent, T cell-independent type 2
TLR............................Toll-like receptor
TNFR ....Tumor necrosis factor receptor

USA............................United States of America

V_H3.............................Immunoglobulin variable region gene family 3

WHO..........................World Health Organization

Chapter 1

Challenges in Pneumococcal Disease Prevention

1.1 Burden of pneumococcal disease

In 1880, microbiologists George Sternberg and Louis Pasteur were the first to independently isolate the Gram-positive bacterium *Streptococcus pneumoniae* by animal passage [1, 2]. This pathogen has been informally referred to as the pneumococcus since the late 1880s when it was established as the principal cause of bacterial pneumonia. Further investigation over subsequent decades has revealed that the clinical spectrum of pneumococcal infection ranges from mild disease, including otitis media and sinusitis, to invasive forms, including bacteremia and meningitis. *S. pneumoniae* continues to be a significant cause of morbidity and mortality worldwide.

Pneumococcal disease is preceded by asymptomatic colonization of the nasopharynx (Figure 1-1). Carriage rates vary with age and the environment, but are highest in children 2-3 years of age [3, 4]. The duration of carriage is generally longer in children than adults. Thus, young children serve as the primary reservoir and source of transmission of pneumococci. *S. pneumoniae* may be isolated from up to 60% of asymptomatic children and 10% of healthy adults, although carriage rates are increased in adults with young children in the same household [4]. Pneumococci are transmitted by

direct contact with aerosols or respiratory secretions from colonized individuals. Similar to other respiratory diseases, rates of pneumococcal infections are highest during the winter and early spring. Infection with the influenza virus increases the risk of pneumococcal pneumonia and invasive disease (IPD).
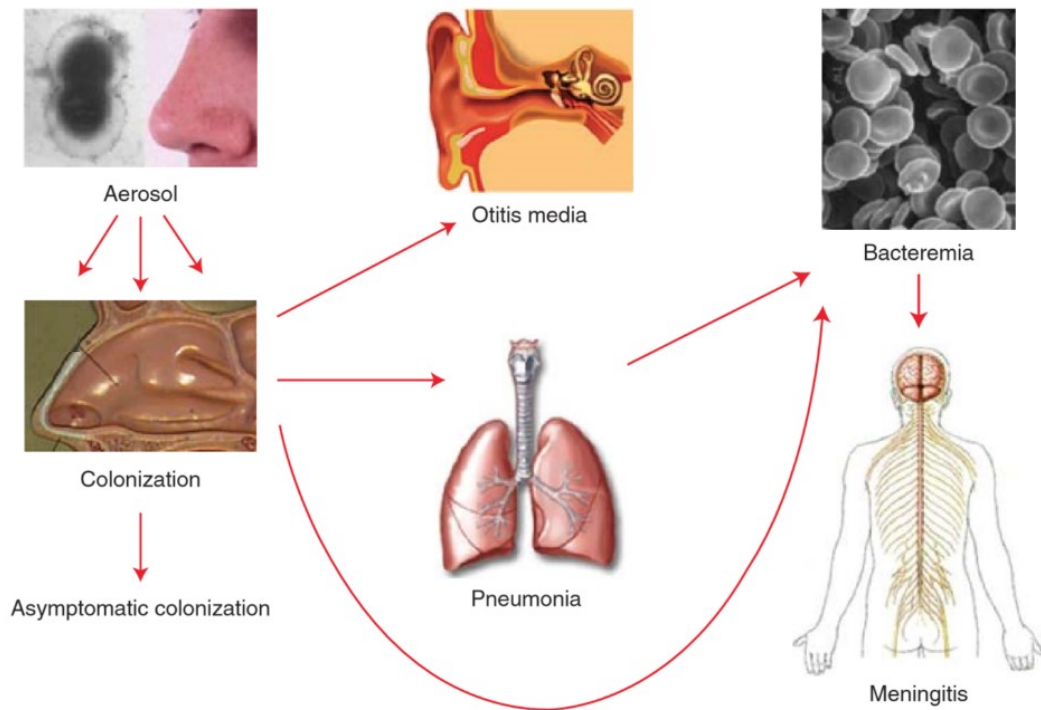
Figure 1-1. Progression of pneumococcal disease. Reprinted from reference [4] by permission of Cold Spring Harbor Laboratory Press, copyright 2013.

In children <5 years of age, an estimated 14.5 million episodes of pneumococcal infections occur each year worldwide, resulting in 500,000 deaths, particularly in low- and middle-income countries [5]. In the USA, conjugate vaccination has resulted in substantial reductions in pneumococcal disease rates in immunized young children [6-11]. Incidence of pneumococcal disease is historically highest at the extremes of age, typically children <2 years of age and older adults aged ≥65 years. Immunocompromised individuals, such as those infected with human immunodeficiency virus (HIV), are also

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highly susceptible to pneumococcal infections. Other risk factors include geographical location; splenectomy; chronic conditions, including heart disease, lung disease, and diabetes; and high risk behaviors such as smoking and alcohol abuse.

Over the past century, significant increases in life expectancy have occurred due to clinical and technological advances. By 2040, the number of individuals aged ≥65 years living in the USA are estimated to double and represent up to 20% of the total population [12]. The aging HIV-infected (HIV+) population has also rapidly expanded due to the introduction of antiretroviral therapy (ART) and increased rates of new infections in older adults [13-15]. Based on the current availability of vaccines and therapeutic options, hospitalizations due to pneumococcal disease in older individuals are projected to grow almost 100% by 2040 and total healthcare costs will increase by $2.5 billion annually [12]. These demographic shifts emphasize the need for continued efforts in controlling pneumococcal disease in adult populations.

The most common clinical presentation of pneumococcal disease in adults is pneumonia. *S. pneumoniae* accounts for approximately 30% of adult community-acquired pneumonia (CAP) and up to 50% of hospital-acquired pneumonia, with a case-fatality rate of 5-7% [3]. Bacteremia occurs in as many as 30% of patients with pneumococcal pneumonia. Invasive forms of pneumococcal disease are less common, but are more severe with higher mortality. As many as 50,000 cases of pneumococcal bacteremia and 6,000 cases of meningitis occur yearly in the USA with overall case-fatality rates of 20% and 30%, respectively. Mortality rates for all forms of pneumococcal disease are increased in elderly persons. Due to pediatric immunization, vaccine-type IPD rates have declined by approximately 50% in adults ≥65 years old

between 2010 and 2013 [16]. However, 33,500 cases and 3,500 deaths from IPD are estimated to have occurred in 2013, primarily in adults aged ≥50 years [17]. Thus, older individuals continue to be significantly impacted by pneumococcal disease.

Similar to the elderly, *S. pneumoniae* is the most common respiratory pathogen in the HIV+ population [6]. In the USA, an estimated 1.2 million people are currently living with HIV infection, with approximately 50,000 new infections occurring each year [18]. Higher rates of recurrent and severe pneumococcal infections are associated with HIV infection [19, 20]. Prior to the introduction of ART, IPD incidence was estimated to be 100-fold higher than in HIV-uninfected (HIV−) individuals [21]. Widespread use of ART has led to a significant disease in pneumococcal disease rates [22, 23]. However, HIV+ individuals remain at 20-40 fold increased risk for IPD compared to age-matched HIV− subjects [24, 25]. Pneumococcal disease therefore remains a significant cause of HIV-associated morbidity and mortality.

1.2 Therapeutic interventions

Studies conducted in rabbits in the late 19th century demonstrated early on the importance of humoral immunity in conferring protection to *S. pneumoniae* [1, 2]. Serum from rabbits who recovered from pneumococcal infection contained protective factors that prevented re-infection and also protected against primary infection with the same strain when injected into other animals. Before antibiotics were available, antiserum therapy was widely used to treat pneumococcal infections in humans with some success [2]. Current strategies to treat pneumococcal disease utilize either monotherapy or a combination of available antibiotics. Penicillin has been the standard treatment for

decades, but other β-lactams, macrolides, and fluoroquinolones are also commonly used [26]. However, the emergence of antibiotic resistance complicates and may reduce the effectiveness of recommended treatment regimens.

Penicillin-resistant *S. pneumoniae* strains were first noted in the 1970s and have steadily increased in incidence worldwide along with strains resistant to other antibiotics [6, 27]. However, a simultaneous rise in case-fatality rates for IPD has not been observed [28, 29]. Since the introduction of conjugate vaccines containing serotypes with high incidence of antibiotic resistance, rates of drug-resistant pneumococcal infections have decreased. In 2013, however, the Centers for Disease Control and Prevention (CDC) classified the threat of antibiotic-resistant *S. pneumoniae* infections a serious concern requiring increased focus on utilizing preventative measures and reducing overuse of antibiotics [30]. Approximately 50% of pneumococcal isolates from IPD cases in the USA were reported to be fully resistant to one or more antibiotics, with the highest prevalence being erythromycin resistance (about 30%) [17]. Thus, continued surveillance and monitoring of the prevalence of antibiotic-resistant serotypes is important for the future development of pneumococcal vaccines.

1.3 Virulence factors: targets for vaccines

The polysaccharide capsule is the most important virulence determinant of *S. pneumoniae* and the target of current vaccine formulations (Figure 1-2). It is critical to pneumococcal colonization, invasion, and dissemination from the respiratory tract. To date, over 90 structurally distinct capsular pneumococcal polysaccharide (PPS) serotypes have been recognized [31]. The majority of serotypes can cause disease, but only a

limited number are known to be responsible for IPD. Pneumococci regulate the amount of capsular material produced during colonization and invasion using phase variation. During the initial stages of colonization, transparent (thin) capsules are favored that promote adherence to host tissues, whereas opaque (thick) capsules prevail during invasion into the bloodstream to prevent opsonophagocytosis. In addition, some PPS are negatively charged, which play a role in preventing mucosal clearance and inhibiting activation of complement [27]. Effective clearance of *S. pneumoniae* by the host requires production of serotype-specific anticapsular antibodies by B cells that opsonize and promote complement-mediated phagocytosis of the organism. Infants and adults with defective humoral responses, therefore, are more susceptible to pneumococcal infections.
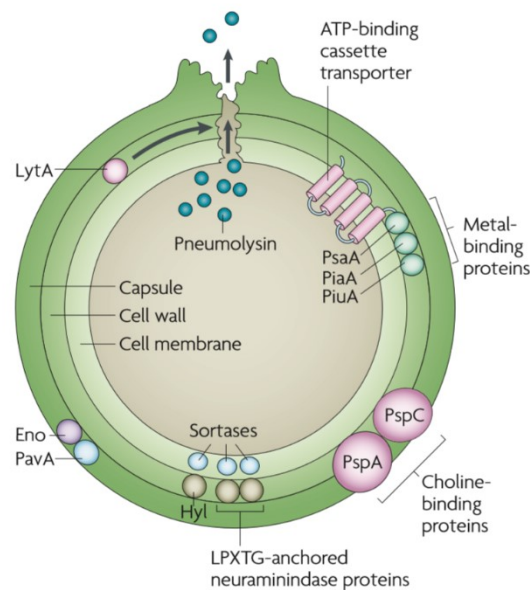
Figure 1-2. Pneumococcal virulence factors. Important pneumococcal virulence factors of note include: the capsule; choline-binding proteins; pneumococcal surface proteins A and C (PspA and PspC); and pneumolysin. Re-printed by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, reference [33], copyright 2008.

Several pneumococcal proteins important for virulence are currently being investigated as potential vaccine antigens due to their ability to stimulate opsonic antibodies (Figure 1-2) [32-34]. These virulence factors have roles in promoting adhesion to and invasion of mucosal tissues, or suppressing host immune defenses. The pore-forming cytotoxin pneumolysin is produced by almost all invasive strains of *S. pneumoniae* and is released during autolysis, resulting in disruption of epithelial and endothelial barriers. In addition to its cytolytic functions, pneumolysin inhibits the respiratory burst and ciliary action of epithelial cells and also activates complement and chemokine production at sub-lytic concentrations. Pneumolysin is thought to be critical for dissemination from the lungs to the bloodstream.

Pneumococcal surface proteins (Psp) A and C both interfere with complement-mediated opsonization. PspC also binds to the polymeric immunoglobulin (Ig) receptor that typically transports secretory IgA during invasion. Pneumococcal choline-binding protein A (PcpA), like pneumolysin, is expressed by almost all virulent serotypes. PcpA appears to play an important role in adhesion to nasopharyngeal and lung epithelium. Vaccination strategies utilizing protein virulence factors, such as pneumolysin, PspA, PspC, and PcpA, are desirable over current vaccines due to the ability to confer serotype-independent protection. However, protein-based pneumococcal vaccines are not likely to be available for clinical use for many years, as the majority of potential candidates are still being evaluated in animal studies.

1.4 Pneumococcal vaccines

The earliest formulations of pneumococcal vaccines containing whole-cell, heat-treated bacteria were marketed in the early 1900s and distributed into the 1930s [2]. The first pneumococcal vaccine was licensed for use in the USA in 1909. Initial clinical trials using the whole-cell pneumococcal vaccine were conducted in South Africa beginning in 1911. Recognition of the importance of serotype specificity resulted in the development of serotype-specific whole-cell vaccines in 1914. Subsequent trials using multivalent versions were performed during World War I. Overall, whole-cell vaccines demonstrated moderate success in preventing pneumococcal infections, but concerns over efficacy prevented widespread use.

PPS were first isolated in 1917 and later established as critical virulence factors responsible for inducing antibody production in humans. Early PPS vaccines were developed, and heptavalent versions were licensed for use in the 1940s based on clinical evidence of efficacy. They were poorly utilized, however, because clinicians preferred to treat pneumococcal infections using newly introduced antibiotics. Interest in pneumococcal vaccine development was rekindled in the 1970s by clinician-scientist Robert Austrian, who noted high pneumococcal case-fatality rates despite antibiotic therapy [2]. Several multivalent forms of PPS vaccines were tested amongst South African gold miners. A 13-valent formulation, for instance, was found to be approximately 80% efficacious against bacteremia and pneumonia [35]. Success with these early vaccines, combined with increased rates of antibiotic-resistant infections, led to the license of a 14-valent PPS vaccine in the USA in 1977 indicated for adults ≥50 years old and children ≥2 years with underlying medical conditions [2, 36]. The 14

serotypes included in the vaccine were estimated to cover up to 68% of disease-causing isolates [37].

Due to a global need to expand serotype coverage, the currently available 23-valent PPS vaccine (PPV, Pneumovax 23®; Merck & Co., Inc.) was licensed for use in the USA in 1983, increasing IPD coverage at that time to approximately 85% [2, 38]. PPV contains the following serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. Since its introduction, vaccine recommendations for adults ≥50 years old and individuals ≥2 years of age with certain medical conditions or other indications have included PPV. Several studies have demonstrated that PPV is highly effective in preventing IPD in young, healthy adults [6]. In contrast, effectiveness of PPV is significantly reduced in elderly individuals and immunocompromised adults, including HIV+, with about 50% efficacy in preventing IPD [39-41]. A recent meta-analysis conducted by Moberley *et al.* supports use of PPV for preventing IPD in adults, but clear evidence of reducing all-cause pneumonia or mortality is lacking [42].

Poor immunogenicity of PPV in children <2 years old led to the development of third-generation pneumococcal vaccines which contain PPS conjugated to a protein carrier. The covalent coupling of PPS to an immunogenic carrier protein, such as the non-toxic mutant of diphtheria toxin (CRM197), stimulates a T-cell dependent (TD) response, leading to improved immunogenicity and booster response in children [6]. The complexity of the conjugation process severely limits the number of serotypes that can be included in these conjugate vaccines, as each PPS is individually conjugated. In 2000, the first 7-valent conjugate vaccine was licensed in the USA, covering approximately 80-

90% of IPD- and 65% of otitis media-causing serotypes in young children [43, 44]. The 7-valent formulation contained serotypes 4, 6B, 9V, 14, 18C, 19F and 23F. Conjugate vaccination in the pediatric population led to substantial, sustained reductions in vaccine-type IPD among vaccinees with an efficacy of over 90% (Figure 1-3) [6-10]. Reduced rates of pneumonia and otitis media occurred to a lesser extent in immunized children. Remarkably, introduction of the conjugate vaccine also resulted in decreased rates of vaccine-type IPD in unvaccinated adult populations [8, 45]. This "herd immunity" was likely a result of reduced transmission of vaccine-type serotypes due to decreased nasopharyngeal carriage in vaccinated children [46-50].
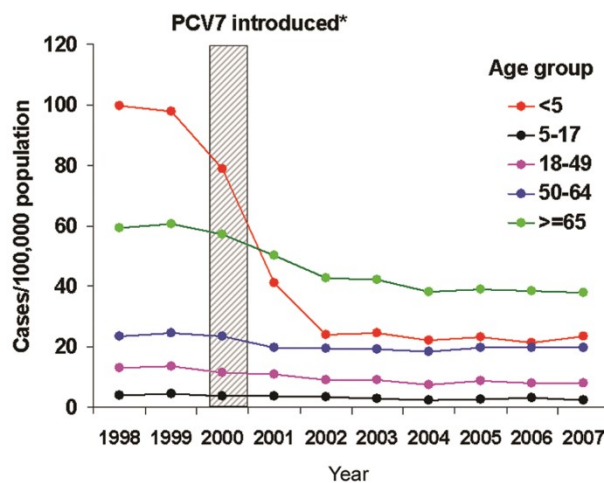
Figure 1-3. Changes in overall invasive pneumococcal disease (IPD) incidence rates by age group, 1998–2007. *Seven-valent pneumococcal conjugate vaccine (PCV7) was introduced in the United States for routine use among young children and infants in the second half of 2000. Reprinted from reference [7] by permission of Oxford University Press, copyright 2010.

The observed benefits of the 7-valent conjugate vaccine were limited by a shift in serotype prevalence [6]. Emergence of infections due to non-vaccine serotypes occurred as rates of vaccine-type disease declined. Significant increases in serotype 19A isolates

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were of particular concern due to the high prevalence of multidrug resistance in clinical specimens [45, 51, 52].   Growing fears over serotype replacement and antibiotic resistance led to the development and licensure of the currently available 13-valent conjugate vaccine (PCV, Prevnar 13®; Wyeth Pharmaceuticals., Inc.) in the USA in 2010. PCV contains 6 additional serotypes (1, 3, 5, 6A, 7F, and 19A) to those included in PCV7. Similar to the 7-valent conjugate vaccine, further reductions in vaccine-type IPD have been observed with PCV in both vaccinated and unvaccinated individuals [11, 53, 54].   PCV is predicted to prevent approximately 168,000 cases of IPD in the USA between 2011 and 2020 [55].   Continued surveillance in countries where PCV is available will provide a more accurate assessment of its impact on reducing pneumococcal disease and the extent to which serotype replacement will occur.

The success of conjugate vaccines in the pediatric population has prompted consideration of its use in adults. Approximately 20-25% of IPD cases in adults ≥65 years old and 50% of IPD cases in immunocompromised adults are caused by serotypes contained in PCV [16, 56]. In 2011, the Food and Drug Administration (FDA) approved the use of PCV in adults ≥50 years old based on immunogenicity studies demonstrating increased or similar antibody responses to PCV compared to PPV in adults [57]. ACIP vaccination recommendations now include use of both PCV and PPV in adults with immunocompromising conditions, as well as in those ≥65 years old [16, 56]. Use of PCV in adult populations is controversial [58-70].   A large scale clinical trial in the Netherlands recently reported PCV had 75% efficacy for vaccine-type IPD and 45% efficacy for vaccine-type nonbacteremic pneumonia compared to placebo in approximately 85,000 adults ≥65 years old [71].   Unfortunately, this study did not

include comparison with PPV. A trial conducted in HIV+ adults observed 74% efficacy against recurrent vaccine-type IPD with the 7-valent conjugate vaccine, but protection declined after the first year [72]. Limitations in serotype coverage and vaccine efficacy in adults with the currently available vaccines emphasize the need to develop novel strategies for pneumococcal disease prevention [73].

1.5 Current vaccination recommendations

Pneumococcal vaccination policies are largely influenced by the epidemiology of pneumococcal infections and trends in antimicrobial resistance. Since 2010, the ACIP has updated vaccination recommendations to include PCV for all high risk populations in the USA. Routine vaccination with PCV is recommended for all young children 2-59 months of age. Infants should receive a 3 dose primary series of PCV at 2, 4, and 6 months, followed by a PCV booster at 12-15 months; alternative dosing schedules are recommended for those previously vaccinated with the 7-valent conjugate vaccine [74, 75]. Unvaccinated children aged 24-71 months with underlying medical conditions that increase risk for pneumococcal disease should receive 2 doses of PCV ≥8 weeks apart [74, 75]. A single dose of PCV is recommended for unvaccinated children 6-18 years with immunocompromising conditions, functional or anatomic asplenia, cerebrospinal fluid leaks, or cochlear implants [76]. Children 2-18 years old with underlying medical conditions should receive a single dose of PPV at least 8 weeks after completing all recommended doses of PCV [74, 75]. Use of PPV is recommended to prevent vaccine-type infections not covered by PCV. Revaccination with PPV ≥5 years after the first

PPV is recommended in children with asplenia, HIV infection, or other immunocompromising conditions [74-76].

Despite the impact of "herd immunity" from widespread pediatric immunization, IPD rates remain high in adults ≥19 years old with underlying medical conditions that increase risk for pneumococcal disease, such as HIV infection [24, 25, 56]. Current ACIP recommendations for unvaccinated adults ≥19 years old with immuno-compromising conditions, asplenia, cerebrospinal fluid leaks, or cochlear implants should receive one dose of PCV followed by a single dose of PPV ≥8 weeks later (Table 1.1) [56]. A second dose of PPV at least 5 years after the first PPV is recommended in adults 19-64 years old with asplenia or immunocompromising conditions. Adults with other medical conditions that increase risk for disease, including chronic illnesses, alcoholism, and cigarette smoking, should receive a single dose of PPV [56]. Adults who received PPV before 65 years of age for any indication should receive another dose of PPV at age 65 years, or later if the previous PPV was ≤5 years prior [56].

In 2014, the ACIP issued new recommendations for adults ≥65 years of age to include routine use of PCV based on the findings of the clinical trial conducted in the Netherlands [16, 71]. Previously, it was recommended that individuals receive a single dose of PPV at age 65 [77]. All unvaccinated adults ≥65 years old should now receive a single dose of PCV first, followed by a dose of PPV 6-12 months later [16]. Continued surveillance of the impact of PCV on serotype replacement and also on vaccine-type IPD burden and CAP will determine if modifications in vaccination policy need to occur. Towards this goal, this recommendation will be reevaluated in 2018 by the ACIP and revised as needed.

Table 1.1. Current ACIP recommendations for administration of PCV and/or PPV in adults ≥19 years old with medical conditions or other indications.

Risk group	Underlying medical condition	PCV13 Recommended	PPSV23 Recommended	PPSV23 Revaccination 5 yrs after first dose
Immunocompetent persons	Chronic heart disease[†]		✓	
	Chronic lung disease[§]		✓	
	Diabetes mellitus		✓	
	Cerebrospinal fluid leak	✓	✓	
	Cochlear implant	✓	✓	
	Alcoholism		✓	
	Chronic liver disease, cirrhosis		✓	
	Cigarette smoking		✓	
Persons with functional or anatomic asplenia	Sickle cell disease/other hemaglobinopathy	✓	✓	✓
	Congenital or acquired asplenia	✓	✓	✓
Immunocompromised persons	Congenital or acquired immunodeficiency[¶]	✓	✓	✓
	Human immunodeficiency virus infection	✓	✓	✓
	Chronic renal failure	✓	✓	✓
	Nephrotic syndrome	✓	✓	✓
	Leukemia	✓	✓	✓
	Lymphoma	✓	✓	✓
	Hodgkin disease	✓	✓	✓
	Generalized malignancy	✓	✓	✓
	Iatrogenic immunosuppression**	✓	✓	✓
	Solid organ transplant	✓	✓	✓
	Multiple myeloma	✓	✓	✓

* All adults aged ≥65 years should receive a dose of PPSV23, regardless of previous history of vaccination with pneumococcal vaccine.
† Including congestive heart failure and cardiomyopathies, excluding hypertension.
§ Including chronic obstructive pulmonary disease, emphysema, and asthma.
¶ Includes B- (humoral) or T-lymphocyte deficiency, complement deficiencies (particularly C1, C2, C3, and C4 deficiencies), and phagocytic disorders (excluding chronic granulomatous disease).
** Diseases requiring treatment with immunosuppressive drugs, including long-term systemic corticosteroids and radiation therapy.

Source: CDC, reference [56].

Licensure of and recommendations for new pneumococcal vaccines in target populations include assessment of immunogenicity. Large scale efficacy trials will likely not be conducted in the future for a number of reasons [78]. It is no longer ethical to conduct placebo-controlled trials in high risk individuals due to the high efficacy of conjugate vaccines. In addition, the substantial reductions in IPD incidence that have occurred in unvaccinated populations due to widespread vaccination practices will make it difficult to detect differences between control and experimental patient groups. As a result, efficacy trials will require large sample sizes and substantial resources to be conducted.

Two serological parameters are used to estimate protective immunity. A third-generation enzyme-linked immunosorbent assay (ELISA) is currently used for detection

of anticapsular serum antibody levels. The functional characteristics of serotype-specific antibodies are assessed using the opsonophagocytic killing assay (OPA). In 2003, the World Health Organization (WHO) published serological criteria for evaluation of pneumococcal vaccines in infants. An anticapsular IgG concentration of $\geq$0.2-0.35 μg/ml, which corresponded to an OPA titer of 1:8, was recommended as a reference value that correlates to protection [6, 79]. Serological correlates of protection have not been defined in adults, severely limiting the ability to make clinical decisions based on immunogenicity data. Higher threshold values may be more appropriate for high-risk adult populations [6]. Discrepancies between ELISA and OPA assays have been reported in the elderly and immunocompromised adults, but the OPA assay is generally regarded as a better measure of protection as it mimics the host phagocytic response and is a better predictor of vaccine failure [78]. Continued efforts aimed at defining correlates of vaccine protection in adults are critical for determining effective vaccination strategies.

1.6 Immune response to pneumococcal vaccination

In contrast to proteins, PPS do not require cognate T cell-B cell interactions to stimulate an immune response and are classified as T cell-independent type 2 (TI) antigens. Each PPS molecule consists of multiple repeating epitopes that activate mature B cells through extensive cross-linking of surface Ig receptors, resulting in PPS-specific antibody production (Figure 1-4a) [80, 81]. TI antigens are generally regarded as poor inducers of immunological memory due to a lack of direct T cell interactions and productive germinal center responses [82]. Thus, immunization with PPV, which contains purified PPS, generates anticapsular antibodies through TI mechanisms. In

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contrast, the conjugation of PPS to protein, as in PCV, provokes a TD response comprised of major histocompatibility complex (MHC) class II-mediated peptide presentation to carrier peptide-specific CD4+ T cells (Figure 1-4b). Conjugate vaccines are therefore thought to induce memory responses upon repeated immunization [83].

The B cell subset(s) that respond to PPS in humans remain a topic of debate, although evidence suggests multiple candidates are likely involved. The spleen serves as a critical site for immune surveillance of blood-borne pathogens and contains innate-like marginal zone (MZ) B cells responsible for TI responses [84]. Individuals lacking a spleen are at higher risk of infection caused by encapsulated bacteria, including *S. pneumoniae*. Several studies indicate that peripheral IgM memory B cells phenotypically represent circulating splenic MZ B cells [85-88]. In addition, blood and splenic IgM memory B cells possess a prediversified Ig repertoire distinct from naïve or switched memory B cells thought to correspond with TI responses [86, 89, 90]. Peripheral serotype-specific B cells generated after PPV immunization are predominantly IgM memory in young adults, further emphasizing the importance of this B cell subset in the immune response to PPS [91]. Evidence also suggests that MZ B cells exhibit functional plasticity, as they can undergo class-switching in response to both TD and TI antigens [82]. IgM memory B cells transplanted in humanized severe combined immunodeficiency mutation (*SCID/SCID*) mice produced PPS-specific IgM and IgG antibodies after immunization with PPV [92]. It is unlikely, however, that IgM memory B cells are the only subset that responds to PPS, as transplanted switched memory B cells in the same study produced PPS-specific IgG postvaccination [92]. In addition,

individuals at increased risk for pneumococcal disease also exhibit reduced frequencies of switched memory B cells [93].
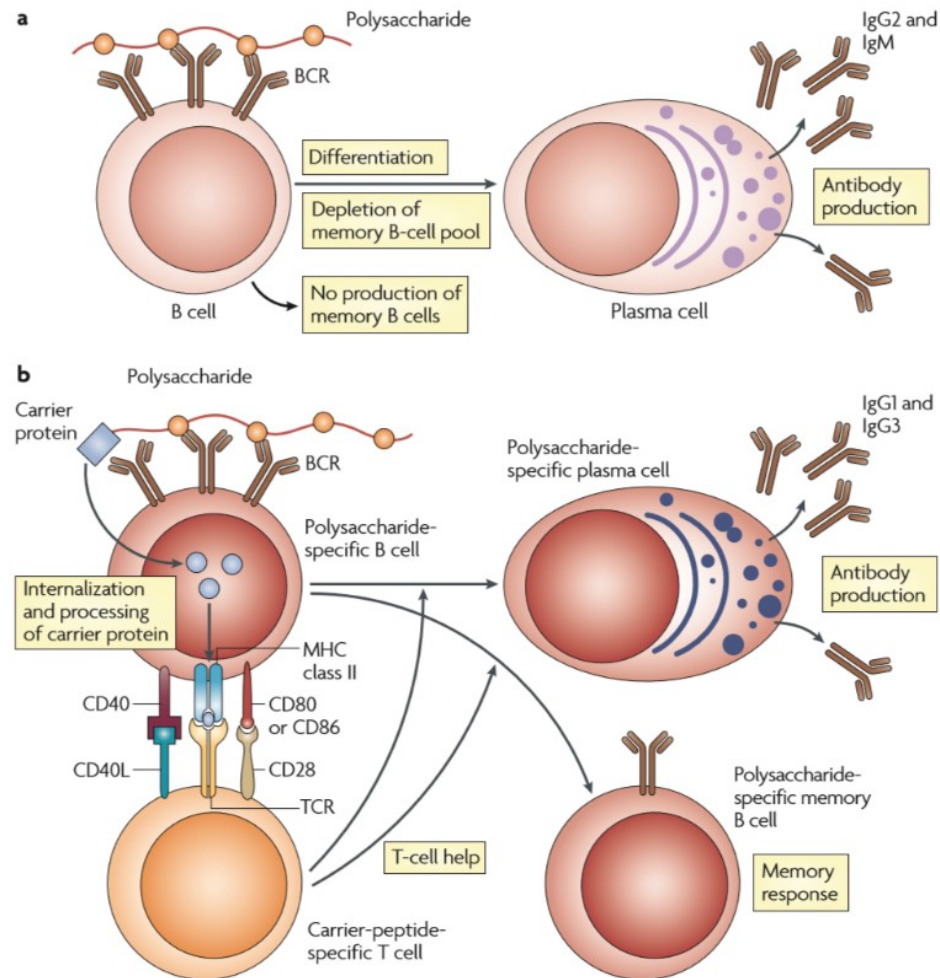
Figure 1-4. The immune response to polysaccharide and protein–polysaccharide conjugate vaccines. a) Polysaccharides from encapsulated bacteria stimulate B cells by crosslinking the B cell receptor (BCR) and driving immunoglobulin production.
b) The carrier protein from protein–polysaccharide conjugate vaccines is processed by polysaccharide-specific B cells, and peptides are presented to carrier peptide-specific T cells, resulting in production of both plasma cells and memory B cells. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, reference [82], copyright 2009.

It is postulated that TI antigens may require secondary signals, such as innate stimuli and B cell receptors, to generate a rapid and effective antibody response [80, 81]. Engagement of Toll-like receptors (TLRs) by microbial products enhance antibody responses to TI antigens, and TLR ligands are potential adjuvants for pneumococcal vaccines [80, 94]. In addition to TLRs, complement also serves as a critical link between innate and adaptive immune systems. Murine studies indicate the complement system is required for innate immunity to *S. pneumoniae* [95, 96]. Complement receptor CD21 is highly expressed on MZ B cells and interacts with complement fragments bound to the surface of TI antigens. CD21 ligation lowers the threshold for antigen-specific B cell activation and thus enhances humoral responses [80, 97]. CD21-deficient mice exhibit impaired antibody responses to *S. pneumoniae*, further supporting a role for this receptor in TI responses [98, 99].

Growing evidence indicates that several members of the tumor necrosis factor receptor (TNFR) superfamily members and their ligands are important for humoral responses to TI and TD antigens [94, 100]. The ligand B cell activating factor (BAFF) is secreted by macrophages and dendritic cells and binds to BAFF receptor (BAFF-R), transmembrane activator and calcium-modulating cyclophilin ligand modulator (TACI), and B cell maturation antigen (BCMA). A proliferation-inducing ligand (APRIL) binds to only TACI and BCMA. BAFF-R, TACI, and BCMA regulate B cell differentiation and survival. TACI is critical for CD40-independent Ig production. Mice deficient in TACI are unable to produce antibodies in response to TI antigens, but generate normal responses to TD antigens [94, 101, 102]. BAFF knockout mice also exhibit reduced TI responses [103]. Murine MZ B cells demonstrate enhanced responsiveness to BAFF, and

surface TACI expression is upregulated in the presence of microbial TLR ligands [100]. In humans, increased TACI and loss of BAFF-R expression is associated with B cell differentiation into plasmablasts in response to various stimuli *in vitro* and after influenza vaccination [104, 105]. Moreover, subsets of patients with common variable immune deficiency (CVID) exhibit reduced antibody responses due to mutations in TACI [106]. These data indicate that TACI is critical for humoral responses in humans. Regulation of TNFR family members in response to pneumococcal vaccines is unknown.

1.6.1. Infants and young children

Unlike TD responses which are present soon after birth, responses to TI antigens develop late in ontogeny. The delay in antibody responses to PPS renders infants and young children <2 years of age nonresponsive to PPV immunization and highly susceptible to *S. pneumoniae* infections. The ability to respond to TI antigens coincides with maturation of the splenic MZ compartment [107]. Circulating IgM memory B cells are nearly undetectable at birth but increase in frequency with age, reaching 10-20% of the total B cell population by 2 years of age [85]. Infants exhibit reduced CD21 expression on MZ B cells and low levels of complement which may result in hyporesponsiveness to TI antigens [81, 94]. In addition, B cells isolated from preterm cord blood express decreased levels of TACI, BAFF-R, and BCMA compared to adult B cells, resulting in diminished antibody secretion [108]. Conjugate vaccines improve the immunogenicity of PPS in infants and young children, overcoming some of the immunological limitations with plain PPS, and have been highly successful in reducing overall pneumococcal disease burden in this population [6-8, 83].

1.6.2. Elderly individuals

A decline in overall humoral and cellular responses occurs in an age-dependent manner, posing a significant challenge for development of effective vaccines containing either TD or TI antigens in older adults. Numerous studies have observed decreased efficacy with PPV in the elderly [6, 32, 39, 41, 42]. Although elderly individuals generate concentrations of serotype-specific IgG similar to young adults after vaccination with PPV, they exhibit diminished functional antibody activity [109-112]. Lower serotype-specific IgM production and altered repertoire diversity of antibodies generated in response to immunization observed in older adults may all contribute to functional differences [112-116]. Age-dependent reductions in the percentage or absolute number of IgM memory B cells may also result in decreased responsiveness to pneumococcal vaccination in the elderly [117-119]. In contrast to young adults, serotype-specific B cells in older individuals are primarily switched memory B cells postvaccination with PPV [117]. Collectively, these findings suggest that age-associated reductions in serotype-specific IgM production and IgM memory B cells are responsible for decreased efficacy of PPV in the elderly. How other aspects of cellular responses to pneumococcal vaccination, such as CD21 expression or TNFR signaling pathways, are impacted by aging is unknown. It is also unclear how PCV influences responding B cell populations. Overall, there is no consistent evidence that PCV is superior to PPV in regards to quantitative and functional antibody responses in elderly individuals [32, 39, 61, 120-122].

1.6.3. HIV-infected adults

HIV infection leads to dysregulation of several immune cell populations, including B cells. Viremia causes functional and phenotypical B cell defects beginning early in infection and a reduction in overall B cell numbers [123, 124]. B cell subpopulations are dramatically altered, resulting in loss of memory B cells and increased mature activated and immature transitional B cells [124]. Viremic patients also exhibit expansion of abnormal peripheral $CD21^{lo}$ B cell populations associated with exhaustion and increased turnover. Increased serum BAFF levels, resulting from viremia-induced production by macrophages and dendritic cells, are thought to correlate with HIV-associated B cell disease progression and contribute to B cell hyperactivation [124-126]. Decreased B cell survival has been linked to reduced BAFF-R expression in apoptosis-prone B cell subpopulations [124, 127]. These HIV-associated B cell alterations likely disrupt humoral responses to pneumococcal vaccination.

After vaccination with PPV, HIV+ adults exhibit reduced antibody responses compared to HIV− controls [128-131]. In contrast to elderly individuals, both serotype-specific IgG and IgM levels, in addition to functional titers, are diminished. Antibody responses to PPV are predominantly derived from B cells expressing the Ig variable region gene family 3 (V_H3) which are depleted during HIV infection [132-134]. Loss of V_H3 may contribute to impaired antibody responses to PPV. In addition, depletion of total or serotype-specific circulating memory B cell subsets is associated with impaired serological responses to PPS [128, 129, 135-137]. Initiation of ART leads to a significant improvement in B cell counts and normalization of subpopulations in parallel with an increase in $CD4^+$ T cells [123, 138]. However, studies indicate that restoration of

memory B cells is incomplete, and ART-treated HIV+ individuals continue to exhibit impaired responses to vaccination [136, 137]. Both ART-untreated and –treated HIV+ individuals exhibit reductions in serotype-specific IgM memory B cells compared to HIV– controls [128, 129]. Reconstitution of $CD4^+$ T cells with ART may also be incomplete, particularly in patients who delay treatment until CD4 <200 [139]. Thus, it remains unclear whether PCV can elicit greater antibody responses compared to PPV in HIV+ individuals.

1.7 Study Objectives

By 2015, more than half of all HIV+ individuals living in the USA will be ≥ 50 years of age (Figure 1-5) [140]. The most significant factor responsible for this demographic shift is the development of ART. HIV infection is now a chronic condition in patients who are able to control viremia using ART. A 20-year-old HIV+ individual currently living in the USA initiating ART can expect to live into their early 70s, a life expectancy close to that of the general population [15]. In addition to ART, the rate of newly diagnosed HIV infections in older adults has been steadily increasing. The CDC reported that approximately 20% of HIV diagnoses were among Americans aged 50 and older in 2013 [141]. Growing evidence indicates that age influences the course of HIV infection. The time from acquisition of HIV infection to the development of acquired immune deficiency syndrome (AIDS) or death is shorter in older patients, even in the ART era [140]. While use of ART prevents AIDS-related complications, it does not fully restore health, as a number of immunological defects persist despite suppression of viremia. Moreover, the onset of age-associated conditions, including cardiovascular

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disease, neurocognitive disorder, renal disease, and cancer, is accelerated in HIV+ individuals [142, 143].
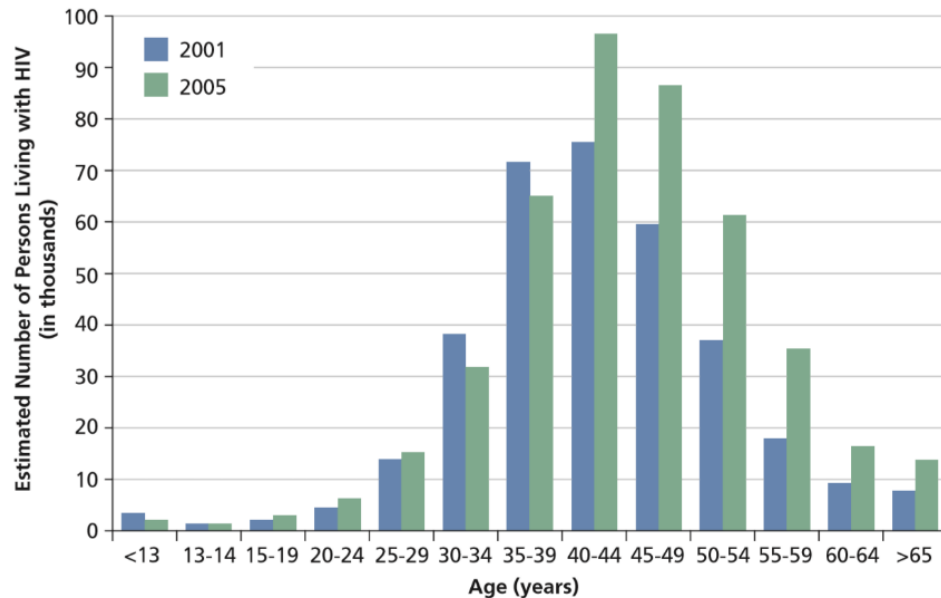
Figure 1-5. Age distribution of HIV-infected individuals living in the United States. Reprinted from reference [143] by permission of the International Antiviral Society-USA, copyright 2009.

As previously discussed, both elderly and HIV+ individuals are at increased risk for pneumococcal disease due in part to B cell dysfunction. Older HIV+ persons are likely to be at very high risk for IPD, as advanced age is a significant risk factor within the HIV+ population [25]. Aging and HIV infection share in common many features of B cell alterations, including polyclonal activation, hypergammaglobulinemia, and defective responses to antigen stimulation [144]. However, these populations exhibit distinct perturbations in B cells responding to PPV immunization [117, 128, 129]. Aging HIV+ individuals, due to their combination of risk factors, may therefore possess unique responses to pneumococcal vaccination which reflect synergism of B cell defects between aging and HIV infection. It is currently recommended that HIV+ adults receive

a single dose of PCV followed by one dose of PPV 8 weeks later (PCV/PPV) [56]. It remains unclear whether this combined regimen enhances responses compared to a single dose of PPV, as aging HIV+ individuals exhibit persistent defects in both T cells and B cells.

The overall goal of the current study was to assess humoral and cellular responses to the recommended PCV/PPV regimen compared to a single dose of PPV in ART-treated aging HIV+ individuals 50-65 years old with CD4$^+$ T cells/µl (CD4) >200. This work is highly significant because development of effective vaccination strategies in the aging HIV+ population will likely be very challenging to the presence of multiple co-morbidities that increase risk for pneumococcal disease. This is also the first study, to our knowledge, to characterize antigen-specific B cells responding to PCV versus PPV in HIV+ subjects.

The objectives of the current study are summarized below:

1. **To determine whether PCV/PPV improves immunogenicity in HIV+ subjects 50-65 years old compared to PPV alone (Chapter 2).**
   a. Assessment of quantitative antibody responses
   b. Assessment of functional antibody responses
   c. Comparison of antibody responses to HIV− controls after PCV/PPV

2. **To determine whether HIV+ subjects 50-65 years old exhibit distinct perturbations in B cells responding to PPV alone (Chapter 3).**
   a. Phenotypic analysis of serotype-specific memory B cells postvaccination with PPV

3. **To determine whether PCV/PPV alters the frequency and composition of circulating serotype-specific B cells compared to PPV alone in HIV+ subjects 50-65 years old (Chapter 3).**

    a. Assessment of serotype-specific B cell frequency postvaccination with PCV/PPV or PPV

    b. Phenotypic analysis of serotype-specific memory B cells postvaccination with PCV/PPV or PPV

    c. Characterization of surface expression of complement receptor CD21 and TNFRs CD40, BAFF-R, and TACI on serotype-specific B cells postvaccination with PCV/PPV or PPV

    d. Comparison of serotype-specific B cell responses to HIV− controls after PCV/PPV

Chapter 2

Quantitative and Functional Antibody Responses to the 13-Valent Conjugate and/or 23-Valent Purified Polysaccharide Vaccine in Aging HIV-Infected Adults*

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2.1. Introduction

Streptococcus pneumoniae infections, including pneumonia and invasive disease (IPD), remain a significant cause of HIV-associated morbidity and mortality despite several clinical advances. Widespread pediatric pneumococcal immunization, due to indirect effects, and use of antiretroviral therapy (ART) have resulted in substantial reductions in IPD incidence [1, 2]. However, disease burden persists in HIV+ individuals despite higher CD4 counts and is 20-40 fold higher than in age-matched HIV− individuals [1, 3]. Increased rates of recurrence and severe infections are also associated with HIV infection [4, 5].

The population of aging HIV+ individuals has rapidly expanded due to the success of ART in reducing mortality combined with the increased rate of new diagnoses in older adults [6-8]. Evidence suggests age influences the course of HIV infection by accelerating the development of comorbidities and decreasing the duration of clinical latency in older patients [9, 10]. Approximately one-half of HIV+ individuals living in the United States will be ≥50 years old as of this year [9]. Advanced age is a significant risk factor for pneumococcal disease in HIV+ and HIV− individuals [3, 11]. Both aging and HIV infection contribute to B cell dysfunction, resulting in decreased responses to vaccination.

Recommendations for prevention of bacterial pneumonia in HIV+ adults include use of ART, smoking cessation, and vaccination against influenza and S. pneumoniae [12]. Prior vaccination guidelines for HIV+ adults in the United States recommend a single dose of PPV at diagnosis, followed by revaccination 5 years later, and again after age 65 [13]. However, the effectiveness of PPV in HIV+ adults has been a subject of

debate [14, 15]. Several factors impacting efficacy, including timing of vaccination and degree of immunocompromise, remain ill defined.

In an effort to improve protection against pneumococcal infection, PCV was added to the vaccination recommendations for adults with immunocompromising conditions [13]. For HIV+ pneumococcal vaccine-naïve individuals and those vaccinated with a primary dose of PPV ≥5 years prior, the Advisory Committee on Immunization Practices (ACIP) recommends a single dose of PCV followed by a dose of PPV at least 8 weeks later [13]. Immunogenicity studies conducted in HIV+ adults have thus far, however, yielded inconsistent results regarding the superiority of PCV alone or in combination with PPV over the prior recommendation [16-22]. Thus, the potential value of PCV in the HIV+ population remains to be established.

The combined impact of immunosenescence and HIV infection on responses to pneumococcal vaccines may be an important consideration in the clinical management of older HIV+ adults. The goal of the current study was to compare quantitative and functional antibody responses generated from the recommended PCV/PPV regimen to a single dose of PPV in HIV+ adults 50-65 years old.

2.2. Methods

Design and Study Population

Volunteers 50-65 years old were recruited between April 2012 and January 2015 at the University of Toledo Medical Center. Written, informed consent was obtained from all subjects. The study was monitored and approved by the Institutional Review Board at the University of Toledo. Exclusion criteria included: active infection (except HIV), PPV <5 years prior, pregnancy, immunosuppressive medications, and history of cancer, autoimmune disease, bleeding disorders, immunoglobulin therapy, organ transplantation, splenectomy, and end stage renal or liver disease. Volunteers were questioned about any prior hospitalizations consistent with pneumococcal infection. Eligibility criteria for HIV+ participants were further defined as current CD4 >200, HIV viral load ≤400 copies/ml, and ART for ≥1 year. Adherence to ART was confirmed by patient's self-report and review of pharmacy records.

HIV− individuals received a single dose of PCV (Prevnar 13®; Wyeth Pharmaceuticals, Inc.) followed by one dose of PPV (Pneumovax 23®; Merck & Co., Inc.) 8 weeks later (PCV/PPV). HIV+ individuals were randomized at enrollment to receive either PCV/PPV or a single dose of PPV. All participants who received PCV/PPV were followed up 2 months (8 weeks; PPV immunization) and 3 months after study enrollment. Participants who received PPV were followed up 1 month after enrollment.

Laboratory

Blood samples were collected at each study visit. Serum samples were used to measure capsular pneumococcal polysaccharide-specific antibody responses pre- and 1 month postvaccination with PPV (post-PPV) to serotypes 14 and 23F (included in PCV and PPV). These serotypes were selected due to their historically high prevalence in the HIV+ population and inclusion in our previous studies [23, 24]. Serotype-specific IgG and IgM serum levels were detected by enzyme-linked immunosorbent assay (ELISA) as previously described [25, 26] using 89SF or 007SP as standards. Opsonophagocytic killing assay (OPA) was performed as previously described [25, 26] to determine functional antibody responses. Data were analyzed using the Opsotiter1 software program (University of Alabama at Birmingham). OPA titers were defined as the reciprocal of the serum dilution that killed 50% of target bacteria (compared to serum-free control) during 45 minutes of incubation at 37ºC.

Statistical Analysis

Participant characteristics were represented as mean (range) for numerical values and number (percentage) for categorical values. Serotype-specific serum IgG and IgM levels (µg/ml) and OPA titers were reported as geometric mean concentrations or titers (95% confidence interval), respectively. Responders to vaccination were determined as previously defined [16, 17, 19]. Serotype-specific IgG and IgM responses were defined as a ≥2-fold increase from baseline and postvaccination levels of ≥1 µg/ml. A positive OPA response was defined as a ≥4-fold increase from baseline. IgG and IgM levels and OPA titers were log-transformed to approximate normal distribution prior to statistical

analysis. Pre- to 1 month post-PPV comparisons were calculated by analysis of covariance (ANCOVA) with Bonferroni correction. Post-PPV antibody responses were compared using analysis of variance (ANOVA) with Dunnett's post-hoc test, with HIV+ PCV/PPV designated as the control group. The number of responders from each group were compared using the Fisher's exact test. Correlations were determined by Pearson's correlation coefficient. All statistical analyses were performed using the SAS software package (version 9.3; SAS Institute). P values <0.05 were considered significant.

2.3 Results

Subjects

Baseline characteristics of the 51 participants (37 HIV+ and 14 HIV−) included in this study are reported in Table 2.1. CD4 counts at enrollment were similar between HIV+ groups. Other clinical characteristics, including nadir CD4 counts and HIV viral load, did not differ between HIV+ groups. All HIV+ subjects were adherent to ART ≥ 1 year. Differences in the distributions of sex and race in the HIV− group compared to the HIV+ groups were noted. A larger proportion of HIV+ participants had been immunized with PPV ≥ 5 years prior (83.8%) compared to HIV− (6.3%).

Serum antibody levels to serotypes 14 and 23F

There were no significant differences in baseline serotype-specific IgG and IgM serum levels between study groups (Tables 2.2 and 2.3). Pre- to 1 month post-PPV IgG levels were significantly higher in all groups for both serotypes (P <0.009; Table 2.2). Significant pre- to post-PPV increases in IgM levels were observed for serotype 14 in the HIV+ PPV group and for serotype 23F in the HIV+ PCV/PPV group (P <0.05; Table 2.3). IgM levels significantly increased pre- to post-PPV in the HIV− PCV/PPV group for both serotypes (P <0.001).

Post-PPV antibody levels were compared between HIV+ PPV and PCV/PPV groups and between HIV+ and HIV− PCV/PPV groups. In HIV+ PPV and PCV/PPV groups, post-PPV IgG levels for both serotypes were similar. Post-PPV IgM levels for serotype 23F, but not 14, were significantly lower in the HIV+ PPV compared to HIV+ PCV/PPV groups (P <0.05). Post-PPV IgG and IgM levels were significantly reduced

for serotype 14 only in HIV+ compared to HIV– PCV/PPV groups (P <0.05). The number of subjects that had positive IgG or IgM responses (defined as ≥2-fold increase and post-PPV levels ≥1 µg/ml) were similar for all groups, although more frequent in HIV– individuals (Table 2.5). Positive responses were lower for IgM than IgG for both serotypes.

Serum OPA titers to serotypes 14 and 23F

Baseline OPA titers were similar between groups (Table 2.4). Significant increases in serotype-specific OPA titers from pre- to 1 month post-PPV occurred in all groups (P <0.0001). Post-PPV OPA titers were compared between HIV+ PPV and PCV/PPV groups and between HIV+ and HIV– PCV/PPV groups. Post-PPV OPA titers were similar between HIV+ PPV and PCV/PPV groups. In HIV+ compared to HIV– PCV/PPV groups, post-PPV OPA titers were significantly reduced for serotype 23F only (P <0.05). Positive OPA responses (defined as ≥4-fold increase) were similar between all study groups (Table 2.5).

Correlations between post-PPV antibody levels and OPA titers

For all groups, there were significant correlations between post-PPV serotype-specific IgG levels and OPA titers. There was a strong correlation for serotype 23F (r = 0.64, P = 0.001), but not for serotype 14 (r = -0.36, P = 0.10) in the HIV+ PPV group. In the HIV+ PCV/PPV group, there was a strong correlation for serotype 14 (r = 0.88, P <0.0001), but no correlation for serotype 23F (r = 0.20, P = 0.48). Moderate correlations for both serotypes 14 and 23F (r = 0.68, P = 0.008; r = 0.58, P = 0.03, respectively) were

observed in the HIV– PCV/PPV group. There were no significant correlations between post-PPV serotype-specific IgM levels and OPA titers.

2.4 Discussion

The current study is the first to our knowledge to assess the impact of a combined PCV/PPV regimen in older HIV+ individuals. Our findings suggest that this approach offers no clear improvement in serum antibody titers or opsonophagocytic activity compared to a single dose of PPV. Immunization of HIV+ subjects with either PPV or PCV/PPV resulted in significant but similar increases in serotype-specific IgG levels and OPA titers compared to baseline levels. However, the magnitude of antibody responses in the HIV+ PCV/PPV group was diminished compared to those observed in age-matched HIV− PCV/PPV controls.

HIV disease progression results in extensive defects in humoral immunity. Loss of memory B cell subsets is associated with impaired antibody responses to pneumococcal vaccination that are incompletely restored by ART [27-29]. We have previously shown that both ART-treated and −untreated HIV+ adults exhibit reduced serotype-specific IgM memory B cells after PPV compared to HIV− individuals [25, 26]. In addition, a substantial proportion of patients on ART do not achieve normalization of CD4 counts [30]. These data, in combination with the current study, suggest that despite higher CD4 counts and use of ART, persistent cellular defects likely contribute to diminished antibody responses after vaccination in our HIV+ subjects.

Independent of HIV infection, increased age is associated with reduced responses to pneumococcal vaccination [11]. Impairment of functional responses in older adults may result from reduced serotype-specific IgM levels post-PPV [11, 23, 31]. We have demonstrated that serotype-specific IgM memory B cells are also reduced in elderly individuals post-PPV compared to younger volunteers [23]. A strength of our study is

the use of age-matched uninfected controls. Minimal increases in the frequency of serotype-specific IgM responses were observed for all groups. Therefore, although HIV− participants had higher IgG levels and OPA titers, immunosenescence likely impacted humoral responses in all of our subjects. We investigated peripheral B cell subpopulations responding to PCV and/or PPV in these individuals to further delineate the impact of aging and HIV infection on vaccine responses (accompanying manuscript).

The finding that PPV and PCV/PPV elicited similar responses is consistent with some [18, 21], but not all [17, 19], previous studies in HIV+ individuals. Increased immunogenicity with PCV observed in other studies may be of limited duration, however, as the number of responders decline as early as 6 months postvaccination [16, 19]. The transient nature of antibody responses has also been a longstanding issue with PPV in HIV+ individuals [14]. Potential differences in duration of antibody responses in our study subjects are currently being assessed.

Immunological hyporesponsiveness to repeated vaccination is a potential concern [32]. The majority of our HIV+ participants were previously vaccinated with PPV, in contrast to our HIV− subjects. Although the number of individuals analyzed was limited, we found no differences in antibody responses between HIV+ participants who were vaccine naïve and those vaccinated with PPV ≥5 years prior, regardless of whether they received PPV or PCV/PPV. This finding is consistent with other revaccination studies [16, 20, 25, 33]. The diminished antibody responses in our HIV+ subjects are therefore unlikely due to prior vaccination.

Similar to previous findings, we observed increases in antibody responses after PCV that were not significantly enhanced by the subsequent dose of PPV (data not

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shown) [17, 18]. As an alternative to single doses or combinations of PCV and PPV, several studies have evaluated antibody responses in HIV+ individuals given consecutive doses of PCV [17, 20, 22, 33]. Additional doses of PCV after an initial dose appear to have limited impact on antibody responses. Several issues regarding this approach remain, including optimal dosing intervals, number of PCV boosters, and limited serotype coverage of PCV compared to PPV. Increased incidence of non-PCV serotypes continues to be a concern, particularly for high-risk populations [2, 34]. Thus, it remains unclear how PCV may be utilized in aging HIV+ individuals to improve antibody responses and protection against disease.

Vaccine responses were assessed in the current study using established immunological parameters. We found significant correlations between serotype-specific IgG concentrations and OPA titers post-PPV; however, we also observed OPA responses in individuals lacking a positive IgG response. Discrepancies between these assays have been reported in several adult populations including the elderly and immunocompromised. The OPA assay is generally regarded as a better measure of protection compared to antibody concentrations as it mimics the host phagocytic response [35]. Serological criteria for evaluation of pneumococcal vaccines in infants have been established, but correlates of protective pneumococcal immunity in adult populations are lacking [36]. Thus, it is possible that PCV/PPV elicited better protection in HIV+ subjects compared to PPV even though conventional assays did not indicate it, emphasizing that defined criteria to predict protection are urgently needed in adults.

Pneumococcal disease is preceded by asymptomatic nasopharyngeal carriage. PPV does not appear to affect pneumococcal colonization [37]. Several studies indicate

that conjugate vaccines reduce acquisition of vaccine-type carriage in vaccinated children, resulting in decreased transmission of vaccine-type serotypes to adults [37-39]. Similarly to children, PCV may directly reduce nasopharyngeal colonization in adult populations. Immunological parameters currently utilized in immunogenicity studies exclude any potential impact of PCV on mucosal defenses that could contribute to colonization and protective immunity. Increases in serotype-specific IgG or IgA concentrations have been detected in the lung fluid of HIV+ subjects and saliva of immunocompetent adults following conjugate vaccination [40, 41]. Presently, mucosal antibody levels and nasopharyngeal colonization are not routinely measured in immunogenicity studies or efficacy trials, but should be considered as possible measures of protection in addition to ELISA and OPA assays.

Clinical trials evaluating the efficacy of PPV in HIV+ adults have failed to demonstrate a clear reduction in pneumococcal disease [14, 15]. While one study demonstrated vaccine efficacy of 49% against IPD [42], another trial in Uganda reported possible detrimental effects [43]. The only trial among HIV+ adults examining the conjugate vaccine to date found 74% efficacy against recurrent vaccine-type IPD; however, protection was greatest within the first year only [44]. A 9-valent conjugate vaccine administered to HIV+ children also reduced vaccine-type IPD, but had no significant impact on pneumonia [45]. In elderly individuals, PPV reduced the risk of IPD with an estimated 55% efficacy, but its effectiveness in preventing nonbacteremic pneumonia is controversial [46, 47]. Recently, a large randomized trial conducted in the Netherlands examining the impact of PCV in older adults reported a vaccine efficacy of 75% for vaccine-type IPD and 45% for vaccine-type nonbacteremic community-acquired

pneumonia [48]. However, the study did not include comparison with PPV. Large scale efficacy trials evaluating PCV versus PPV in aging HIV+ individuals are unlikely. Lack of clear, direct clinical evidence that PCV or PPV provides protection against all vaccine-type pneumococcal disease in older and HIV+ adults further emphasizes the need for studies investigating immunological mechanisms responsible for increased risk and development of alternative vaccination approaches.

We recognize that our study has several limitations. Our sample size was small, limiting the power of the current study. Addition of HIV+ individuals 21-40 years old in our analysis to increase statistical power did not reveal any significant differences in functional antibody responses, supporting our overall conclusion of noninferiority between regimens (data not shown). Our study evaluated only 2 serotypes, and thus it is unknown what impact a priming dose of PCV may have on other serotypes. We selected serotypes 14 and 23F based on their differences in immunogenicity and inclusion in both PCV and PPV. In addition, we did not measure serum IgA concentrations, as IgA levels in respiratory mucosal tissues, and not serum, are likely to confer protection. It has been shown that serum IgA levels do not correlate with salivary IgA levels in adults immunized with conjugate vaccine [41].

In conclusion, we determined that PCV/PPV did not demonstrate a clear immunological advantage compared to PPV alone in older HIV+ individuals, and antibody responses to PCV/PPV were reduced compared to HIV− PCV/PPV controls. Development of effective vaccination strategies in the aging HIV+ population may be very challenging due to the presence of multiple comorbidities that increase risk of pneumococcal infection. Continued efforts aimed at defining correlates of vaccine

protection in adults and immunological mechanisms that reduce vaccine responses are critical.

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Table 2.1. Baseline characteristics of study participants.

	HIV-infected		HIV-uninfected
	PPV	PCV/PPV	PCV/PPV
Characteristic	(N = 22)	(N = 15)	(N = 14)
Demographic			
Mean age (range)	55.2 (50-64)	54.8 (49-63)	55.6 (50-64)
Male (%)	20 (90.9)	12 (80.0)	6 (42.9)
Race (%)			
Black	7 (31.8)	8 (53.3)	1 (7.1)
White	15 (68.2)	5 (33.3)	12 (85.7)
Other	0 (0.0)	2 (13.3)	1 (7.1)
Clinical History			
Prior PPV ≥5 years (%)	18 (81.8)	13 (86.7)	1 (7.1)
Receiving ART ≥1 year (%)	22 (100.0)	15 (100.0)	N/A
Nadir CD4$^+$ T cell count (cells/μl)			
>200 (%)	10 (45.5)	5 (33.3)	N/A
≤200 (%)	12 (54.5)	10 (66.7)	N/A
Laboratory Data at Enrollment			
HIV viral load (copies/ml)			
≤400 (%)	22 (100.0)	15 (100.0)	N/A
CD4$^+$ T cell count (cells/μl)			
Mean (range)	652 (230-1599)	717 (331-1298)	N/A

PCV/PPV groups received PCV followed by PPV 8 weeks later. Data are no. (%) of subjects, unless otherwise noted. Abbreviations: HIV, human immunodeficiency virus;

PPV, 23-valent pneumococcal polysaccharide vaccine; PCV, 13-valent pneumococcal conjugate vaccine.

Table 2.2. Geometric mean concentrations (GMC) and 95% confidence intervals (CI) of IgG (µg/ml) to indicated serotypes in aging HIV-infected and HIV-uninfected adults pre- and postvaccination.

	Serotype 14 GMC (95% CI)	Serotype 23F GMC (95% CI)
HIV-infected PPV		
Prevaccination	3.02 (1.56–5.85)	0.54 (0.27–1.06)
1 month post-PPV	5.25 (2.67–10.33)*	0.99 (0.47–2.10)*
HIV-infected PCV/PPV		
Prevaccination	0.52 (0.19–1.40)	0.29 (0.14–0.61)
1 month post-PPV	1.79 (0.51–6.33)*	1.86 (0.99–3.50)*
HIV-uninfected PCV/PPV		
Prevaccination	2.36 (1.06–5.24)	0.92 (0.56–1.53)
1 month post-PPV	11.14 (4.30–37.72)*, [#]	5.92 (2.94–11.94)*

PCV/PPV groups received PCV followed by PPV 8 weeks later. Abbreviations: IgG, immunoglobulin G; HIV, human immunodeficiency virus; PPV, 23-valent pneumococcal polysaccharide vaccine; PCV, 13-valent pneumococcal conjugate vaccine. * $P < 0.05$ compared to prevaccination level. [#] $P < 0.05$ compared to postvaccination HIV-infected PCV/PPV level.

Table 2.3. Geometric mean concentrations (GMC) and 95% confidence intervals (CI) of IgM (µg/ml) to indicated serotypes in aging HIV-infected and HIV-uninfected adults pre- and postvaccination.

	Serotype 14 GMC (95% CI)	Serotype 23F GMC (95% CI)
HIV-infected PPV		
Prevaccination	0.20 (0.12–0.32)	0.10 (0.06–0.16)
1 month post-PPV	0.26 (0.16–0.44)*	0.11 (0.06–0.19)[#]
HIV-infected PCV/PPV		
Prevaccination	0.28 (0.19–0.40)	0.12 (0.08–0.19)
1 month post-PPV	0.44 (0.25–0.77)	0.34 (0.23–0.49)*
HIV-uninfected PCV/PPV		
Prevaccination	0.49 (0.36–0.66)	0.26 (0.17–0.41)
1 month post-PPV	1.39 (0.81–2.38)*, [#]	0.49 (0.30–0.80)*

PCV/PPV groups received PCV followed by PPV 8 weeks later. Abbreviations: IgM, immunoglobulin M; HIV, human immunodeficiency virus; PPV, 23-valent pneumococcal polysaccharide vaccine; PCV, 13-valent pneumococcal conjugate vaccine. * $P <0.05$ compared to prevaccination level. [#] $P <0.05$ compared to postvaccination HIV-infected PCV/PPV level.

Table 2.4. Geometric mean of OPA titers (GMT) and 95% confidence intervals (CI) to indicated serotypes in aging HIV-infected and HIV-uninfected adults pre- and postvaccination.

	Serotype 14	Serotype 23F
	GMT (95% CI)	GMT (95% CI)
HIV-infected PPV		
Prevaccination	10.44 (3.72–29.32)	4.32 (3.06–6.10)
1 month post-PPV	977.97 (539.33–1773.37)*	124.17 (60.68–254.09)*
HIV-infected PCV/PPV		
Prevaccination	12.83 (3.84–42.83)	4.76 (2.75–8.25)
1 month post-PPV	585.64 (211.26–1623.41)*	111.26 (41.19–300.52)*
HIV-uninfected PCV/PPV		
Prevaccination	10.75 (3.05–37.82)	12.99 (5.11–33.00)
1 month post-PPV	1300.41 (381.69–4430.49)*	484.35 (256.70–913.87)*, #

PCV/PPV groups received PCV followed by PPV 8 weeks later. Abbreviations: OPA, opsonophagocytic killing assay; HIV, human immunodeficiency virus; PPV, 23-valent pneumococcal polysaccharide vaccine; PCV, 13-valent pneumococcal conjugate vaccine. * $P < 0.05$ compared to prevaccination titer. # $P < 0.05$ compared to postvaccination HIV-infected PCV/PPV titer.

Table 2.5. Number (percentage) of responders to the indicated number of serotypes after vaccination.

	HIV-infected PPV	HIV-infected PCV/PPV	HIV-uninfected PCV/PPV
IgG			
0	10 (45.5)	4 (26.7)	2 (14.3)
1	6 (27.3)	5 (33.3)	2 (14.3)
2	6 (27.3)	6 (40.0)	10 (71.4)
IgM			
0	20 (90.9)	12 (80.0)	8 (57.1)
1	2 (9.1)	3 (20.0)	5 (35.7)
2	0 (0)	0 (0)	1 (7.1)
OPA Titer			
0	2 (9.1)	1 (6.7)	0 (0)
1	3 (13.6)	4 (26.7)	1 (7.1)
2	17 (77.3)	10 (66.7)	13 (92.9)

PCV/PPV groups received PCV followed by PPV 8 weeks later. Responders were defined as ≥2-fold increase and ≥1 µg/ml in IgG (top panel) and IgM (middle panel) levels or ≥4-fold increase in OPA titers (bottom panel) 1 month postvaccination with PPV. Abbreviations: OPA, opsonophagocytic killing assay; HIV, human immuno-deficiency virus; PPV, 23-valent pneumococcal polysaccharide vaccine; PCV, 13-valent pneumococcal conjugate vaccine. $P \geq 0.05$ for all comparisons between groups.

Chapter 3

Alterations in Serotype-Specific B Cell Responses to the 13-Valent Pneumococcal Conjugate Vaccine in Aging HIV-Infected Adults*

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3.1. Introduction

The success of antiretroviral therapy (ART) has led to a substantial increase in life expectancy for HIV+ individuals [1]. As a result, the population of older HIV+ adults is rapidly growing. Approximately one-half of HIV+ individuals living in the United States are now ≥ 50 years old [2]. Aging and HIV infection both result in profound immunological changes due to chronic antigenic stress [2-5]. Similarities and differences, as well as the synergism, between these processes remain poorly understood.

Elderly and HIV+ individuals are at increased risk for pneumococcal infections due in part to humoral dysfunction. PPV immunization generates impaired antibody responses in both populations, and effectiveness in preventing pneumococcal disease is controversial [6-9]. The Advisory Committee on Immunization Practices (ACIP) currently recommends that HIV+ adults receive a single dose of PCV followed by a dose of PPV 8 weeks later (PCV/PPV) [10]. Superiority of PCV, however, is also questionable, as immunogenicity studies comparing single doses or combined regimens of PCV and PPV have yielded variable results [11-16].

Further investigation into the underlying cellular mechanisms responsible for these suboptimal responses is urgently needed. In humans, evidence suggests that both IgM ($CD19^+CD27^+IgM^+$) and switched ($CD19^+CD27^+IgM^-$) memory B cells generate antibodies to pneumococcal antigens [17]. Several studies have reported an association between reduced memory B cell subsets and impaired responses to pneumococcal vaccination in elderly and HIV+ individuals [18-21]. However, these analyses were performed on total rather than antigen-specific B cell populations.

We have previously characterized the phenotype of serotype-specific B cells responding to PPV in young adults, the elderly, and HIV+ [22-25]. The majority of serotype-specific B cells consist of IgM memory B cells in young, immunocompetent adults [22, 23]. Both elderly and HIV+ adults exhibit significant reductions in this subset that may contribute to decreased vaccine responsiveness [23-25]. However, unlike the elderly, HIV+ adults lack an increase in the proportion of serotype-specific switched memory B cells. Our data suggest that elderly and HIV+ individuals exhibit distinct perturbations in B cell subsets critical for protection against pneumococcal disease.

Older HIV+ individuals may possess a unique cellular response to vaccination reflecting the combined effects of aging and HIV infection. In the present study, we assessed the phenotype of serotype-specific B cells responding to the recommended PCV/PPV regimen compared to a single dose of PPV in HIV+ adults aged 50-65 years. We also sought to identify potential differences in surface expression of B cell receptors, including complement receptor CD21 and tumor necrosis factor superfamily receptors (TNFRs) CD40, TACI, and B cell-activating factor receptor (BAFF-R), on serotype-specific B cells that may contribute to vaccine responses.

3.2. Methods

Study design

Volunteers aged 50-65 years were recruited between April 2012 and January 2015 in this University of Toledo Institutional Review Board-approved study. Written, informed consent was obtained from all subjects. Exclusion criteria included: active infection (except HIV), PPV <5 years prior, pregnancy, immunosuppressive medications, and prior history of splenectomy or other immunocompromising conditions as defined by ACIP vaccination recommendations [10]. Volunteers were questioned about any past hospitalizations. Eligibility criteria for HIV+ participants were further defined as current CD4>200, HIV viral load ≤400 copies/ml, and ART for ≥1 year. HIV− controls received a single dose of PCV (Prevnar 13®; Wyeth Pharmaceuticals, Inc.) followed by one dose of PPV (Pneumovax 23®; Merck & Co., Inc.) 8 weeks later (PCV/PPV). HIV+ individuals received either PCV/PPV or a single dose of PPV.

Characterization of serotype-specific B cells

Blood samples were obtained from all participants on the day of each vaccination and 1 week postvaccination (post-PPV or post-PCV). Total lymphocyte counts were determined using complete blood counts (CBC) with differential. Peripheral blood mononuclear cells (PBMCs) were isolated and stained as previously described [22-25]. Fluorescently conjugated PPS or monoclonal antibodies (BD Biosciences or eBioscience) to the following antigens were used: PPS14-CB, PPS23F-DTAF, CD19 (APC-Cy7), CD27 (PerCP-Cy5.5), IgM (APC), BAFF-R (PE), TACI (PE), CD40 (PeCy7), and CD21 (BV421). Total and serotype-specific IgM memory B cells (CD19$^+$CD27$^+$IgM$^+$) and

switched memory B cells ($CD19^+CD27^+IgM^-$) were identified as previously described by our laboratory [22-25]. Total and serotype-specific B cells were also characterized for expression of CD21, CD40, BAFF-R, and TACI. Flow cytometric analysis was performed on a FACSAria with FACSDiva software (BD Biosciences) and data files were analyzed using FlowJo software (version 7.6.5, Tree Star).

Serotype-specific antibody responses

Quantitative and functional antibody responses to vaccination in subjects were assessed in the accompanying manuscript. Serotype-specific IgM and IgG serum levels and opsonophagocytic killing assay (OPA) titers were determined as previously described [22-25].

Statistical analysis

Participant characteristics are represented as median (interquartile range, IQR) for numerical values and number (percentage) for categorical values. Summary statistics for B cell percentages and counts are expressed as median (IQR). Absolute numbers of total CD19+ B cells (cells/µl) for each subject were calculated by multiplying the percentage as determined by flow cytometry with the lymphocyte count. Baseline comparisons between groups were analyzed by Kruskal Wallis analysis of variance (ANOVA) with Bonferroni correction. Comparisons of pre- to postvaccination serotype-specific B cell percentages were performed using the paired Wilcoxon signed-rank test. Postvaccination comparisons between groups (HIV+ PPV and HIV+ PCV/PPV or HIV+ PCV/PPV and HIV− PCV/PPV) were analyzed using pair-wise multiple comparison with Mann-

Whitney U test. Correlations between serotype-specific B cell subsets and post-PPV antibody responses were determined by Spearman's correlation coefficient. All statistical analyses were performed using the SAS software package (version 9.3; SAS Institute). *P* values <0.05 were considered significant.

3.3 Results

Subjects

Baseline characteristics are reported in Table 3.1. Differences in the distributions of sex and race in HIV− individuals compared to HIV+ subjects were noted. Clinical characteristics, including CD4 count at enrollment and use of ART, did not differ between HIV+ groups. A larger proportion of HIV+ participants had been immunized with PPV ≥5 years prior (85.3%) compared to HIV− (6.3%). Quantitative and qualitative antibody responses to pneumococcal vaccination in study subjects were assessed in the accompanying manuscript.

Baseline median frequencies of circulating total B cells and IgM memory and switched memory B cell subsets were assessed by flow cytometry (Table 3.1). Total B cell percentages and counts were significantly higher in the HIV+ PCV/PPV group compared to HIV+ PPV or HIV− PCV/PPV groups (P <0.006). IgM memory B cell percentages in HIV+ PPV and PCV/PPV groups were significantly reduced compared to the HIV− PCV/PPV group (P <0.0001). Switched memory B cell percentages were similar between groups.

Peripheral serotype-specific B cells are reduced in subjects vaccinated with PCV/PPV compared to PPV

Using fluorescently labeled PPS, we evaluated circulating serotype-specific B cell percentages in subjects pre- and 1 week post-PCV or -PPV (Table 3.2). Serotype-specific B cells were identified by flow cytometry as previously described [22-25]. No significant differences in prevaccination median serotype-specific B cell percentages

were observed between groups. Serotype-specific B cell percentages significantly increased post-PPV for both serotypes in the HIV+ PPV group ($P <0.0001$). In the HIV+ PCV/PPV group, significant increases in serotype-specific B cells were observed for serotype 23F only post-PCV ($P = 0.02$) and for both serotypes post-PPV ($P<0.01$) compared to prevaccination levels. In the HIV− group, serotype-specific B cells increased significantly post-PCV for serotype 23F only ($P = 0.03$) and post-PPV for both serotypes ($P <0.02$). No significant differences between post-PCV compared to post-PPV serotype-specific B cell percentages were observed within HIV+ or HIV− PCV/PPV groups.

Comparisons of postvaccination serotype-specific B cell percentages were evaluated between HIV+ PPV and PCV/PPV groups or HIV+ and HIV− PCV/PPV groups. Both post-PCV and post-PPV serotype-specific B cell percentages were significantly reduced in the HIV+ PCV/PPV group compared to the HIV+ PPV group post-PPV ($P <0.007$). In contrast, post-PCV and post-PPV serotype-specific B cell percentages were similar between HIV+ and HIV− PCV/PPV groups.

Peripheral serotype-specific memory B cell subset percentages are similar in HIV-infected and HIV-uninfected subjects

We compared the phenotypic distribution of serotype-specific B cells between HIV+ PPV and PCV/PPV groups and between HIV+ and HIV− PCV/PPV groups 1 week post-PPV (Figure 3-1). Serotype-specific B cells were subdivided into IgM memory ($CD19^+CD27^+IgM^+$) and switched memory ($CD19^+CD27^+IgM^-$) subsets as previously described [22-25]. No significant differences in median serotype-specific IgM and

switched memory B cell percentages were observed between HIV+ PPV and HIV+ PCV/PPV groups. Serotype-specific IgM and switched memory B cell percentages were also similar post-PPV in HIV+ and HIV– PCV/PPV groups. Serotype-specific IgM memory and switched memory B cell percentages were evenly distributed within study groups for both serotype 14 (HIV+ PPV, 27.3% and 33.3%; HIV+ PCV/PPV, 27.7% and 24.0%; HIV– PCV/PPV, 39.0% and 33.3%) and serotype 23F (HIV+ PPV, 37.1% and 35.8%; HIV+ PCV/PPV, 26.8% and 25.0%; HIV– PCV/PPV, 38.6% and 38.8%) post-PPV. A similar pattern was observed in serotype-specific B cell memory subsets post-PCV in HIV+ and HIV– PCV/PPV groups (data not shown).

There were significant correlations between post-PPV serotype-specific IgM memory B cell percentages and antibody responses in the HIV+ PPV group. PPS14-specific IgM memory B cells correlated with serotype 14 OPA titers ($r = 0.75$, $P = 0.0006$), and PPS23F-specific IgM memory B cells correlated with PPS23F-specific IgM levels ($r = 0.52$, $P = 0.02$). No significant correlations were observed in HIV+ or HIV– PCV/PPV groups between post-PPV or post-PCV serotype-specific memory B cells and antibody responses.

Serotype-specific TACI$^+$ B cell percentages are reduced in subjects after PCV/PPV compared to PPV

We evaluated surface expression of complement receptor CD21 and TNFRs CD40, BAFF-R, and TACI on total B cells at baseline in all study groups. Median percentages of total BAFF-R$^+$, CD21$^+$, and CD40$^+$ B cells were similar between study groups (data not shown). Total TACI$^+$ B cells percentages were also similar between

HIV+ groups. However, TACI$^+$ B cell percentages were decreased in HIV+ compared to HIV− PCV/PPV groups ($P = 0.003$, data not shown).

We then compared the expression of these receptors on serotype-specific B cells between HIV+ PPV and PCV/PPV groups and between HIV+ and HIV− PCV/PPV groups 1 week post-PPV (Figure 3-2). Median percentages of PPS23F-specific CD21$^+$, CD40$^+$, and BAFF-R$^+$ B cells post-PPV were similar between HIV+ PPV and HIV+ PCV/PPV groups. In contrast, PPS23F-specific TACI$^+$ B cell percentages were significantly higher in HIV+ PPV compared to HIV+ PCV/PPV groups ($P = 0.03$). PPS23F-specific TACI$^+$ B cell percentages were similar in HIV+ compared to HIV− PCV/PPV groups. No significant differences in PPS23F-specific BAFF-R$^+$ and CD40$^+$ B cell percentages were observed between HIV+ and HIV− PCV/PPV groups. PPS23F-specific CD21$^+$ B cell percentages were significantly reduced in HIV+ compared to HIV− PCV/PPV groups ($P = 0.02$). No significant differences in these receptors were observed on post-PCV PPS23F-specific B cells in HIV+ compared to HIV− PCV/PPV groups (data not shown).

3.4 Discussion

The B cell subsets responsible for humoral responses to PPS and their dysregulation in high risk populations remain poorly understood. Elderly and HIV+ subjects in our prior studies exhibited distinct perturbations in serotype-specific memory B cell subsets compared to young, immunocompetent adults [22-25]. In the current study, we assessed the combined impact of aging and HIV infection on serotype-specific B cells responding to the recommended PCV/PPV regimen compared to a single dose of PPV in HIV+ adults 50-65 years old.

We identified serotype-specific B cells using fluorescently conjugated PPS as previously described by our laboratory [22-25]. Serotype-specific B cell percentages post-PPV were significantly reduced in the HIV+ PCV/PPV group compared to the HIV+ PPV group, suggesting that a priming dose of PCV limits the frequency of circulating responding B cells after a subsequent dose of PPV. In contrast, vaccination with PPV or PCV/PPV in HIV+ subjects resulted in similar increases in antibody responses (accompanying manuscript). No significant differences in serotype-specific B cell percentages were observed between HIV+ and HIV− PCV+ PPV groups. However, post-PPV IgG levels and OPA titers were significantly reduced in the HIV+ PCV/PPV group, further emphasizing a discrepancy between serotype-specific cellular and antibody responses. We assessed peripheral blood samples at 1 week post-PCV or -PPV when the frequency of antigen-specific B cells is highest after vaccination [22, 23, 26]. Analysis of circulating B cell populations excludes other B cell compartments, such as lymph node or spleen, where antigen-specific B cells may be located. PPS contained in PPV, due to their repetitive nature, activate B cells without direct T cell interactions and promote

extrafollicular proliferation. Conjugation of PPS to an immunogenic carrier protein, as in PCV, is thought to preferentially drive antigen-specific B cells towards germinal center (GC) responses [27]. Thus, assessment of circulating serotype-specific B cells in HIV+ and HIV– PCV/PPV groups may not accurately represent the total population of responding B cells.

We characterized serotype-specific B cells memory B cell subsets to determine whether distinct phenotypic alterations occur in aging HIV+ individuals, reflecting synergism between immunosenescence and HIV infection. IgM and switched memory B cell subsets each accounted for approximately one-third of serotype-specific B cells in the HIV+ PPV group. This phenotype distribution was similar to that observed in ART-treated and –untreated HIV+ individuals who were not stratified according to age [24, 25]. In contrast, serotype-specific B cells in elderly individuals are predominantly switched memory B cells post-PPV [23]. Whether older HIV+ individuals will exhibit an increase in the proportion of serotype-specific switched memory B cells as they continue to age, like the elderly, remains to be investigated. However, this is questionable as some studies have observed an HIV-associated reduction in the proportion or function of switched memory B cells [19, 20, 28]. Our findings suggest that HIV infection is the predominant factor in determining the phenotype of B cells responding to PPV in aging HIV+ individuals, although its impact on serotype-specific B cells with advanced age remains unclear.

The phenotype distribution was similar in HIV+ subjects regardless of whether they received PPV or PCV/PPV, suggesting that both vaccines similarly influence circulating serotype-specific memory B cell subsets. Significant variations in serotype-

specific antibody titers and antibody-secreting cells still occur with conjugate vaccines [29] despite induction of strong carrier-protein specific memory T cell responses in HIV+ and HIV– adults [29, 30]. Thus, the phenotype of serotype-specific B cells generated in response to either PCV or PPV may be influenced by the existing B cell repertoire more so than the presence of T cell responses. In addition, no significant differences were observed in serotype-specific IgM and switched memory B cell percentages between HIV+ and HIV– PCV/PPV groups post-PCV or -PPV. In HIV– subjects, this pattern likely reflects a transition occurring from predominantly serotype-specific IgM memory B cells to switched memory B cells with advanced age [23]. Further investigation is needed to determine if alternative vaccination regimens including multiple doses of PCV will have the same impact on circulating serotype-specific B cells subsets.

Other studies have also assessed B cell populations after conjugate vaccination in HIV+ or elderly subjects [31-33]. Reduced frequencies of circulating serotype-specific B cells in PCV/PPV participants observed in our study contrasts the findings of Baxendale et al. [32] and Clutterbuck et al. [33] who reported no change or increases in antigen-specific B cell populations with PCV compared to PPV, respectively. Marked differences in study design and time points, methodologies, vaccination regimens, and subjects render these studies incomparable. However, collectively these findings suggest memory B cell subsets are important for humoral responses to pneumococcal vaccination.

Evidence suggests IgM memory B cells in humans are critical for defense against pneumococcal infection [34]. We have previously observed significant correlations between serotype-specific IgM memory B cells and OPA titers in both HIV– and ART-treated HIV+ adults post-PPV [22, 24]. In the current study, post-PPV PPS14-specific

IgM memory B cell percentages also significantly correlated with serotype 14 OPA titers in the HIV+ PPV group. In contrast, serotype-specific IgM memory B cells poorly correlated with post-PPV antibody responses in the HIV+ PCV/PPV group. The lack of correlation may be associated with the lower frequency of circulating serotype-specific B cells post-PCV and post-PPV, as serotype-specific B cells in compartments other than the peripheral blood may better correlate with antibody responses. Postvaccination serotype-specific memory B cell subsets also poorly correlated with antibody responses in the HIV– PCV/PPV group. It remains to be determined whether testing total or serotype-specific memory B cell populations in high risk individuals, such as the elderly or HIV+, can be used to predict vaccine responses. Our findings suggest that associations between post-PPV serotype-specific B cells and antibody responses may be difficult to determine after a priming dose of PCV.

Complement receptor CD21 and TNFRs, including CD40, BAFF-R, and TACI, are critical in the generation of antibody responses to T cell-dependent (TD) and/or –independent (TI) antigens. BAFF-R and TACI are innate mediators of B cell differentiation, activation, and class switching [35]. We evaluated whether a priming dose of PCV alters surface expression of these receptors on PPS23F-specific B cells post-PPV. No significant differences in PPS23F-specific CD21$^+$, CD40$^+$, or BAFF-R$^+$ B cell percentages were observed between HIV+ PPV or PCV/PPV groups. In contrast, PPS23F-specific TACI$^+$ B cells percentages post-PPV were significantly higher in HIV+ PPV compared to HIV+ PCV/PPV groups, suggesting that a priming dose of PCV influences TACI expression after subsequent vaccination with PPV. Moreover, no

significant differences were observed in TACI[+] PPS23F-specific B cells percentages post-PPV in HIV+ and HIV− PCV/PPV groups.

TACI is essential for humoral responses to TI antigens including PPS [35, 36]. TACI-deficient mice are unable to produce antibodies in response to TI antigen while TD responses remain intact [36, 37]. Individuals at high risk for pneumococcal disease, including infants and subgroups of common variable immunodeficiency (CVID) patients, exhibit decreased antibody secretion due to reduced [38] or mutated [39] TACI expression. Higher percentages of PPS23F-specific TACI^{+} B cells post-PPV observed in the HIV+ PPV group further support a role for TACI in TI responses. TACI is also thought to be a negative regulator of CD40-mediated antibody production [35, 40]. We observed a significant, negative correlation between TACI^{+} and CD40^{+} PPS23F-specific B cells in the HIV+ PPV group (r = -0.63, P = 0.04), but not PCV/PPV group (r = -0.17, P = 0.54). To our knowledge, this is the first description of TNFRs on serotype-specific B cells responding to pneumococcal vaccination in humans and the distinct impact of PCV priming on TACI expression. The molecular mechanisms responsible for differential expression remain to be investigated. Alternatively, our findings may represent PPS-induced hyporesponsiveness to PPV and not a direct effect of prior PCV. However, this is unlikely as PPS23F-specific TACI[+] B cell percentages post-PCV were similar to post-PPV in both HIV+ and HIV− PCV/PPV groups (data not shown).

CD21 provides a critical secondary signal for B cell activation in response to TI antigens, and reduced expression is associated with impaired antibody responses to PPS [41, 42]. HIV viremia results in expansion of abnormal peripheral CD21^{lo} B cells associated with exhaustion and increased cell turnover [43, 44]. Control of viremia with

ART appears to significantly reduce these populations [45]. Consistent with this finding, no difference was observed in CD21 expression on total B cells between HIV+ and HIV– PCV/PPV groups. However, PPS23F-specific CD21$^+$ B cell percentages post-PPV were significantly reduced in HIV+ compared to HIV– PCV/PPV groups. Reduced expression of CD21 on serotype-specific B cells may contribute to the diminished antibody responses generated in our HIV+ subjects (accompanying manuscript).

We recognize that the current study has limitations. Although our sample size was limited, we observed distinct differences in serotype-specific B cell responses to PPV compared to PCV/PPV. It is unclear whether PCV priming has a similar impact on other vaccine serotypes. In addition, we were unable to evaluate TNFR expression on serotype-specific IgM and switched memory B cell subpopulations due to the low frequency of cells available for analysis. It thus remains to be determined whether TNFR expression is differentially regulated on serotype-specific B cell subpopulations.

Collectively, our findings indicate that PCV priming alters the overall frequency and surface TACI expression of serotype-specific B cells post-PPV. It remains unclear whether the alterations due to PCV observed in the current study confer any immunological advantage, as postvaccination antibody responses in HIV+ PPV and PCV/PPV groups were similar (accompanying manuscript). In contrast, PCV had no impact on the phenotypic distribution of serotype-specific B cells. In aging HIV+ individuals, HIV infection appears to be the predominant factor in determining the phenotype of serotype-specific B cells, while in aged-matched, HIV– controls, age is the principal factor. Analysis of serotype-specific B cell populations in young HIV+ and

elderly HIV– subjects vaccinated with PPV or PCV/PPV will provide further insight into the impact of immunosenescence and HIV infection on humoral immunity.

Our study emphasizes the need for continued investigation into signaling mechanisms that regulate B cell responses. Utilization of TI and/or TD stimuli to enhance B cell responses may be the key to improving pneumococcal vaccination strategies in high risk adult populations, such as the aging HIV+, where vaccine responses are suboptimal. Evidence from murine studies indicates that Toll-like receptors (TLRs) can serve as effective adjuvants to both TI and TD components of encapsulated bacteria [35, 36]. In support of this concept, addition of a TLR9 agonist to the 7-valent conjugate vaccine increased the proportion of HIV+ patients with high antibody responses [46].

3.5 References

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Table 3.1. Baseline characteristics.

Characteristic	HIV-infected		HIV-uninfected
	PPV	PCV/PPV	PCV/PPV
	(n = 19)	(n = 15)	(n = 14)
Demographic			
Median age (IQR)	55.0 (51-59)	54.0 (52-58)	55.5 (52-58)
Male (%)	17 (89.5)	12 (80.0)	6 (42.9)
Race (%)			
Black	7 (36.8)	8 (53.3)	1 (7.1)
White	12 (63.2)	5 (33.3)	12 (85.7)
Other	0 (0.0)	2 (13.3)	1 (7.1)
Clinical History			
Prior PPV $\geq$5 years (%)	16 (84.2)	13 (86.7)	1 (7.1)
Receiving ART $\geq$1 year (%)	19 (100.0)	15 (100.0)	N/A
Nadir CD4$^+$ T cell count			
>200 (%)	9 (47.4)	5 (33.3)	N/A
$\leq$200 (%)	10 (52.6)	10 (66.7)	N/A
Immunological parameters			
HIV viral load (copies/ml)			
$\leq$400 (%)	19 (100.0)	15 (100.0)	N/A
CD4$^+$ T cells (cells/μl)			
Median (IQR)	525 (336-976)	576 (503-990)	N/A
CD19$^+$ B cells (cells/μl)			
Median (IQR)	165 (76-242)*	297 (216-382)	137 (85-185)*
CD19$^+$ B cells (%)			
Median (IQR)	7.0 (5.3-13.3)*	12.7 (10.0-16.9)	7.9 (5.5-10.5)*
CD19$^+$ IgM Memory B cells (%)			

|			
Median (IQR)	5.4 (3.9-8.3) [#]	5.4 (2.2-9.8) [#]	10.3 (6.9-14.7)
CD19$^+$ Switched Memory B cells (%)			
Median (IQR)	12.1 (5.8-21.5)	15.3 (9.6-20.8)	11.8 (9.0-14.6)

PCV/PPV groups received PCV followed by PPV 8 weeks later. Data are no. (%) of subjects, unless otherwise noted. Abbreviations: HIV, human immunodeficiency virus; PPV, 23-valent pneumococcal polysaccharide vaccine; PCV, 13-valent pneumococcal conjugate vaccine; IQR, interquartile range. * P <0.05 compared to HIV-infected PCV/PPV. [#] P <0.05 compared to HIV-uninfected PCV/PPV.

Table 3.2. Pneumococcal polysaccharide-specific CD19+ B cell percentages to the indicated serotypes in HIV-infected and HIV-uninfected subjects.

	Serotype 14 Median (IQR)	Serotype 23F Median (IQR)
HIV-infected PPV (%)		
Prevaccination	0.80 (0.45-1.30)	0.80 (0.60-1.00)
1 week post-PPV	2.80 (1.50-3.95)*	2.80 (1.50-3.90)*
HIV-infected PCV/PPV (%)		
Prevaccination PCV	0.70 (0.60-0.90)	0.60 (0.40-0.70)
1 week post-PCV	0.70 (0.60-1.20) [#]	0.70 (0.60-1.00)*, [#]
Prevaccination PPV	0.70 (0.50-0.90)	0.60 (0.30-0.80)
1 week post-PPV	1.10 (0.90-1.50)*, [#]	1.10 (0.70-1.40)*, [#]
HIV-uninfected PCV/PPV (%)		
Prevaccination PCV	0.90 (0.75-1.35)	0.70 (0.50-0.90)
1 week post-PCV	1.90 (0.75-2.30)	1.00 (0.60-1.13)*
Prevaccination PPV	0.70 (0.60-1.00)	0.70 (0.50-0.90)
1 week post-PPV	1.70 (1.25-2.00)*	1.10 (0.70-1.60)*

PCV/PPV groups received PCV followed by PPV 8 weeks later. Abbreviations: HIV, human immunodeficiency virus; PPV, 23-valent pneumococcal polysaccharide vaccine; PCV, 13-valent pneumococcal conjugate vaccine; IQR, interquartile range. * $P <0.05$ compared to prevaccination level within group. [#] $P <0.05$ compared to postvaccination HIV-infected PPV level between groups.
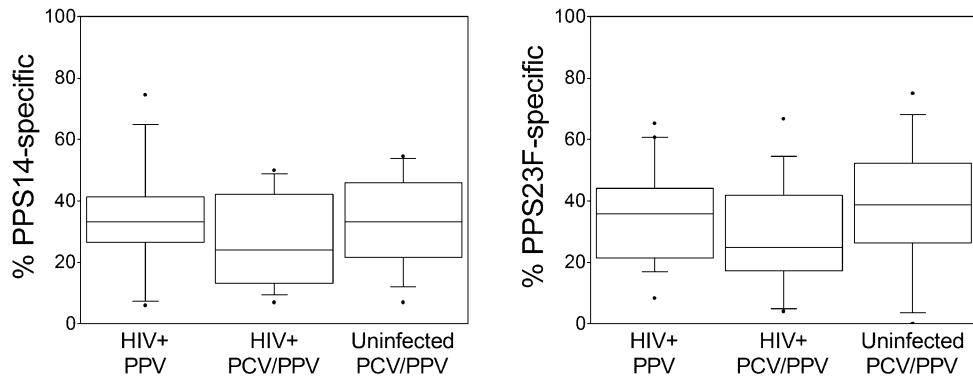
IgM Memory B cells
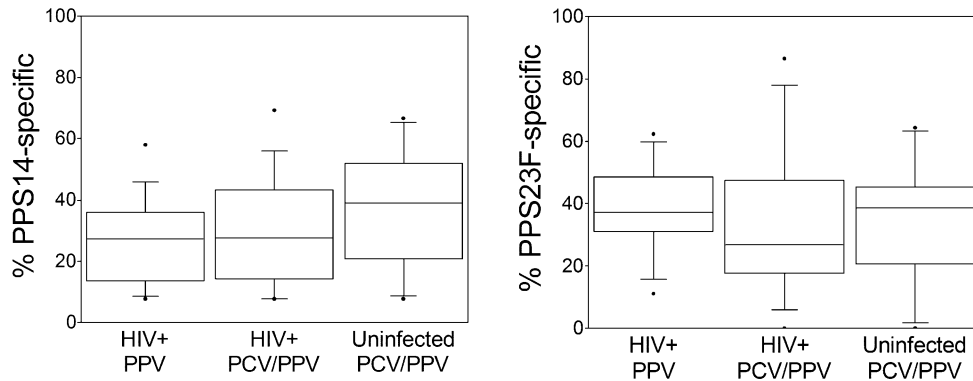
Switched Memory B cells

Figure 3-1. Pneumococcal polysaccharide-specific memory CD19$^+$ B cell subset percentages to the indicated serotypes in HIV-infected and HIV-uninfected subjects. Percentages of serotype-specific IgM memory (CD19$^+$CD27$^+$IgM$^+$) and switched memory (CD19$^+$CD27$^+$IgM$^-$) B cell subsets measured by flow cytometry in HIV-infected PPV (PPS14, n=17; PPS23F, n=19), HIV-infected PCV/PPV (PPS14 and PPS23F, n=15), and HIV-uninfected PCV/PPV (PPS14, n=13; PPS23F, n=14) groups. PCV/PPV groups received PCV followed by PPV 8 weeks later. Graphs represent percentages 1 week after vaccination with PPV. Left panels represent PPS14-specific B cell percentages and right panels represent PPS23F-specific B cell percentages. Box plots include median (horizontal black line) with 25th and 75th percentile borders and error bars indicate 10th and 90th percentiles. Abbreviations: PPS, pneumococcal polysaccharide; HIV, human immunodeficiency virus; PPV, 23-valent pneumococcal polysaccharide vaccine; PCV, 13-valent pneumococcal conjugate vaccine.
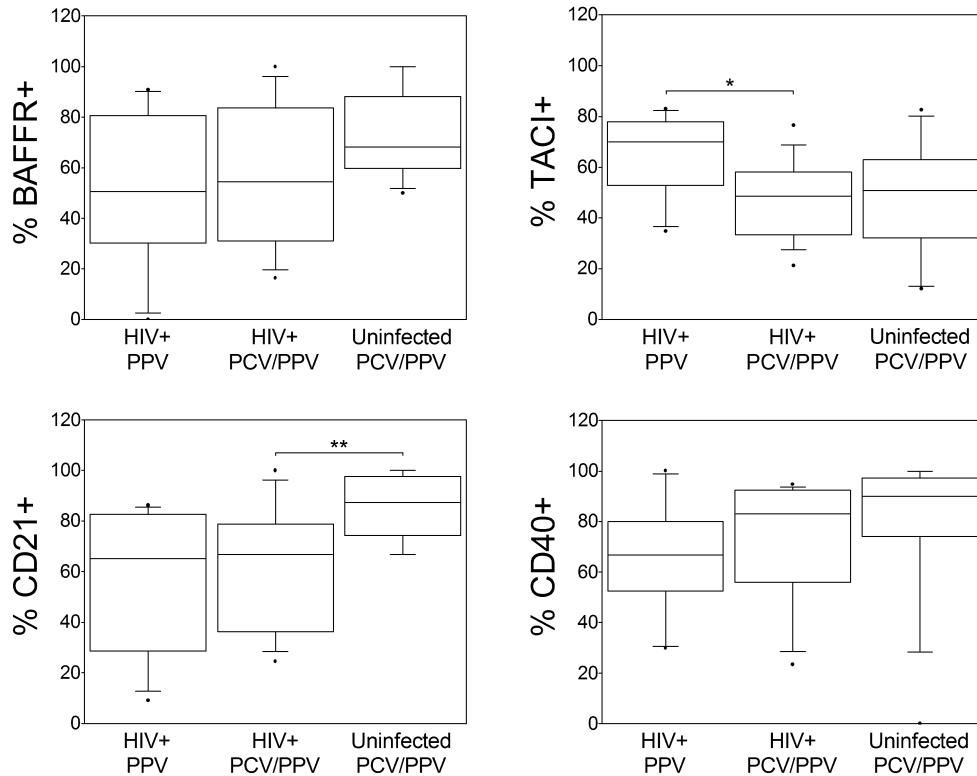
PPS23F-specific B cells

Figure 3-2. Surface expression of B cell receptors on pneumococcal polysaccharide-specific CD19^{+} B cells to the indicated serotype in HIV-infected and HIV-uninfected subjects. Percentages of BAFF-R, TACI, CD21, and CD40 positive PPS23F-specific CD19^{+} B cells postvaccination with PPV. Surface expression of receptors were measured by flow cytometry in HIV+ PPV (n=11), HIV+ PCV/PPV (n=15), and HIV–PCV/PPV (n=16) subjects. Box plots include median (horizontal black line) with 25th and 75th percentile borders and error bars indicate 10th and 90th percentiles. Abbreviations: PPS, pneumococcal polysaccharide; HIV, human immunodeficiency virus; PPV, 23-valent pneumococcal polysaccharide vaccine; PCV, 13-valent pneumococcal conjugate vaccine. *P <0.05, **P <0.01.

Chapter 4

Summary of Findings and Future Directions

HIV+ individuals are at increased risk for *S. pneumoniae* infections due to humoral dysfunction caused by viremia. Pneumococcal disease remains a significant cause of morbidity and mortality in this population; therefore, vaccination is recommended. PPV immunization in HIV+ adults results in antibody responses of reduced magnitude and duration compared to uninfected controls, and effectiveness in preventing pneumococcal disease remains a controversial subject [1-6]. The ACIP currently recommends that HIV+ adults receive a single dose of PCV followed by one dose of PPV 8 weeks later (PCV/PPV) [7]. However, immunogenicity studies evaluating single or combined regimens of PCV and PPV have thus far failed to demonstrate a clear advantage of using PCV over PPV alone [8-14]. The impact of several factors that limit efficacy, such as timing of vaccination, dosing schedule, degree of immunocompromise, and influence of other risk factors on vaccine responses, remain poorly understood. Advanced age is a significant risk factor for IPD in the HIV+ population [15]. As previously discussed, the aging HIV+ population is likely to be at higher risk for pneumococcal disease due to the presence of multiple factors that increase susceptibility. The goal of the current study was to assess humoral and cellular responses to

pneumococcal vaccination in aging HIV+ individuals 50-65 years old on ART ≥1 year with a current CD4 >200 to determine whether the recommended PCV/PPV regimen offers any immunological benefit to PPV alone.

We assessed quantitative and qualitative antibody responses to pneumococcal serotypes 14 and 23F in our aging HIV+ subjects using surrogate markers of protection. In PCV/PPV and PPV groups, significant increases in serotype-specific IgG serum levels and OPA titers were observed for both serotypes pre- to postvaccination. Serotype-specific IgM levels were significantly higher postvaccination compared to prevaccination for one serotype only in both groups. However, no significant differences were observed between PCV/PPV and PPV groups postvaccination. Our findings indicate that PCV/PPV offers no clear improvement in the magnitude or quality of antibody responses compared to PPV in aging HIV+ individuals, and that alternative strategies are needed to increase vaccine responses. We are currently investigating whether these vaccination regimens differ in regards to the duration of antibody responses. In addition, we are expanding the scope of the present study to other populations, including younger HIV+ and those with CD4 <200. It is possible that PCV/PPV may enhance immunogenicity in certain subsets of HIV+ patients.

Antibody responses generated in response to PCV/PPV were reduced in our HIV+ subjects compared to age-matched, HIV− controls despite higher CD4 counts and use of ART. Previous studies have shown that ART does not fully restore immunological defects caused by HIV infection, and ART-treated patients continue to exhibit impaired responses to pneumococcal vaccination [5, 16-20]. A major limitation of immuno-genicity studies conducted in adults is a lack of defined correlates of protective immunity.

Thus, although HIV+ subjects exhibited significantly higher antibody levels and functional titers from pre- to postvaccination, it is unclear whether they achieved adequate levels of protection. Studies are urgently needed to determine threshold concentrations/titers or alternative immunological parameters in adults that can be used to predict protection, as large scale efficacy studies comparing PCV to PPV are unlikely. Without well-defined correlates, clinical decisions regarding vaccine recommendations, such as whether to include conjugate vaccines and determination of optimal dosing intervals, will become increasingly difficult based on immunogenicity data.

In contrast to antibody responses, we observed significant differences in B cell responses between PCV/PPV and PPV in our aging HIV+ subjects. Serotype-specific B cell percentages were significantly reduced postvaccination with PCV/PPV compared to PPV in HIV+ individuals. No significant differences were observed between HIV+ and HIV− subjects vaccinated with PCV/PPV, suggesting that a priming dose of PCV limits the frequency of circulating serotype-specific B cells regardless of HIV infection. Analysis of the peripheral blood does not take into account other tissues where antigen-specific B cells could be located after vaccination, such as the spleen or lymph nodes. Due to the TD nature of PCV, it is thought that responding B cells may form GCs [21, 22]. Thus, assessment of peripheral blood samples postvaccination with PCV/PPV may not accurately reflect the total population of responding B cells. It remains to be determined whether pneumococcal vaccines differentially activate antigen-specific B cells in spleen and lymph node tissue in humans.

PCV had no significant impact on the phenotypic distribution of serotype-specific memory B cells in aging HIV+ subjects. In both PCV/PPV and PPV groups, IgM and

switched memory subsets each accounted for approximately one-third of serotype-specific B cells postvaccination. This phenotype distribution is similar to that observed in ART-treated and –untreated HIV+ individuals postvaccination with PPV [5, 6]. Our findings suggest that PCV and PPV similarly influence the existing B cell repertoire, and HIV infection is the primary factor in determining the phenotype of responding B cells in aging HIV+ individuals. There were no significant differences in IgM and switched memory serotype-specific B cell percentages between HIV+ and HIV– individuals who received PCV/PPV. For our controls, age is most likely the predominant factor responsible for this phenotypic shift. Continued effort is needed to define the impact of immunosenescence and HIV infection on cellular responses to pneumococcal vaccination. We are currently investigating whether younger HIV+ individuals and those with CD4 <200 are similarly impacted by PCV/PPV and PPV. It also remains to be determined if HIV+ individuals ≥65 years of age will exhibit an increase in the proportion of serotype-specific switched memory B cells similar to uninfected elderly subjects [23].

We evaluated whether a priming dose of PCV alters the surface expression of several receptors, including CD21, CD40, BAFF-R, and TACI, on serotype-specific B cells that may influence vaccine responses. No significant differences were observed in CD21, CD40, and BAFF-R expression between aging HIV+ subjects vaccinated with PCV/PPV and PPV. In contrast, serotype-specific TACI$^+$ B cells were significantly reduced postvaccination with PCV/PPV compared to PPV. Several studies suggest that TACI is essential for humoral responses to TI antigens [24, 25] and differentiation of human B cells into plasmablasts [26]. Our findings support a role for TACI in TI

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responses in humans, as serotype-specific TACI[+] B cell percentages were significantly higher in the PPV group. However, it remains to be determined whether differential regulation of TACI by PCV on serotype-specific B cells confers any immunological advantage or disadvantage, as PCV/PPV and PPV elicited antibody responses of similar magnitudes. Decreased TACI expression on B cells was associated with HIV+ individuals who were nonresponders to influenza immunization [27]. Future studies should therefore assess whether use of adjuvant can increase TACI expression on serotype-specific B cells postvaccination with PCV or PPV to enhance vaccine responses.

There were no significant differences in CD40, BAFF-R, and TACI expression on serotype-specific B cells between HIV+ and HIV− individuals postvaccination with PCV/PPV, indicating that HIV infection does influence these receptors during responses to pneumococcal vaccines. However, serotype-specific CD21^{+} B cell percentages were significantly reduced in HIV+ compared to HIV− subjects. As previously mentioned, CD21 is thought to be an important secondary signal for B cell activation in response to TI antigens such as PPS [28, 29]. Decreased expression of CD21 on serotype-specific B cells may therefore contribute to the diminished antibody responses observed in our aging HIV+ subjects compared to controls. Further investigation is needed to assess the impact of CD21 expression on responses to pneumococcal vaccination in the context of HIV infection.

Collectively, our findings indicate that PCV/PPV alters the responding B cell population in aging HIV+ individuals compared to PPV. However, these changes appear to have no significant impact on antibody responses as measured using surrogate markers of protection. The combined PCV/PPV regimen as currently recommended therefore

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offers no clear clinical benefit compared to PPV alone. Due to the presence of multiple risk factors that increase susceptibility to infection, development of effective vaccination strategies in the aging HIV+ population may be challenging. It remains to be determined how PCV may be utilized to effectively enhance antibody responses and protective immunity in this high risk population. While PCV has been remarkably effective in children, it has not achieved the same success in adults, indicating that the immunological defects that increase susceptibility to *S. pneumoniae* infections are not the same. Our study emphasizes the need for future studies evaluating alternative vaccination strategies in adult high risk populations. Continued investigation regarding the immunological mechanisms that govern humoral and cellular responses to pneumococcal vaccines, and how immunosenescence and/or HIV infection influences these processes, is necessary. Towards this goal, we are currently assessing serotype-specific B cell populations in other subsets of HIV+ patients to further delineate the impact of immunosenescence and HIV infection on vaccine responses.

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Chapter 1

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Chapter 3

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