Humoral Immunity to the Opportunistic Pathogen, *Pneumocystis*, in a Simian Model of HIV Infection

by

Heather M. Kling

B.S., Biology, University of Georgia, 2002

Submitted to the Graduate Faculty of

the School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy in Molecular Virology and Microbiology

University of Pittsburgh

2010

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Heather M. Kling

It was defended on

April 8, 2010

and approved by

Lisa Borghesi, Ph.D., Associate Professor, Department of Immunology

Jay K. Kolls, M.D., Professor, Departments of Pediatrics and Immunology

Ronald C. Montelaro, Ph.D., Professor, Department of Microbiology and Molecular Genetics

Kelly Stefano-Cole, Ph.D., Associate Professor, Department of Immunology

Dissertation Advisor and Committee Chair:

Karen A. Norris, Ph.D., Professor, Department of Immunology

Humoral Immunity to the Opportunistic Pathogen, *Pneumocystis*, in a Simian Model of HIV Infection

Heather M. Kling, PhD

University of Pittsburgh, 2010

The fungal opportunistic pathogen, *Pneumocystis jirovecii* (formerly *Pneumocystis carinii* f. sp. *hominis*) (Pc) is the causative agent of *Pneumocystis* Pneumonia (PcP) in immunocompromised persons. Despite improvements in anti-retroviral treatments and Pc prophylaxis, Pc remains an important pathogen in immunocompromised populations. Pc colonization, the presence of Pc in subjects without clinical signs or symptoms of PcP, is common in HIV+ subjects; however, the clinical consequences of colonization are undefined. The non-human primate model of Pc infection in simian immunodeficiency virus (SIV)- or chimeric simian-human immunodeficiency virus (SHIV)-infected macaques has been developed to study Pc colonization pathogenesis in the context of AIDS immunosuppression.

Using this model, immunologic parameters associated with natural Pc colonization of macaques were evaluated to gain understanding of protective immune responses to Pc. Humoral immunity to the recombinant Pc-antigen, kexin (KEX1), correlated with protection from subsequent Pc colonization, despite declining CD4+ T cells. Furthermore, macaques that remained Pc-negative were protected against lung injury observed in macaques that became Pc-colonized, supporting a role for Pc in pulmonary obstruction development. These experiments suggest KEX1-specific antibodies may provide protection of immunocompromised individuals from developing obstructive pulmonary disease.

Because B cell deficits and dysfunctions are reported in HIV+ subjects, we examined peripheral blood B cell populations in SHIV-infected macaques. We report declines in total (CD20+), memory (CD20+CD27+) and IgM+ memory B cell numbers, increased percentages of activated (CD95+) B cells, and hypergammaglobulinemia in SHIV-infected macaques, similar to what has been reported for HIV+ patients, suggesting the relevance of this model for studying HIV-related B cell dysfunctions. Pc colonization status did not correlate with deficits in total B cell populations. Rather, protection from Pc-colonization appears associated with a KEX1-specific memory B cell pool, despite early loss of total CD27+ B cells. These results suggest exposure to Pc prior to immunosuppression, resulting in high levels of circulating antibodies/plasma cells, contributes to maintenance of a Pc-specific memory B cell pool following immunosuppression.

These results demonstrate importance of a Pc-specific humoral response in protection from Pc colonization and pulmonary damage, thereby providing a rationale for Pc-KEX1 vaccine development to protect at-risk populations against this opportunistic pathogen.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

Many individuals have contributed to my personal and professional growth over the course of my graduate training. I would like to thank my mentor, Karen Norris, first and foremost for giving me the opportunity to work on such a multi-faceted and challenging, yet rewarding project. She has provided me with the guidance and support necessary for successful completion of my thesis work. I have learned a great deal working in her laboratory, and I am grateful for everything she has taught me.

I would also like to thank the other members of my thesis committee, Lisa Borghesi, Jay Kolls, Ron Montelaro, and Kelly Stefano-Cole, for their insights, critiques, and assistance in strengthening and improving my thesis work. Members of the Norris lab, both past and present, have also aided me in my graduate studies. I would like to thank especially, Sangita Patil, for her instrumental role in training me on the *Pneumocystis* project and for all of her guidance. She was always a patient teacher, and I am grateful for all of her assistance. Tim Shipley is another member of the Norris lab who deserves special acknowledgment. We have worked together on the *Pneumocystis* project for nearly the entirety of our graduate training. This is a challenging project, and it was comforting and helpful having someone with Tim's experience with whom to work. I am also grateful for his sense of humor and friendship. I would like to thank also Jan Kristoff, for the technical support with which she provided us for several years on the *Pneumocystis* project. Thank you as well to Siobhan Guyach, for excellent technical assistance,

and Margie Beucher, for guiding me through my first laboratory experience at the University of Pittsburgh, and to both of them for their friendship. Special acknowledgement also goes to Eustace Fernandes, who made very important contributions through his knowledge of pulmonology, and who assisted the lab a great deal in initial set up of our plethysmograph. I am also grateful to previous lab technicians and post-doctoral researchers, including Lynnette Robinson, Jennifer Roguskie, Ruth Saxl, Wendell Meira, Mahesh Netravali, Marianne Bryan, and Xiuping Shao, for all of their assistance and advice, but also for great conversations and for their friendship. I would like to thank our excellent veterinary staff, including former and current veterinarians, Anita Trichel and Chris Janssen, and our veterinary technician, Nicole Banichar, for their excellent veterinary care, and without whose assistance this project would not have been possible.

A project of this nature includes many collaborators who have provided us with key assistance. Acknowledgements go to Ron Montelaro and Tim Sturgeon for the gift of the viral stock and for generating viral load and titer data. Also to be acknowledged are our collaborators in the Division of Allergy, Pulmonary and Critical Care Medicine, including Bob Rogers, Frank Sciurba, Paul Thompson, Tom Richards, and especially Alison Morris. I am also grateful to the other member of the Morris lab, for their input and advice, both during and outside our shared lab meetings.

Acknowledgements also go to all the staff who have assisted me in navigating through the various administrative aspects of graduate school. I am grateful to the Molecular Virology and Microbiology program staff, especially the former coordinator, Mary Ann Merranko and current coordinator, Kristin DiGiacomo, as well as the Graduate Studies office staff, but especially Cindy Duffy, who is always helpful in answering my many questions. Dr. Olja Finn

and the Immunology Department office staff also deserve special thanks. The Immunology Department has been my home at the University of Pittsburgh for the last several years, and Matt Barry, Tess Petropoulos, Dolores Davis, Mike Damico, Ryan Moeslein, Darlene Porter, and Debra Welsh have all assisted in keeping things run smoothly in the department. Additionally, the department's flow cytometry facility would not be the same without the knowledge, assistance and patient instruction from Dewayne Falkner.

I would also like to express my gratitude for the National Institutes of Health training grant (T32 AI49820), which supported me during a portion of my graduate training, and to the investigator responsible for its administration, Neal DeLuca. Additionally, Neal DeLuca also deserves acknowledgement as the Molecular Virology and Microbiology program director, as do the previous directors during the time I have been in graduate training, Michael Parniak and Joanne Flynn.

I am also especially grateful to my family for all the ways they have helped me through my graduate training, providing me with encouragement and support. Thank you to my parents and my siblings for their support through my various endeavors, including graduate school, and for remaining interested in my work and my life, despite the distances that separate us. Finally, I would like to thank my husband, Kurt, for his unwavering love and support. He shares every day with me, comforting me during difficult times, celebrating the successes, and always encouraging me. I am more grateful than I can express to have him in my life.

1.0 INTRODUCTION

The fungal opportunistic pathogen, *Pneumocystis jirovecii* (formerly *Pneumocystis carinii* f. sp. *hominis*) (Pc) is the causative agent of *Pneumocystis* pneumonia (PcP), which is a major cause of morbidity and mortality among immunocompromised persons. Mistakenly first reported as phase in the *Trypanosoma cruzi* lifecycle by Carlos Chagas in 1909 (Chagas, 1909), *Pneumocystis* was recognized as a unique organism in 1912 (Delanoe and Delanoe, 1912) but was mostly considered clinically irrelevant for the next 40 years (Kovacs and Masur, 2009). In the 1940s and 1950s Pc was identified as the cause of a particular pneumonia that was causing epidemics in premature and malnourished infants (Gajdusek, 1957). With the expansion of immunosuppressive therapy use, clinicians began to recognize PcP as a life-threatening disease in immunosuppressed hosts, especially in children receiving chemotherapy for treatment of leukemia (Walzer et al., 1974). Then in June of 1981, a case series in the CDC's *Morbidity and Mortality Weekly Report* described incidence of PcP in five homosexual male patients, all without any previously known immunodeficiency. The occurrence of PcP and mucosal candidiasis in previously healthy patients was subsequently reported to be evidence of a new cellular immunodeficiency (Gottlieb et al., 1981) and would later be recognized as the onset of the AIDS epidemic.

PcP is now considered an AIDS-defining illness and remains one of the most common serious opportunistic infections in those with AIDS (Kaplan et al., 2000; Morris et al., 2004b),

even with the availability of effective Pc treatment and anti-retroviral therapies in the developed world (Kaplan et al., 2000; Morris et al., 2004b). One study reported mortality from PcP as high as 25 percent in patients receiving ART, which although lower than the reported 63 percent in those not on ART, is still significant (Morris et al., 2003). Several studies have shown that subpopulations of HIV-infected individuals remain at risk for development of PcP despite anti-retroviral therapy (Connors et al., 1997; Morris et al., 2004b), although the basis of susceptibility is poorly understood. In the developing world, PcP still occurs frequently, affecting as many as 40 percent of the HIV+ population in Thailand (Wannamethee et al., 1998) and has been reported at a prevalence as high as 50 percent of HIV-infected persons in Brazil (Weinberg and Duarte, 1993). In sub-Saharan Africa, PcP is thought to be rare among HIV+ adults (Abouya et al., 1992), but this may be due to underreporting because of limited diagnostic resources. This hypothesis is supported by high rates of anti-Pc antibodies as well as high rates of PcP among African children (Lucas et al., 1996; Ikeogu et al., 1997; Graham et al., 2000; Ruffini and Madhi, 2002). Decline of CD4+ T cell levels below 200 cells/μl is a major risk factor for HIV+ patients; however, other factors such as cigarette smoking and environmental factors contribute to development of disease (Morris et al., 2004a).

Several hypotheses exist regarding the development of PcP in immunocompromised persons, including reactivation of latent infection acquired during childhood, active acquisition of infection from environmental exposure, and active infection acquired from person-to-person transmission (Morris et al., 2002). Although major declines in PcP incidence as well as increased survival from PcP (Morris et al., 2003) have occurred in the post-ART era, this disease remains a significant source of morbidity, even in industrialized countries. Possible reasons for this include lack of medical care, noncompliance with treatment regimens, possible drug resistance

resulting in decreased efficacy of prophylaxis, and lack of prophylactic prescription (Morris et al., 2004b).

1.1 BIOLOGY OF *PNEUMOCYSTIS*

The taxonomy of Pc has changed continuously over the years. It was originally named *P. carinii*, for its lung tropism and crediting the investigator, Antonio Carinii, who identified the organism in the lungs of rats in 1910 (Thomas and Limper, 2007; Krajicek et al., 2008). Pc was originally classified as a protozoan based on histological characteristics of the two life-cycle forms, the trophozoite and the cyst. Additionally, successful treatment of Pc-infected persons with the anti-protozoan medication, pentamidine, lent further support to this hypothesis. However, in 1988, following the sequencing of its small ribosomal RNA subunit, Pc was phylogenetically placed in the fungal kingdom (Edman et al., 1988), and is now classified within the phylum Ascomycota. It resides in a unique class (Pneumocystidomycetes), order (Pneumocystidales) and family (Pneumocystidaceae). Pc possesses a high level of host-specificity, such that a unique species, each with different genetics, has been identified in virtually every mammal. The various forms were historically designated with forma specialis (e.g., *Pneumocystis carinii* forma specialis *hominis*, the form that infects humans); however, in a recent nomenclature shift, the form that infects humans has been renamed *P. jirovecii*, in deference to the pathologist Jirovec, who initially described the organism in humans (Stringer, et al 2003).

Pc exists almost exclusively within the alveoli of the lung of an infected host. Transient proliferation of Pc is possible in the presence of lung epithelial cells in tissue culture medium;

however, sustained growth has yet to be achieved. This obstacle has been an impediment to studying the genetics and life cycle of Pc species, and life cycle events have been described only by microscopic observation. The trophic form and the cyst form are the two predominant life-cycle forms (Figure 1). During infection, trophic forms greatly predominate over cyst forms. Trophic forms during infection are mostly haploid, though a few diploid forms are also present (Wyder et al., 1998). The trophic forms may be able to conjugate and develop into cysts. Electron microscopy has revealed the existence of three intermediate cyst stages: early, intermediate and late precysts, which contain 2, 4 and 8 nuclei, respectively (Matsumoto and Yoshida, 1984). Eight nuclei are contained within the mature cyst, which ruptures to generate trophic forms emanating from the nuclei. As a form of sexual reproduction, trophic forms can then conjugate to re-form the cyst, or undergo vegetative growth (Matsumoto and Yoshida, 1984). Asexual reproduction can also be achieved by trophic forms, which may conjugate by binary fission.

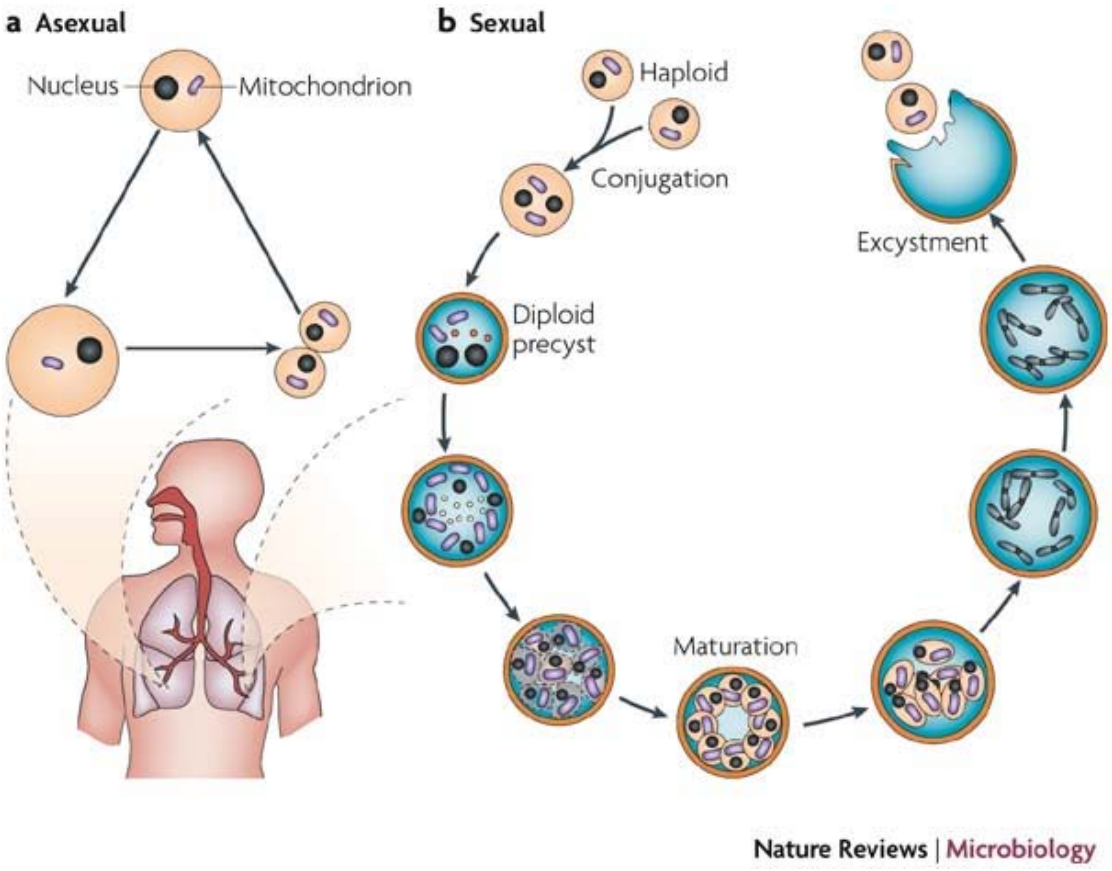

Figure 1. The hypothesized life cycle of *Pneumocystis* sp.

Copyright permission was obtained from Nature Publishing Group for use of this figure for reproduction within this dissertation.

The Pc cell wall is composed of polymerized carbohydrates, chitins and associated proteins, with the main structural component being β-1,3-glucan. This component not only contributes structural stability, but also elicits an inflammatory response during infection of the host. This inflammation contributes to alveolar epithelial damage, which is associated with Pc infection.

1.2 *PNEUMOCYSITS* INFECTION

1.2.1 *Pneumocystis* Pneumonia – Clinical Disease, Diagnosis and Treatment

Pneumocystis Pneumonia is a common AIDS-defining illness, but is also diagnosed in patients with other immunodeficiencies, such as those receiving chronic immunosuppressive medications, those with hematologic or solid malignancies, transplant recipients, and others with immune system alterations (Thomas and Limper, 2004). PcP diagnosis is a challenge for physicians, compounded by the observation that PcP symptoms in HIV-negative patients differ from those seen in HIV-infected persons. HIV+ patients typically present with progressive dyspnea, non-productive or productive cough (with clear sputum), low-grade fever, and malaise. In contrast, non-HIV-infected patients usually develop acute, fulminant respiratory failure, which is associated with dry cough and fever, and may frequently require mechanical ventilation. The preferred clinical sample used for diagnosis of PcP is bronchoalveolar lavage (BAL) fluid (Huang et al., 2006). However, some clinicians rely on induced sputum or oropharyngeal wash samples, which, although have decreased sensitivity compared with BAL fluid samples, require less invasive procedures (Wang et al., 2007). Pc organisms, in the trophic or cyst forms, from patient samples can be stained using standard histochemical methods, such as modified Giemsa stains for visualizing the trophic form and Grocott-Gomori methenamine stain (GMS) for visualization of the cystic form (Krajicek et al., 2008). Immunofluorescent staining, which has been reported to be the most sensitive but least specific visualization method, is also available for direct staining of both trophic and cyst forms of Pc (Procop et al., 2004; Krajicek et al., 2008). Molecular techniques including PCR for a variety of targets, nested PCR (Wakefield et al., 1990a; Wakefield et al., 1990b), real-time PCR (Alvarez-Martinez et al., 2006; Huggett et al.,

2008), and reverse transcriptase PCR for Pc messenger RNA (mRNA) detection (de Oliveira et al., 2007), offer the advantage of increased sensitivity and objectivity over visualization methods for Pc detection, especially for patients or experimental animals with low organism burden. These techniques also have other advantages, such as the quantitative capacity of real-time PCR and the use of reverse transcriptase PCR for determination of organism viability. However, less invasive methods for diagnosis of Pc infection are desirable, and several studies have investigated the predictive capacity of various serum antigens that may be used as indicators of Pc infection. Preliminary studies of β-D-Glucan, a major component of the cell wall of most fungi, reported high sensitivity; however, other studies report that levels decrease during PcP treatment (Kawagishi et al., 2007; Marty et al., 2007; Krajicek et al., 2008). Therefore, the diagnostic capacity of this test and investigation into other potential serum markers of Pc infection warrant further research.

Prior to the AIDS epidemic, the major chemoctherapeutic agents available for treatment of PcP were parenteral pentamidine and trimethoprim-sulfamethoxazole (TMP-SMX). To date, TMP-SMX remains the drug of choice for both treatment and prophylaxis of PcP, because of its established safety and efficacy, as well as low cost. Intravenous pentamidine has equivalent efficacy; however, adverse drug reactions, which may limit treatment, are more common than in patients treated with TMP-SMX. TMP-SMX is recommended as first-line prophylaxis against PcP in HIV+ patients with CD4+ T cell counts less than 200 cells/µl, those with oral candidiasis, and those with PcP after completion of PcP treatment regimen (Kaplan et al., 2002). Prophylaxis is also recommended for non-HIV-infected patients who are receiving immunsuppressive medications or who have an underlying acquired or inherited immunodeficiency. Development of resistance to TMP-SMX is also a concern. The two targets of TMP-SMX, the dihydrofolate

reductase (DHFR), the target of TMP, and dihydropteroate synthase (DHPS), the target of SMX and other sulfa drugs, are both enzymes involved folate biosynthesis. Mutations in the DHPS gene have been identified in Pc, and evidence from the literature suggests that these mutations may be associated with prophylaxis failures in HIV-infected patients (Kazanjian et al., 1998). In the case of adverse reactions to TMP-SMX, which are relatively common, or in patients with known allergies to sulfa, alternative choices for treatment include dapsone, a sulfone which also targets the DHPS, or atovaquone, which targets the mitochondrial cytochrome *b*. Mutations in the mitochondrial cytochrome *b* have been demonstrated to confer resistance to atovaquone. Because mutations in target genes, which may be associated with drug resistance, have already been reported in Pc, and because of the prevalence of patients' adverse reactions to anti-Pc medications, it remains important for researchers to explore other avenues for treating or preventing PcP.

1.2.2 *Pneumocystis* Colonization

Despite abundant research, a general lack of understanding regarding Pc transmission and life cycle still exists, mostly due to the inability to culture this organism. Pc appears to be ubiquitous, and the identity of the reservoir(s) is still unknown. Although it is known that most children develop anti-Pc antibodies early in life (Pifer et al., 1978; Vargas et al., 2001), the rate of asymptomatic carriage in adults is unknown. The hypothesis of environmental exposure is supported by studies which have reported detection of Pc in samples of pond water (Casanova-Cardiel and Leibowitz, 1997), air filters (Wakefield, 1994), and soil (Navin et al., 2000).

Pc colonization or carriage, defined as the presence of Pc in respiratory samples from subjects without signs of clinical disease, has been reported to be uncommon in healthy subjects

(Wakefield et al., 1990c; Peters et al., 1992; Leigh et al., 1993; Tamburrini et al., 1997). However, recently, the prevalence of *Pneumocystis* in an immunocompetent, HIV-negative adult population was shown to be as high as 20 percent (Medrano et al., 2005), with some month-to-month variability in tested samples, supporting the idea of transient colonization. This suggests that healthy persons may be able to transmit organisms to other immunocompetent hosts. Transient colonization of healthy individuals also occurs in macaque populations (Demanche et al., 2005) and in colonies of healthy, immunocompetent rodents (Vestereng et al., 2004). In a healthy population of macaques, the prevalence of Pc as detected by nested PCR is highly variable from one month to the next, as is the duration of Pc carriage (Demanche et al., 2005). Rates of Pc colonization, though variable (Huang et al., 2003; Larsen et al., 2004; Morris et al., 2004b), have been reported as relatively common in HIV+ patients (Morris et al., 2002; Morris et al., 2004a; Morris et al., 2004b; Demanche et al., 2005) despite the use of anti-Pc prophylaxis. In one cohort of HIV+ subjects who died from causes other that PcP, Pc was detected by nested PCR in 46 percent of lung samples (Morris et al., 2004a). Another study reported Pc colonization rates as high as 69 percent (Huang et al., 2003). Pc colonization rates are also somewhat variable in those with pulmonary diseases (Sing et al., 1999; Morris et al., 2004c; Respaldiza et al., 2005), perhaps a consequence of the detection limitations of PCR or the transient nature of Pc colonization. There may also exist geographic regional differences in colonization rates (Morris et al., 2004a; Nevez et al., 2006). Although the clinical consequences of Pc colonization are undefined, it is likely that even at low levels, Pc colonization of the lung can result in inflammatory changes, such as increases in neutrophils, CD8+ T cells or macrophages, which may release potentially destructive inflammatory mediators.

1.3 ANIMAL MODELS OF *PNEUMOCYSTIS* INFECTION

1.3.1 Mouse Models of *Pneumocystis* Infection

To date there is no reliable *in vitro* culture system for propagation and proliferation of Pc organisms. The lack of an *in vitro* culture system for Pc necessitates the use of animal models for the study of host immune responses, transmission, and other factors of disease. Traditional animal models of Pc have been rodents, for which immunosuppression is achieved through corticosteroid treatment or genetic knock-outs (Steele et al., 2002), as in severe combined immune deficiency (SCID) mice (Walzer et al., 1989; Harmsen and Stankiewicz, 1990) or nude mice (Powles et al., 1992), or through CD4+ T cell depletion (Shellito et al., 1990; Nevez et al., 1997; Hanano and Kaufmann, 1998). In these models, mice or rats are either infected through intranasal or intratrachial inoculation or naturally acquire Pc through co-housing with infected animals (Gigliotti et al., 2002). Although the vast majority of information known about host immune responses to Pc has been generated through the use of these rodent models, there are a number of inadequacies. Corticosteroid treatment generates broad immunosuppressive effects that may mask immune effector mechanisms of interest. Additionally, although CD4+ T cell depletion is a central mechanism for AIDS immunosuppression, there are other immune dysfunctions caused by HIV infection, which may be relevant to the study of host immune responses to Pc in the context of AIDS-associated immunosuppression. Finally, given recent evidence regarding the genetic variation among Pc species, and high degree of host specificity of these species, it may be more relevant to study Pc disease in an animal model that can be infected by a Pc species more closely related to that which infects humans. Phylogenetically, Pc derived from non-human primates is more closely related to human-derived Pc (Norris et al.,

2003). For these reasons, it may be more relevant to examine immune responses to Pc infection in a model that more closely approximates human disease.

1.3.2 Non-human Primate Model of *Pneumocystis* Infection

To address these concerns, the Norris lab has developed a non-human primate model of Pc in the context of AIDS immunosuppression (Croix et al., 2002; Board et al., 2003; Patil et al., 2003). SIV-infected macaques naturally develop opportunistic infections associated with AIDS (Reimann et al., 1996). SIV-infected macaques co-infected with Pc exhibit lymphocyte and neutrophil infiltration in bronchoalveolar lavage (BAL) fluid, eosinophilic exudates in alveolar spaces, and an increase in CD8+ to CD4+ T cell ratio in BAL fluid (Croix et al., 2002; Board et al., 2003; Patil et al., 2003) as well as increased levels of pro-inflammatory cytokines (interferon (IFN)-γ, tumor necrosis factor (TNF), and interleukin (IL)-8 in the lungs (Croix et al., 2002). The disease course observed in this model closely resembles that of PcP in AIDS patients (Dei-Cas et al., 1998), making this a useful model for studying this disease. An alternative to SIV-induced immunosuppression is the use of a chimeric Simian-Human Immunodeficiency Virus (SHIV) to infect macaques. Researchers have developed a chimeric virus, composed of SIV, expressing HIV-1 env, and usually various combinations of HIV-1 auxiliary genes (Li et al., 1992; Dunn et al., 1996; Reimann et al., 1996). It has been shown that these chimeric viruses replicate efficiently in macaques (Li et al., 1992), induce lymphadenopathy, CD4+ T cell depletion, and AIDS-like disease, including wasting and occurrence of opportunistic infections (Dunn et al., 1996; Reimann et al., 1996). Additionally, the virulence of SHIV for rhesus and cynomolgus macaques appears to closely resemble that of HIV-1 for humans in the acute phase

of infection (Dunn et al., 1996). These aspects of clinical relevance lend support to the use of the SIV/SHIV macaque model of immunosuppression for the study of immune responses to Pc.

1.4 HOST IMMUNOLOGIC DEFENSE AGAINST *PNEUMOCYSTIS* INFECTION

1.4.1 Innate Immune Response to *Pneumocystis*

Alveolar macrophages are the first line of host defense against Pc infection (Hidalgo et al., 1992; Laursen et al., 2003). This cell type is ultimately responsible for clearing Pc infection from the lungs of an infected host. The macrophage mannose receptor has been shown to mediate uptake of Pc organisms (Ezekowitz et al., 1991) via the ligand the major surface glycoprotein (gpA) on the surface of Pc organisms (O'Riordan et al., 1995). The importance of the mannose receptor in facilitating uptake and subsequent clearance of Pc organisms is further supported by reports of down-regulation of mannose receptor expression on alveolar macrophages from HIV-infected subjects, which correlated with impaired phagocytosis of Pc, compared with alveolar macrophages from healthy subjects (Koziel et al., 1998). This impairment may therefore contribute to the high incidence of Pc colonization and PcP in the HIV+ population. However, this is in contrast to studies from mouse experiments, which report that mannose receptor-deficient mice are able to clear Pc infection (Swain et al., 2003). Beta-glucan receptors, notably Dectin-1 (Steele et al., 2003), are also reported to be important for Pc update and clearance by alveolar macrophages. Binding of Pc beta-glucans has been shown to elicit production of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), macrophages

inflammatory protein-2 (MIP-2) (Vassallo et al., 1999), and IL-6 (Vassallo et al., 2001). Additionally, the importance of toll-like receptor (TLR) function for alveolar macrophage recognition of Pc has been suggested by the impaired responses of alveolar macrophages in MyD88-defecient mice (Lebron et al., 2003). In addition to uptake, the importance of alveolar macrophages in host defense against Pc is highlighted by their role in killing of Pc organisms, likely through hydrogen peroxide production (Hidalgo et al., 1992; Laursen et al., 1994; Steele et al., 2003). Supporting this observation are data showing that the respiratory burst of macrophages and neutrophils is impaired in HIV-infected persons, whether they have PcP or are asymptomatic (Koziel et al., 2000), and possibly contributes to the pathogenesis of PcP. Finally, experiments in which mice are depleted of alveolar macrophages, and then challenged with Pc inoculation, demonstrate significantly higher Pc burden in the lungs, compared with alveolar macrophage-replete mice (Limper et al., 1997).

Neutrophils also play a role in Pc infection, although the outcome of their involvement may be more detrimental than beneficial to the host. Neutrophil infiltration of the lung likely occurs in response to increased levels of the chemoattractant, IL-8. Like alveolar macrophages, neutrophils produce superoxide when cultured with Pc organisms (Laursen et al., 2003), and the respiratory burst response of neutrophils from HIV-infected subjects with a history of PcP has been reported to be decreased compared with neutrophils from AIDS patients without previous PcP incident (Laursen et al., 1995). However, a correlation has been suggested between neutrophil counts and compromised pulmonary function in patients with PcP, as well as clinical severity of disease (Jensen et al., 1991; Sadaghdar et al., 1992), suggesting that neutrophils play a role in the pathogenesis of Pc infection.

An important step in establishment of Pc infection is adherence of organisms to alveolar epithelial cells (Limper et al., 1998), which are the predominant cell types for Pc contact. Binding of the Pc organisms, which likely occurs via integrins on the epithelial cell surface (Pottratz and Martin, 1990), induces alveolar epithelial cell production and secretion of inflammatory cytokines, such as IL-6 (Pottratz et al., 1998) IL-8, and monocyte chemoattractant protein-1 (MCP-1) (Benfield et al., 1999). In this way, alveolar epithelial cells mediate immune function and participate in host defense against infection, but may also contribute to lung inflammation and resulting pathogenesis.

1.4.2 T cell Responses to *Pneumocystis*

The role of CD4+ T cells in Pc infection is widely recognized as being critical in host defense. This was initially shown in studies of Pc infection using mice experimentally depleted of CD4+ T cells, which are susceptible to the development of PcP (Shellito et al., 1990). In these experiments, selective CD4+ T cell depletion was achieved through the use of monoclonal antibodies, and when antibody treatment was removed, animals were able to clear Pc infection from the lungs. The use of SCID mice for experimental Pc infection additionally supports the importance of CD4+ T cells (Walzer et al., 1989). SCID mice are highly susceptible to PcP, but are able to resolve the infection following adoptive transfer of splenocytes from naïve mice (Harmsen and Stankiewicz, 1990). When reconstituted SCID mice are then depleted of CD4+ T cells, they are again unable to clear Pc infection (Harmsen and Stankiewicz, 1990). The precise role of this T cell subset has not been defined, but they may provide help for antibody production of Pc, as well as in production of cytokines such as IFN-γ, which has also been shown to promote control of Pc infection (Kolls et al., 1999). In HIV+ patients and in experimental rodent

models of CD4+ T cell depletion, there is an influx of CD8+ T cells in the lungs; however, the role of these cells is somewhat controversial (Beck et al., 1996; Kolls et al., 1999; Wright et al., 1999). Some reports support a role for CD8+ T cells as protective in Pc infection and instrumental in clearance of the pathogen (Beck et al., 1996; Kolls et al., 1999). Researchers have previously demonstrated that mice experimentally depleted of both CD4+ and CD8+ T cells experienced more intense Pc infection than mice depleted of CD4+ T cells alone (Beck et al., 1996). However, this study also reported that mice depleted only of CD8+ T cells were able to clear Pc infection, suggesting that although it appears that CD8+ T cells participate in host defense during Pc infection, they are not required when CD4+ T cells are present. *In vitro* experiments using CD8+ T cells purified from the lungs of CD4+ T cell-depleted mice demonstrated proliferation in response to Pc antigen and IFN-γ production, likely revealing a mechanism of protection (Beck et al., 1996). The importance of IFN-γ in control of Pc infection has been demonstrated elsewhere, through experiments in which IFN-γ was introduced by gene transfer into the lungs of CD4+ T cell-depleted, Pc-infected mice (Kolls et al., 1999). This introduction of IFN-γ promoted clearance of Pc and was associated with increased recruitment of IFN-γ-producing CD8+ T cells and NK cells to the lungs. However, IFN-γ gene transfer in SCID mice and in mice depleted of both CD4+ and CD8+ T cells did not enhance Pc clearance, indicating that IFN-γ itself may not be sufficient for organism clearance. Rather, this study suggests that IFN-γ is likely part of a cytokine response optimal for cell recruitment needed for host defense (Kolls et al., 1999). In contrast, others report that CD8+ T cells contribute to lung injury and impairment of pulmonary function (Wright et al., 1999). It has been demonstrated that although mice depleted of both CD4+ and CD8+ T cells develop PcP, they do not develop lung injury. However, mice depleted of only CD4+ T cells demonstrate severe lung

inflammation and pulmonary complications, suggesting that CD8+ T cells are responsible for this pathology (Wright et al., 1999). It has been suggested that the mechanism for damage from CD8+ T cell-associated lung inflammation may be related to altered surfactant function in the presence of CD8+ T cells (Wright et al., 2001), or increased levels of cytokine or chemokine production.

1.4.3 B cell and Antibody Responses to *Pneumocystis*

It is generally accepted that B cells play an important role in protection against Pc (Beck and Harmsen, 1998), however, the precise mechanism is as yet unknown. Transgenic *μMT* mice, which are highly susceptible to Pc infection, do not develop mature B cells and cannot produce immunoglobulins, but have normal numbers of T cells (Marcotte et al., 1996). Marcotte and colleagues described an outbreak of *P.carinii* in a colony of *μMT* mice, during which greater than 50 percent of animals died of pneumonia (Marcotte et al., 1996). Results from this study suggested a critical role for B cell or antibody responses against Pc infection. Additionally, the report highlighted how easily Pc is transmitted by the airborne route, despite the fact that the mouse colony was maintained under sterile conditions (Marcotte et al., 1996).

Control of Pc infection appears to be enhanced by passive transfer of polyclonal immune sera or monoclonal Pc-specific antibodies (Gigliotti et al., 2002; Empey et al., 2004), as well as immunization with soluble Pc antigens (Pascale et al., 1999). Using a SCID mouse model, it has been shown that topical, intranasal administration of Pc-specific IgM or IgG1 switch variant monoclonal antibodies provide protection against Pc challenge, which was administered by co-housing with Pc-infected mice. In this experiment, Pc organism burden was greatly reduced in mice which were inoculated with antibody, compared with negative control mice (Gigliotti et al.,

2002). In a subsequent study, Wells et al. demonstrated that the Fc region of this monoclonal antibody and functional complement are necessary for the antibody to have optimal prophylactic effect against PcP development in Pc-challenged SCID mice (Wells et al., 2006b). Another study examining passive immunization demonstrated that neonatal mice born to immunized adults and challenged with Pc organisms exhibited enhanced clearance of Pc, compared with neonatal mice born to naïve adults (Empey et al., 2004).

Furthermore, evidence from the literature demonstrates that effective immunization of immunocompetent mice can provide protection against development of PcP even when CD4+ T cells are no longer present. In one study, immunocompetent mice were intratracheally inoculated with Pc organisms, and were then CD4+ T cell-depleted, followed by challenge with a second Pc inoculation. Pc-vaccinated mice produced high levels of Pc-specific serum IgG, and exhibited significantly reduced Pc organism burden, compared with naïve mice that were Pc-challenged (Harmsen et al., 1995). In a subsequent study, again using Pc organisms for immunization, the role of skewing T-helper responses was examined through the use of IL-4(-/-) mice and IFN-γ (-/-) mice (Garvy et al., 1997). It was found that protection was elicited by specific antibodies generated from either T-helper type 1 or type 2 responses; thus, clearance of organisms from the lungs of Pc-challenged mice was independent of the antibody isotype profile produced (Garvy et al., 1997). Additional studies with other Pc antigens, such as nonviable inocula (Pascale et al 1999) and recombinant Pc kexin proteins or proteins with homology to Pc kexin (Gigliotti et al., 2002; Zheng et al., 2005; Wells et al., 2006a), followed by CD4+ T cell depletion and Pc challenge, have also demonstrated protective capacity in the mouse model of PcP. Similar to results observed with immunization with whole Pc organisms, immunized mice in these studies also demonstrate a Pc-specific antibody response and reduced burden upon Pc

challenge, compared with naïve mice that were CD4+ T cell depleted and Pc challenged. Together these studies indicate that in the absence of CD4+ T cell help, B cells that are sufficiently primed are capable of providing protection against the development of PcP.

Human studies have examined antibody production in response to Pc in HIV-infection, and have demonstrated a deficit in IgA and IgG production in BAL fluid of HIV+ patients with PcP, suggesting that development of PcP in these patients may not only be because of declining CD4+ T cell numbers, but also a defect in local humoral immunity (Jalil et al., 2000). Together, these studies indicate that Pc-specific antibodies have a role in protection from Pc infection and against the development of PcP.

Antibody production is an important facet of B cell contribution to host defense; however, it has been suggested that B cells are critical to the host immune response to Pc in other capacities (Lund et al., 2003), as B cells also function as antigen-presenting cells and also produce cytokines (Lund, 2008). CD40 is an important co-stimulatory marker expressed on B cells as well as other antigen-presenting cells. Experiments involving CD40 knock-out mice (Lund et al., 2003) demonstrated that these animals have reduced numbers of activated CD4+ T cells in the lungs and lymph nodes compared with wild-type controls, suggesting that B cells are important for activation of T cells in response to Pc infection. These mice produce normal levels of Pc-specific IgM, but were unable to clear the infection (Lund et al., 2003). Thus, it is hypothesized that functional CD40-expressing B cells, may be responsible for co-stimulation of T cells during Pc infection, thus regulating the strength or quality of the CD4+ T cell response (Lund et al., 2003). Other experiments have used adoptive transfer to demonstrate the importance of B cells in resolving Pc infection. Researchers adoptively transferred CD4+ T cells from draining lymph nodes of Pc-infected, normal mice or B-cell deficient mice into SCID mice.

Mice receiving CD4+ T cells from B cell-deficient mice had a delayed T cell response in the lungs, compared with those that received T cells from normal mice, and were unable to clear Pc infection (Lund et al., 2006). These studies suggest that B cells are necessary for the generation of effector and memory CD4+ T cells in response to Pc infection (Lund et al., 2006).

1.5 ANTIGENS USED IN EVALUATING ANTIBODY RESPONSES TO *PNEUMOCYSTIS*

1.5.1 Antibody Responses to Whole *Pneumocystis* Organisms

The CD4+ T cell-depleted mouse model has also been used to investigate the effect of immunization with different Pc antigens on subsequent development of PcP. In initial investigations of host antibody response to Pc infection, investigators used either viable or nonviable Pc organisms for immunization (Harmsen et al., 1995; Pascale et al 1999). As mentioned above, intratracheal immunization with whole *P. carinii* organisms protects mice against the development of PcP. These whole-cell immunized, CD4+ T cell-depleted mice showed reduced Pc burden, improved development of systemic Pc-specific IgG response, and reduced likelihood of PcP development, upon challenge with Pc organisms (Gigliotti et al., 1998b). These studies examine antibodies against soluble total Pc protein from lungs of Pc-infected mice (usually SCID or CD4+ T cell depleted) as a readout of host humoral response to Pc infection (Harmsen et al., 1995). However, these are crude preparations and require the use of an infected animal for source organisms; therefore, this type of preparation, either for immunization or for antibody detection, is not ideal. The potential for vaccine development of

this type of antigen preparation is further complicated by the high level of host-specificity of Pc; thus, organisms derived from one host species (e.g., mice) may not be capable of eliciting a protective immune response against Pc infection of another species (e.g., humans).

1.5.2 *Pneumocystis* Major Surface Glycoprotein

An abundance of heavily glycosylated antigens with mannose carbohydrates exist on the surface of Pc organisms. The family of glycoproteins referred to as the major surface glycoprotein (MSG) or glycoprotein A (gpA), with a molecular mass of 95-120 kDa, is the most abundant. These surface proteins are involved in Pc attachment to host alveolar epithelial cells, hypothesized to occur via integrin-fibronectin interaction (Pottratz et al., 1994). This is a multigene family of glyoproteins, with approximately 80 MSG genes present in the Pc genome; however, only one isoform of MSG is expressed at any one time during infection of a host. The expressed isoform is also antigenically distinct for any single host-specific Pc species. Expression of a different isoform of MSG may enable Pc to establish infection by evading host immune recognition.

MSG has been explored by researchers for its immunogenic potential. Immunization with a recombinant form of MSG was initially shown to elicit a protective response in the corticosteroid-treated rat model (Theus et al., 1998), with MSG-immunized animals exhibiting lower organism burden and improved survival, compared with ovalbumin- or adjuvant-alone-immunized control rats (Theus et al., 1998). Studies of passive immunoprophylaxis using monoclonal antibodies also suggested that immune recognition of MSG be instrumental in Pc clearance (Gigliotti et al., 1996). However, other studies using the CD4+ T cell depleted-mouse model of infection have reported that although immunization with gpA elicited a strong gpA-

specific antibody response, experimental animals were not protected from developing PcP (Gigliotti et al., 1998a). MSG is also capable of antigenic variation, and may not consistently present the immunodominant epitope recognized by B cells (Gigliotti et al., 1998a), as it was reported that B cells isolated from tracheobronchial lymph nodes were more likely to produce antibodies specific to Pc antigens other than MSG (Theus et al., 1995; Gigliotti et al., 1998b; Theus et al., 1998).

1.5.3 *Pneumocystis* Kexins

The protease PRT1, known as KEX1 in both human- and mouse-derived Pc species, possesses homology to fungal subtilisin-like serine proteases Kex1 from *Kluyveromyces lactis* and Kex2 from *Saccharomyces cervisiae* and *S. pombe* (Lugli et al., 1997; Lee et al., 2000; Kutty and Kovacs, 2003). Kexins, initially described in *S. cerevisiae*, function in processing and/or maturation of hormones and other proteins. The deduced amino acid sequence of mouse Pc Kex1 demonstrated several structural features similar to the subtilisin-like proteases (Lee et al., 2000). Reports also indicate that mouse Pc *kex1* (Lee et al., 2000), as well as *kex1* from *P. jiroveci* are single-copy genes (Lugli et al., 1997; Lee et al., 2000; Kutty and Kovacs, 2003), unlike what had been previously described for rat-derived Pc. Kex1 is hypothesized to be involved in the proteolytic processing of Pc surface antigens, in particular, MSG.

Initial studies suggesting the immunogenicity of kexins were performed using IgM and IgG1 switch variant monoclonal antibodies directed against a Pc kexin-like molecule, successfully generating a specific antibody response and resulted in reduced organism burden in the lungs of immunized SCID mice (Gigliotti et al., 2002). A subsequent study investigated the

use of a thioredoxin-fusion protein, which exhibited homology to Pc kexin, in an active immunization model. Following immunization of mice with the recombinant protein (A12), Pc-specific antibody responses were detectable by Western blot in mouse serum. Serum antibodies also were capable of binding to the surface of Pc organisms, whereas sera from control mice, which were immunized with thioredoxin alone, did not recognize Pc cysts. Immunization with this protein also reduced organism burden in CD4+ T cell-depleted, Pc-challenged mice (Wells et al., 2006a). Finally, a Kexin-DNA vaccination, using CD40 ligand (CD40L) as an adjuvant for elicitation of CD4+ T cell-independent responses, has been shown to generate significant anti-Pc IgG1 and IgG2a titers in CD4+ T cell-depleted mice (Zheng et al., 2005). Additionally, mice immunized with Kexin-DNA and CD40L exhibited significantly lower Pc organism burden, compared with control mice, when challenged with Pc inoculation (Zheng et al., 2005). In these experiments, serum was collected from Kexin-DNA/CD40L-immunized mice and incubated *ex vivo* with Pc organisms, followed by incubation with peritoneal macrophages. Serum from immunized mice resulted in significant opsonic killing of Pc organisms, compared with killing resulting from incubation with serum from naïve mice (Zheng et al 2005). Together these studies demonstrate that Pc Kexin is an important target in murine immunization against Pc infection. However, because of the aforementioned host specificity of Pc, studies examining the immunogenicity and protective capacity of this antigen in primate models of Pc infection are worth pursuing.

1.6 CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic Obstructive Pulmonary Disease (COPD) is a usually progressive disease that is characterized by airflow limitation, which is not fully reversible. Manifestations of COPD include chronic bronchitis, obstructive bronchiolitis, and emphysema. Emphysema pathology is characterized by permanent airspace enlargement and alveolar wall destruction (Snider, 1985). Hallmarks of emphysema pathophysiology include parenchymal destruction resulting in loss of alveolar-capillary units and lung elasticity, as well as largely-irreversible airflow obstruction (Hogg, 2004). The most common risk factor for COPD development is cigarette-smoking, although only 15-20 percent of smokers develop the disease. Other factors likely contributing to disease progression include genetic and environmental factors, as well as infectious agents, which may amplify local inflammation (Hogg, 2001; Morris et al., 2004c).

1.6.1 COPD Association with HIV

HIV-infected patients, especially smokers, are at increased risk for developing emphysema (Diaz et al., 1992; Diaz et al., 2000a). One study that examined HIV-infected smokers reported that 37 percent had emphysema, as determined by pulmonary function testing and chest computed tomography (CT) scan (Diaz et al., 2000a). For comparison, this study examined smoking-history-matched, HIV-negative patients and found that none of these patients exhibited emphysema. Another study presented evidence that even HIV-infected patients who do not smoke are at increased risk for airflow obstruction and respiratory symptoms (Diaz et al., 2003). Importantly, prolonged life expectancy of the HIV+ population because of ART, as well as a high smoking rate in this population, may be associated with increased frequency of chronic

health issues, including HIV-associated COPD (Collins et al., 2001; Gritz et al., 2004). Finally, it has been reported that HIV-infected patients develop permanent COPD-like changes after having PcP (Morris et al., 2000).

1.6.2 *Pneumocystis* Colonization and COPD

Evidence has accumulated suggesting a role for infectious agents as co-factors in the pathogenesis and exacerbation of COPD (Sethi and Murphy, 2008), in which pulmonary inflammation due to cigarette smoke may be amplified by the presence of persistent infectious agents. This chronic inflammation is thought to contribute to the development of COPD in HIV+ patients (Morris et al., 2008b; Sethi and Murphy, 2008).

There is increasing evidence to suggest an association between persistent Pc colonization and the development of chronic obstructive pulmonary disease (COPD). Inflammatory changes associated with Pc colonization and PcP, such as lung infiltration of CD8+ T cells and neutrophils, and increased levels of IL-8, are similar to those in COPD (Keatings et al., 1996; Di Stefano et al., 1998; Saetta et al., 1998). Additionally, studies have reported increased prevalence of Pc colonization among patients with COPD (10-63%) (Calderon et al., 1996; Probst et al., 2000; Helweg-Larsen et al., 2002), and recently, it was reported that Pc colonization is associated with increased airway obstruction in HIV+ subjects (Morris et al., 2009). One study found that Pc colonization was most common in those with severe airway obstruction, and this association was independent of clinical factors such as smoking history or steroid use. Colonization rates were compared in patients with various end-stage lung diseases, and, of subjects colonized with Pc, 73 percent carried a diagnosis of COPD, compared with only 32 percent of non-colonized subjects that had a diagnosis of COPD (Morris et al., 2004a).

Furthermore, animal models also support the role of Pc colonization in the pathogenesis of COPD. In a model of immunocompetent mice, cigarette exposure and Pc colonization resulted in greater pulmonary function deficits compared to cigarette exposure alone (Christensen et al., 2008). Our laboratory has reported that experimental immunosuppression of non-human primates resulting in Pc colonization, without the development of overt clinical PcP, was associated with pulmonary inflammation, airway obstruction and anatomic emphysema, further supporting the association of Pc colonization and development of COPD (Norris et al., 2006; Shipley et al., in press). Recent findings also suggest that antibodies to the recombinant Pc antigen kexin are associated with protection from increased severity of obstruction in HIV-negative COPD patients (Morris et al., 2008a). These reports lend further support to a growing body of evidence that suggests an association between Pc-colonization and the development of COPD.

1.7 HIV-ASSOCIATED EFFECTS ON B CELLS

In primary HIV infection B cell abnormalities represent an important feature of HIV pathogenesis (De Milito, 2004), and may contribute to the increased risk and incidence of opportunistic infections in HIV-infected patients. Such abnormalities include reduced total and memory B cells (De Milito et al., 2001; Nagase et al., 2001), increased B cell activation (Lane, 1983; Mizuma et al., 1988; Shirai et al., 1992; DeMilito, 2001; Titanji et al., 2005; Moir et al., 2008), resulting in hypergammaglobulinemia and spontaneous Ig secretion *in vitro* (Nagase et al., 2001; De Milito et al., 2004; Titanji et al., 2005), higher percentages of apoptotic naïve and memory B cells, decreased circulating antigen-specific Ig, impaired responsiveness to

stimulation (Jiang et al., 2008), germinal center abnormalities (Guarda et al., 1983; Chalifoux et al., 1984; Chalifoux et al., 1987; Margolin et al., 2002; Margolin et al., 2006), and decreased subsets of memory B cells, including IgM+ memory B cells (Titanji et al., 2005; Hart et al., 2007).

In early studies of HIV-associated dysfunctions, researchers reported on abnormal levels of B-cell activation (Lane, 1983) and resulting spontaneous secretion of immunoglobulins (Mizuma et al., 1987; Mizuma et al., 1988), which provide evidence of *in vivo* polyclonal activation. Serum IgD was identified early as a marker of B cell activation during HIV-infection, and appeared to correlate with level of immunosuppression as measured by percentage of peripheral blood CD4+ T cells (Mizuma et al., 1987). Additionally, levels of spontaneous cellular secretion of IgD, IgM and IgG, measured in PBMC culture supernatants, correlate with serum levels of these isotypes, all of which are reported to be elevated in HIV-infected patients (Mizuma et al., 1988). Circulating levels of IgG have been reported as a prognostic marker of infection, because patients with symptomatic infection exhibit higher levels of plasma IgG than asymptomatic patients, which is likely a consequence of chronic immune activation. The observed hypergammaglobulinemia may be related to increased expression of activation markers, which result in elevated rates of differentiation of memory cells to Ig-secreting plasma cell and increased activation of naïve B cells (Nagase et al., 2001; De Milito et al., 2004; Cagigi et al., 2008; Moir and Fauci, 2009). In HIV-infection, this hypergammaglobulinemia has been reported to be both virus-specific and polyclonal; however, it seems that as disease progresses, the HIV-specific response declines, while the polyclonal response is increased (De Milito 2004). Additionally, specific antibody responses to opportunistic pathogens and immunizing antigens, such as *Streptococcus pneumoniae*, *Cryptococcus neoformans*, measles, and influenza, also

appear decreased in HIV+ patients, compared with HIV-negative controls (Titanji et al., 2006, Cagigi et al., 2007). This chronic immune activation is accompanied by decreased proliferative responses to T-cell independent B cell mitogens *ex vivo* (Lane et al., 1983; Jiang et al., 2008), in both the naïve and memory B cell populations (Jiang et al 2008). Thus, B cell function, measured by the B cell response to specific stimuli or by the capacity to provide co-stimulatory signals to CD4+ T cells, appears impaired during HIV-infection (Malaspina et al., 2003; Moir et al., 2003). A mechanism hypothesized to account for poor proliferative responses of B cells from HIV-infected patients is the elevated level of CD21-low/negative B cells in these subjects (Moir et al., 2001; Jiang et al., 2008; Moir and Fauci, 2009). These cells are thought to be terminally differentiated and thus, proliferate poorly in response to mitogenic stimulation. Evidence from the literature suggests that the frequency of CD21+ memory B cells is directly related to the capacity of these cells to proliferate in response to stimulation (Jiang et al., 2008). Of note, these abnormalities of B cell hyperactivation and hypersecretion of Ig have been demonstrated to be reversible by effective anti-retroviral therapy (ART). These types of B cell abnormalities appear to be closely related to subject viremia; thus, with effective reduction in viral load following ART, many such abnormalities are reversed (Moir et al., 2001; Fournier et al., 2002; De Milito, 2004; Moir and Fauci, 2009).

In addition to hyperactivation, HIV-infection is also associated with a loss of memory B cells and decreased memory B cell function, which, in contrast to what has been reported for activation abnormalities, do not seem to be restored by ART (Hart et al., 2007). Studies have demonstrated: a loss of total memory B cells, impairment of long-term serological memory to immunizing antigens (Titanji et al., 2006), decreased proliferative capacity of memory B cells (Jiang et al., 2008), and a selective loss of particular subsets of memory B cells, such as IgM

memory B cells (Hart et al., 2007) and marginal-zone like populations (Morrow et al., 2008). Because IgM+ memory B cells are believed to function in protection against encapsulated bacterial and other T-independent-type antigens, selective depletion of these types of subsets may aid in explaining the increased susceptibility of HIV-infected individuals to infections like pneumococcal disease. Impaired B cell memory may contribute to the observed reduced capacity of HIV-infected individuals to respond effectively to pathogens, diminished responses to vaccination (Malaspina et al., 2005), and may also contribute to the increased susceptibility of HIV-infected individuals to opportunistic pathogens.

1.8 SUMMARY

Despite increased availability of anti-retroviral therapies and of *Pneumocystis* prophylaxis, this opportunistic pathogen continues to be a major source of morbidity and mortality in immunocompromised populations in both industrialized and developing countries. Additionally, a general lack of understanding regarding Pc transmission, life cycle and prevalence in the general population still exits despite abundant research. Although it is know that Pc colonization in HIV-infected patients is quite common, the clinical consequences of colonization with this pathogen are undefined. Mounting evidence suggests that even in subjects who do not present with clinical signs or symptoms of *Pneumocystis* pneumonia, persistent Pc colonization, even at low levels, may elicit a potentially damaging inflammatory response. In this respect, it is plausible that colonization of host lungs with Pc organisms may contribute to or exacerbate the

development of chronic obstructive pulmonary disease (COPD). This hypothesis is supported by reports of high rates of Pc colonization in COPD patients.

The concern for development of Pc resistance to current medications used for PcP treatment and prophylaxis underscores the importance of exploring other avenues of prevention and treatment. Although CD4+ T cells are critical in controlling Pc infection, B cells play a vital role in protection of the host from development of *Pneumocystis* pneumonia, and many experiments in the mouse model demonstrate the importance of B cells and Pc-specific antibodies in host protection from this pathogen. Although the vast majority of information known about host immune responses to Pc infection has been generated through use of the mouse model, this system has some inadequacies, which can be addressed through the use of a non-human primate model of Pc infection. The macaque model uses SIV or SHIV, which bear many similarities to HIV-infection in humans, to induce immunosuppression, which permits natural persistent Pc colonization, or with experimental Pc inoculation, development of PcP. This is a highly relevant system for examining host immune responses to Pc infection, which, in this model, can be examined in the context of AIDS immunosuppression. HIV-infection is associated with various B cell abnormalities, dysregulations and dysfunctions, which may diminish the capacity of the host immune system to respond to a secondary infection. Additionally, further understanding and characterization of SHIV-associated B cell deficits or abnormalities in the macaque model are needed. Finally, the central role of the B cell response during Pc infection coupled with the known HIV-associated abnormalities underscore the importance of investigating B cell responses during a SHIV/Pc co-infection.

1.9 SPECIFIC AIMS

1.9.1 Specific Aim 1. To establish a model of natural *Pneumocystis* (Pc) colonization, and to develop this model in SIV/SHIV-infected macaques.

We hypothesize that SIV- or Simian- HIV (SHIV)-infected macaques immunosuppressed by depressed CD4+ T cell numbers will naturally acquire Pc as a result of co-housing, and are able to generate an immune response to Pc colonization in advance of the development of PCP. The humoral immune response of these animals can be used to track Pc colonization. Using the recombinant Pc antigen Kexin (KEX1), ELISA will be used to evaluate systemic (plasma) and local (bronchoalveolar lavage fluid supernatant) production of Pc-specific immunoglobulins in SIV/SHIV-infected macaques. The model will allow for the characterization of antibody dynamics associated with and following Pc colonization in a model of SIV/SHIV immunosuppression.

1.9.2 Specific Aim 2. To evaluate the capacity of antibodies to the recombinant Pc antigen kexin (KEX1) to protect SHIV-immunosuppressed macaques from naturally acquired Pc infection and subsequent lung injury.

We will characterize the humoral response to Pc in healthy macaques, and examine whether differential Pc-antibody titer levels prior to immunosuppression contributes to the delay or prevention of Pc colonization. We hypothesize that macaques with high plasma anti-KEX1 antibody titers prior to SHIV-induced immunosuppression will be less likely to become naturally

Pc-colonized following SHIV-infection than macaques with low/negative KEX1 titers. High KEX1 titers will correlate with the delay or prevention of Pc colonization and prevention of subsequent pulmonary disease in SHIV-immunosuppressed macaques.

1.9.3 Specific Aim 3. To evaluate the effect of SHIV-infection on peripheral blood B cell populations in cynomolgus macaques, and to determine whether SHIV-immunosuppression results in an impaired memory response to a secondary, opportunistic infection, Pc.

We will investigate whether SHIV-infection results in declines or deficits in B cell populations in macaques similar to what has been reported in HIV+ patients, to determine whether the SHIV-macaque model is appropriate for studying AIDS-associated B cell dysfunctions. We will investigate whether B cell defects due to SHIV-infection result in an impaired humoral response to Pc colonization. We hypothesize that we will observe similar phenotypic changes in B cell populations as have been reported for HIV-infected subjects, such as declines in total and memory populations and increased activation of B cells. However, we anticipate that these changes or defects will not contribute to Pc colonization status because we hypothesize that a strong antibody response to Pc at baseline will be protective following SHIV-infection despite B cell deficits induced by SHIV-infection.

2.0 PNEUMOCYSTIS COLONIZATION IN IMMUNOCOMPETENT AND SIMIAN IMMUNODEFICIENCY VIRUS-INFECTED CYNOMOLGUS MACAQUES

Heather M. Kling[1], Timothy W. Shipley[1], Sangita Patil[1], Alison Morris[2], and Karen A. Norris[1]

[1]Department of Immunology and [2]Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

This chapter was modified with permission from:

Heather M. Kling, Timothy W. Shipley, Sangita Patil, Alison Morris,

and Karen A. Norris

Pneumocystis Colonization in Immunocompetent and Simian Immunodeficiency Virus-Infected Cynomolgus Macaques

Journal of Infectious Diseases 2009: 199(1): 89-96

2.1 ABSTRACT

Pneumocystis (Pc) colonization is common among HIV-infected subjects, though the clinical consequences of Pc carriage are not fully understood. We examined the frequency of asymptomatic carriage in normal and SIV-infected cynomolgus macaques using polymerase chain reaction and changes in the serologic response to a recombinant fragment of the Pc protein, kexin (KEX1). Anti-KEX1 antibodies were detected in 95% of healthy monkeys. To model natural transmission of Pc, SIV-infected monkeys were co-housed with SIV-Pc co-infected macaques. Pc colonization occurred when CD4+ T cells declined below 500 cells/μl, despite anti-Pc prophylaxis with trimethoprim-sulfamethoxazole. Increased anti-KEX1 titers preceded polymerase chain reaction detection of Pc DNA in BAL samples. These results demonstrate the utility of recombinant KEX1 in serologic studies of Pc colonization and will improve the understanding of Pc transmission and the clinical consequences of Pc colonization in HIV-infected patients.

2.2 INTRODUCTION

Pneumocystis pneumonia (PCP) remains a common serious infection in immunocompromised individuals, particularly those with AIDS (Kaplan et al., 2000; Morris et al., 2004b). Although anti-*Pneumocystis* (Pc) prophylaxis and highly active anti-retroviral therapy (HAART) have resulted in a significant decline in PCP frequency, it remains the most common AIDS-defining opportunistic infection in the United States (Kaplan et al., 2000; Morris et al., 2002; Morris et al., 2004b).

A clear understanding of the Pc route of transmission is lacking due to inability to culture the organisms *in vitro*. Several hypotheses exist regarding transmission of PCP in immunocompromised persons, including reactivation of latent infection acquired during childhood, active acquisition of infection from environmental exposure, and person-to-person transmission (Morris et al., 2002). Airborne transmission has been demonstrated in animal models and has been used experimentally to initiate infection in normal rodents and in immunocompromised rodent models of PCP (Powles et al., 1992; Wolff et al., 1993). PCP has been documented in experimental macaque models of AIDS (Baskin et al., 1988; Vogel et al., 1993), but a naturally occurring model of transmission and colonization in primates has not been described.

Evidence from experimental systems and serologic studies in humans suggests exposure to Pc stimulates a humoral response and antibodies play a significant role in host defense against PCP (Gigliotti and Hughes, 1988; Harmsen et al., 1995; Garvy et al., 1997; Bartlett et al., 1998;

Gigliotti et al., 1998a; Pascale et al., 1999; Gigliotti et al., 2002). Clinical application of Pc serology has been of limited use in understanding transmission and progression of Pc infection due to the frequency of antibodies in both healthy and immunocompromised hosts (Walzer, 1999; Walzer, 2005). Recent studies using the major surface glycoprotein (MSG) of Pc have shown a high frequency of serum antibodies among normal and immunosuppressed subjects (Peglow et al., 1990; Wakefield et al., 1990a; Smulian et al., 1993; Lundgren et al., 1997), and changes in MSG-specific antibody levels were detected in sequential serum samples in response to Pc infection and treatment (Daly et al., 2006). These studies suggest recombinant Pc antigens may be useful in distinguishing past and current Pc exposure.

Highly sensitive techniques such as polymerase chain reaction have been used to detect very low levels of Pc DNA in asymptomatic persons (Nevez et al., 1997; Morris et al., 2004a; Medrano et al., 2005). Detection of Pc DNA by PCR in asymptomatic individuals has been defined as colonization or subclinical carriage (Morris et al., 2004b), although clinical consequences of Pc colonization are unknown. Pc colonization may be important for several reasons: it may increase risk for progression to PCP in susceptible individuals, carriers may transmit infection, and organism persistence may induce chronic inflammation resulting in lung damage. Pc colonization is not common in immunocompetent adults, but can be detected in individuals with mild to severe immunosuppression (Rabodonirina et al., 1997; Morris et al., 2004a). In HIV-infected subjects, frequency of Pc colonization has been reported to be between 5 and 69% (Huang et al., 2003). We have previously reported that experimental, intrabronchial inoculation of Pc in simian immunodeficiency virus (SIV)-infected rhesus macaques could lead to persistent, sub-clinical Pc colonization and PCP. Pc colonization in this model resulted in a chronic inflammatory response in the lungs prior to PCP development with prominent CD8+ T

cell infiltration and cytokine production (Board et al., 2003). A shortcoming of this model is lack of purity of the inoculum due to inability to culture Pc *in vitro*, which may induce a transient, non-specific inflammatory response.

In the present study, we aimed to determine the antibody response and frequency of Pc colonization in a cohort of normal monkeys, to establish a model of airborne transmission of Pc colonization in SIV-infected macaques, and to evaluate the dynamics of anti-Pc antibody response in peripheral blood and bronchoalveolar lavage (BAL) fluid during colonization in SIV-infected macaques.

2.3 MATERIALS AND METHODS

2.3.1 Animals.

Cynomolgus macaques (*Macaca fasicularis*, ages 3.5 to 4.5 years old) used in this study were housed in an American Association for Accreditation of Laboratory Animal Care-accredited biosafety level 2+ primate facility at the University of Pittsburgh. Prior to initiation of the study, all animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

2.3.2 Study Design.

Seventy-four healthy, non-SIV-infected monkeys were tested to determine frequency and magnitude of anti-Pc antibody titers using recombinant Pc KEX1 ELISA, described below. Five

monkeys were subsequently SIV-infected and used to examine natural transmission of Pc colonization and the development of a humoral response to Pc. Prior to SIV infection, monkeys underwent BAL, and samples were evaluated for presence of Pc by microscopic examination of Giemsa-stained BAL fluid and by Pc-specific, nested PCR (Board et al., 2003). In addition, baseline serologic analysis was performed for detection of anti-KEX1 antibodies (described below). Monkeys were then infected with a pathogenic strain of SIV (SIV/Delta B670) (Amedee et al., 1995), and clinical evaluation and plasma viral levels were performed as described (Board et al., 2003).

At the time of SIV infection, monkeys were treated daily with trimethoprim (20 mg/kg)-sulfamethoxazole (100 mg/kg) (TMP-SMX) in two divided doses to clear any pre-existing Pc colonization and co-housed with SIV macaques that had not received anti-Pc treatment. When plasma antibody titers declined to background levels (4 months post-SIV infection), TMP-SMX was reduced to 10 mg/kg TMP and 50 mg/kg SMX, 3 times weekly, on alternate days to approximate a prophylactic regimen (2003). Treatment and prophylactic doses were based on the American Academy of Pediatrics Red Book recommendations for *Pneumocystis* (2003). Based on previous studies of experimental Pc infection in SIV-immunosuppressed macaques, we predicted monkeys would be susceptible to natural Pc colonization when peripheral blood CD4+ T cell levels fell below 30% of the total T cells (Board et al., 2003; Norris et al., 2006). Thus, TMP-SMX prophylaxis was discontinued when CD4+ T cell counts declined below this level (approximately 500 CD4+ T cells/µl), to allow natural Pc colonization to occur. Discontinuation of TMP-SMX occurred at 9 months post-SIV infection for monkeys 133, 157, 158 and at 15 months for monkeys 143 and 156.

2.3.3 BAL and blood collection.

Serial plasma, PBMC and BAL samples from normal and SIV-infected monkeys were collected monthly for longitudinal analyses as described (Board et al., 2003). Aliquots were removed for flow cytometric analysis, microscopic and microbiologic analyses and Pc-specific PCR. Plasma and BAL fluid were heat inactivated (56°C, 30 min) and used in ELISA for detection of Pc-specific antibodies. BAL fluid samples were normalized based on plasma urea concentration (Rennard et al., 1986). Percentages and absolute numbers of CD4+ and CD8+ T cells were calculated based on flow cytometry of PBMC (Croix et al., 2002). Pc colonization was defined as a three-fold increase in anti-KEX1 reciprocal endpoint titer over baseline titer and confirmed by detection of Pc DNA by nested PCR in BAL fluid, as described below.

2.3.4 Western blot analysis.

A partial fragment of the macaque-derived, Pc kexin gene in the pBAD expression vector (gift from C.G. Haidaris, University of Rochester, GenBank accession number EU918304) was used to produce recombinant kexin protein (KEX1). Briefly, KEX1 expression was induced in *E. coli* Top10 (Invitrogen Corp., Carlsbad, CA) containing the pBAD-KEX1 plasmid; cells were lysed, centrifuged and supernatant was purified by affinity chromatography. Purified protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, blocked, and incubated with plasma from a macaque with PCP, and with plasma from SIV-infected macaques from this study. Filters were washed, incubated with

horseradish peroxidase-conjugated IgG secondary antibody, and developed according to standard protocols (more details included in Supplementary Methods, Appendix B).

2.3.5 Endpoint antibody titer determination.

Microtiter plates (Immunolon 4HBX, Thermo Fisher Scientific, Inc., Waltham, MA) were coated with purified KEX1 at 5 μg/ml in Na_2CO_3, (pH 9.5). Heat-inactivated plasma samples were diluted 1:100 in blocking buffer (PBS, 5% non-fat milk). Fifty μL of plasma were plated into KEX1-coated wells and serial dilutions were made to determine endpoint titers. Goat anti-monkey immunoglobulin-conjugated horseradish peroxidase (1:10,000 for IgG, 1:2000 for IgM) (Nordic Immunology, Tilburg, Netherlands) was used for detection and plates were developed by standard methods. Normal (uninfected, Pc-negative) macaque plasma was used as negative control and plasma from a monkey with PCP was used for positive control (more details included in Supplementary Methods). The reciprocal endpoint titer was calculated as the highest dilution at which the optical density (O.D.) values for the test sample were the same or less than the normal sample O.D.

2.3.6 Nested PCR of BAL fluid.

BAL fluid cell lysate samples were analyzed for presence of Pc DNA by nested PCR of the mitochondrial large subunit ribosomal RNA gene (mtLSU), as described (Board et al., 2003; Patil et al., 2003). Nested PCR was performed on 5 μL of the first round product using primers P1 and P2 (Savoia et al., 1997). PCR for β-globin was also performed on BAL samples to control for DNA quality (Croix et al., 2002).

2.3.7 Flow Cytometry.

Mouse anti-monkey CD3-fluorescein isothiocyanate (clone SP34), mouse anti-human CD8-Cy-Chrome (clone SK1) and mouse anti-monkey CD4- allophycocyanin (clone L200) were purchased from BD Pharmingen (San Diego, CA). Cells were isolated from whole blood and stained as previously described (Croix et al., 2002).

2.4 RESULTS

2.4.1 Western blot analysis of KEX1.

After confirming reactivity of purified recombinant KEX1 to plasma from a macaque with microscopically-confirmed PCP (data not shown), plasma samples from the SIV-infected monkeys were examined by western blot; a sample from one animal is shown in Figure 2. SDS-PAGE analysis of purified KEX1 demonstrates purity achieved from a typical protein preparation in Figure 2A. Western blot analysis showed that plasma reacted with KEX1 at the expected molecular mass of 30 kDA, but not with proteins encoded by the vector only, demonstrating the specificity of the antigen, (Fig. 2B).

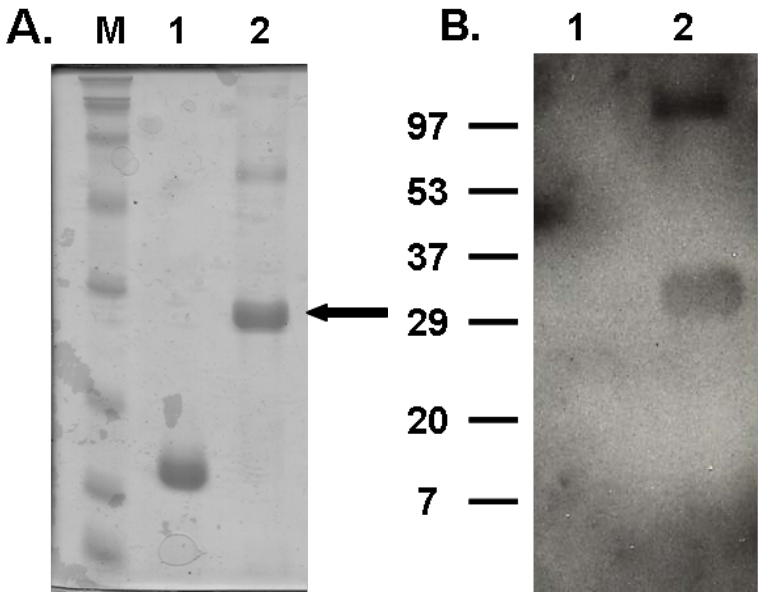

Figure 2. SDS-PAGE and Western blot analysis of recombinant Pc KEX1. A. Coomassie blue-stained SDS- polyacrylamide gel of purified KEX1 protein (lane 2) and preparation of protein purified from the pBAD expression vector not containing the KEX1 insert (lane 1). Equal amounts of protein were applied to both lanes (2.5μg). The migration of molecular mass markers (kilodalton) is indicated at left, and applies to the Western blot (in part B) as well. B. Western blot of recombinant KEX1 (lane 2) or proteins from *E. coli* expression vector (lane 1) using plasma from a Pc-colonized macaque. Equal amounts of protein were applied to both lanes (2.5μg).

2.4.2 Anti-KEX1 serology and Pc colonization in normal cynomolgus macaques.

The majority of healthy cynomolgus macaques (95%, n=74) had detectable reciprocal endpoint titers (RET) (>100). Of those, 59% had RET between 100 and 4800 and the remaining 41% had RET greater than 6400.

We observed transient Pc colonization in 2 healthy monkeys as indicated by an increase in antibody titer and detection of Pc DNA in BAL fluid. At study entry, one monkey had no detectable Pc DNA in BAL, and a plasma anti-KEX1 IgG reciprocal titer of 100. Six weeks later, this animal had detectable Pc DNA (by nested PCR) in the BAL sample and had a 32-fold increase in anti-KEX1 endpoint titer. At subsequent monthly time points, the plasma Pc-specific reciprocal IgG titer declined to 400 and Pc DNA could no longer be detected in BAL samples. Transient colonization was also detected in another monkey by nested PCR of Pc DNA in the BAL fluid at entry into the study, but was not positive by nested PCR 4 weeks later.

2.4.3 Natural Pc colonization of SIV-infected macaques.

To evaluate baseline Pc colonization status prior to SIV infection, nested PCR was performed on BAL samples, and plasma and BAL supernatant samples were analyzed for anti-KEX1 antibodies. Pc DNA was not detected in experimental monkeys prior to SIV infection, though 4 of 5 monkeys (133, 156, 157 and 158) had detectable anti-KEX1 IgG reciprocal titers, ranging from 600 to 1,600 (Fig. 3 and 4). Monkeys were treated with TMP-SMX, and antibody titers declined to background levels. Monthly BAL samples were negative by nested PCR except for a single time point (3 months) in monkeys 156 and 157 (Fig. 3). Treatment doses were then decreased to prophylactic doses and within three months of co-housing with Pc-infected

monkeys, all monkeys experienced a serial rise in anti-KEX1 antibody titers, and Pc DNA was detected in BAL samples from 3 of 5 monkeys, suggesting re-emergence or secondary colonization by Pc despite use of anti-Pc prophylaxis (Fig. 3).

When CD4+ T cells fell below 500 cells/μL, all monkeys eventually experienced an increase in anti-KEX1 plasma antibody titers (Fig 3). Anti-KEX1 IgG did not increase above background in monkey 133; however, by 9 months post-SIV infection serial increases in anti-KEX1 IgM titers were detected, suggesting a primary exposure to Pc (Fig. 3 and 4). After monkeys had seroconverted, Pc DNA was detected by nested PCR in multiple monthly BAL samples confirming Pc colonization. During the course of the study, the SIV-immunosuppressed monkeys were unable to clear Pc colonization.

2.4.4 Anti-KEX1 antibody response in BAL fluid.

Serial BAL samples were analyzed for the presence of anti-KEX1 IgG, IgA and IgM. Increases in anti-KEX1 titers in the BAL fluid occurred at approximately the same time points as in plasma samples (Fig. 4 and 5), and followed a similar dynamic with respect to class switching, despite low peripheral blood CD4+ T cell levels.

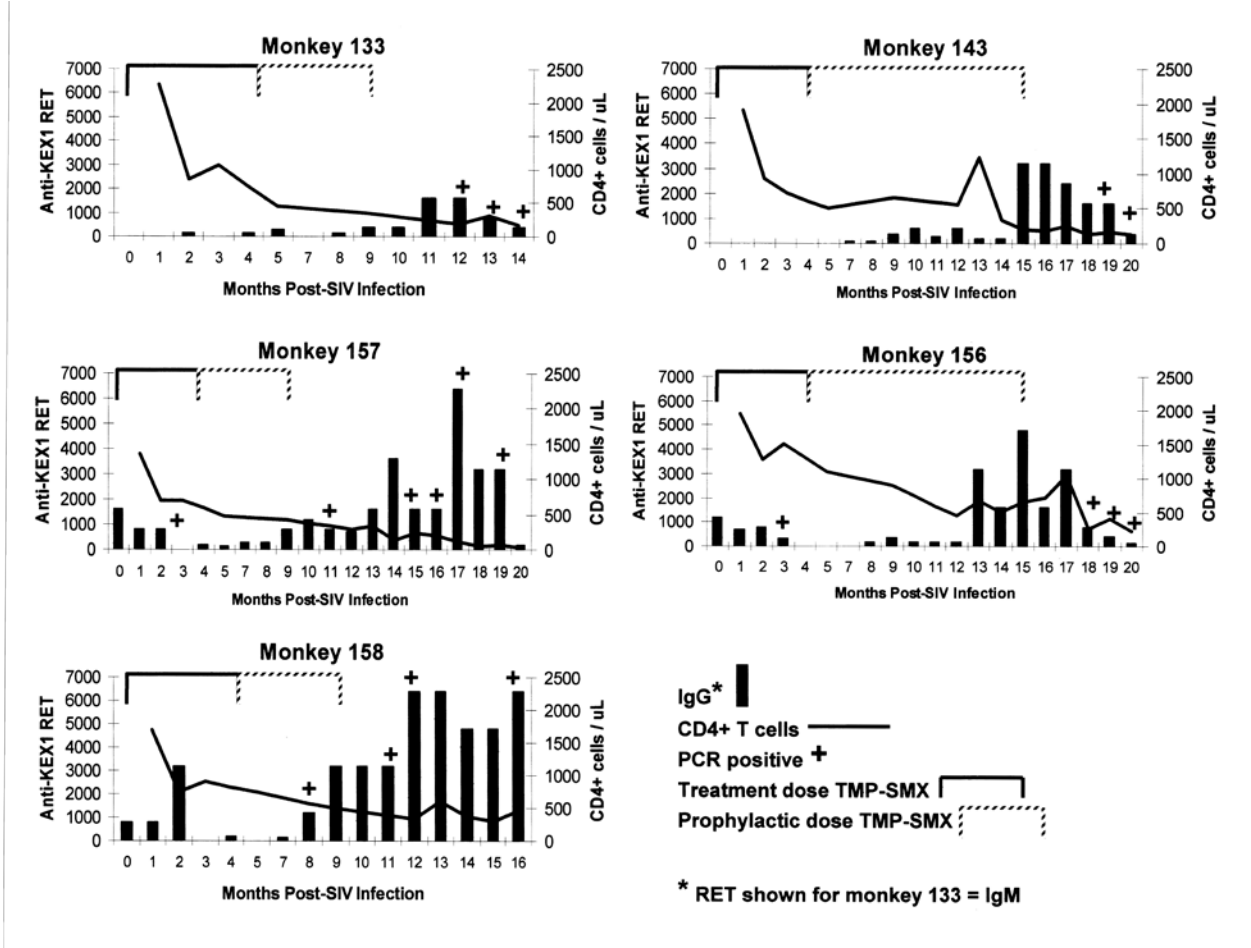

Figure 3. Natural Pc colonization of SIV-infected macaques. Following SIV infection, blood and bronchoalveolar lavage (BAL) fluid were collected at monthly intervals. Anti-KEX1 IgG (for monkeys 143, 156, 157 and 158) or IgM (for monkey 133) (solid black bar) reciprocal endpoint titers (RET) of individual monkeys are shown on the left y-axis and peripheral blood CD4+ T cell numbers (solid black line) are shown on the right y-axis. Detection of Pc DNA by nested PCR of BAL samples is indicated by +. The duration of trimethoprim-sulfamethoxazole (TMP-SMX) treatment (solid black bracket) or prophylaxis (segmented bracket) are shown.

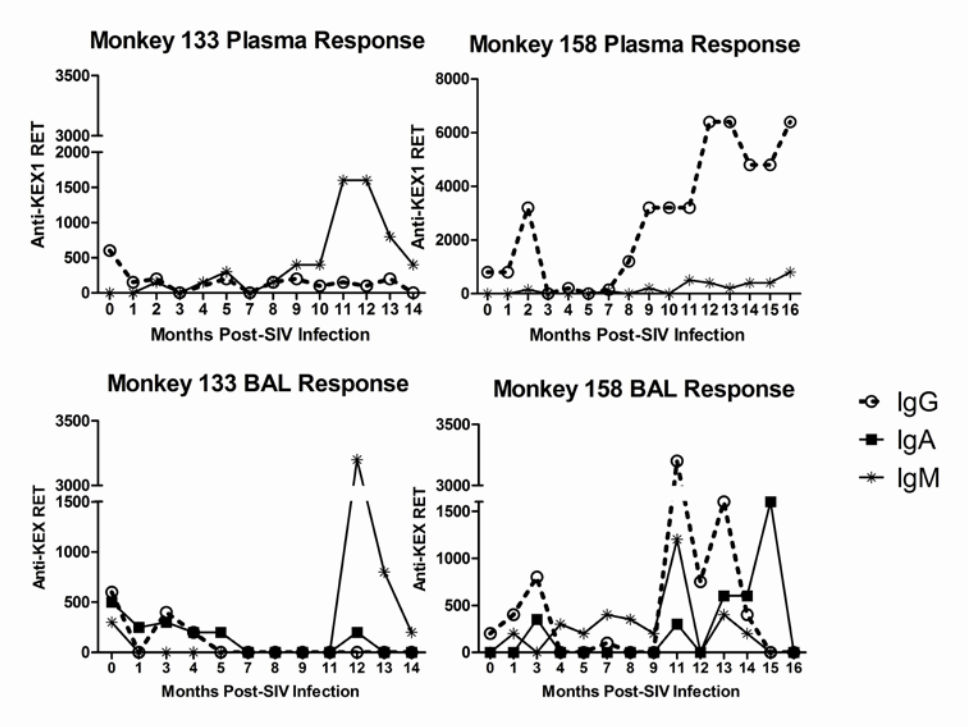

Figure 4. Comparison of anti-KEX1 antibody response in the bronchoalveolar lavage (BAL) fluid supernatant with anti-KEX1 antibody response in the plasma for two representative animals. Blood and BAL fluid supernatant were collected for each animal at monthly time-points following SIV-infection. Anti-KEX1 IgM (-*-), IgG (-○-) and IgA (-■-) reciprocal endpoint titers (RET) were determined.

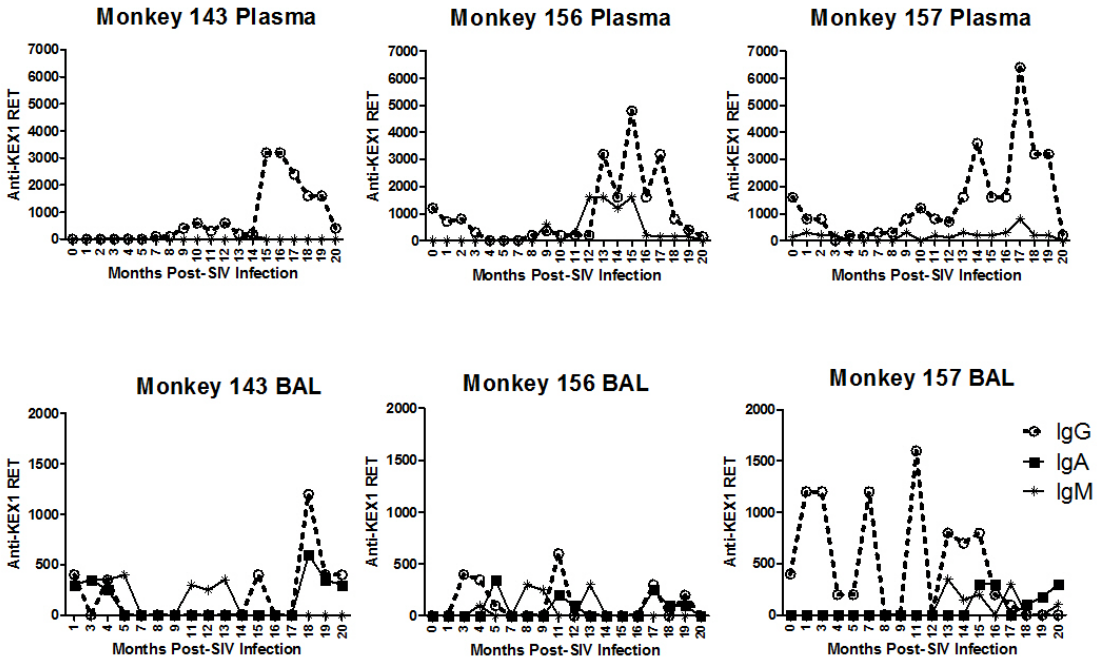

Figure 5. Comparison of anti-KEX1 antibody response in the bronchoalveolar lavage (BAL) fluid supernatant with anti-KEX1 antibody response in the plasma for three SIV-infected macaques. Blood and BAL fluid supernatant were collected for each animal at monthly time-points following SIV-infection. Anti-KEX1 IgM (-*-), IgG (-○-) and IgA (-■-) reciprocal endpoint titers (RET) were determined.

2.5 DISCUSSION

We have developed an anti-Pc antibody ELISA to evaluate Pc colonization and established a co-housing model of Pc colonization in SIV-infected macaques. Using these tools, we found: the majority of immunocompetent adult cynomolgus macaques have detectable anti-Pc antibody titers; transient Pc colonization occurs in healthy macaques and can be monitored by anti-KEX1

serology and nested PCR of BAL samples; co-housing of SIV-infected macaques with SIV/Pc co-infected animals results in Pc colonization which is not prevented by anti-Pc prophylaxis; and anti-KEX1 seroconversion precedes detection of Pc by nested PCR of BAL samples.

In the present study, we used a serologic assay based on a recombinant fragment of Pc kexin. Pc kexin shares sequence homology with a family of fungal serine endoproteases, and in *P. jirovecii* (human-derived Pc), kexin is encoded by a single copy gene (Lugli et al., 1997; Lee et al., 2000; Kutty and Kovacs, 2003). Thus, as a serologic target KEX1 does not present the complexity of multiple gene copies and antigenic variation associated with MSG (Kovacs et al., 1993; Smulian et al., 1997). Immune responses to Pc kexin have been associated with control of Pc infection in experimental models (Zheng et al., 2005; Wells et al., 2006a), thus changes in anti-KEX1 titers and antibody isotypes during and after infection may serve as a useful correlate of protection.Human prevalence studies are limited in ability to correlate antibody levels with Pc colonization and exposure due to difficulty of obtaining serial samples of blood and BAL in subjects. The non-human primate model enables evaluation of anti-Pc antibody responses in serial samples and correlation of results with detection of Pc DNA in BAL samples in both immunocompetent and immunosuppressed animals. We found that approximately 95% of healthy macaques had detectable plasma anti-KEX1 reciprocal endpoint titers, ranging from 100 to >12,800, suggesting the majority of healthy macaques were exposed to Pc. Similar frequencies of anti-Pc antibodies other than kexin have been documented in human epidemiologic studies, with up to 84% of adults having antibodies to Pc (Peglow et al., 1990; Wakefield et al., 1990a; Smulian et al., 1993; Lundgren et al., 1997; Walzer, 1999; Daly et al., 2002; Walzer, 2005). We also found that normal monkeys developed transient colonization as defined by an increase in anti-KEX1 titers followed by detection of Pc DNA by nested PCR of

BAL fluid. Antibody titers subsequently decreased and Pc DNA was no longer detectable, indicating normal monkeys become transiently colonized and are able to clear the organism. Similar results have been reported in immunocompetent rodents when exposed to immunosuppressed rodents with PCP (An et al., 2003; Gigliotti et al., 2003).

Previously, we showed that SIV-infected macaques are susceptible to Pc infection when intrabronchially inoculated with macaque-derived Pc (Board et al., 2003). Because Pc cannot be cultured *in vitro*, one drawback of earlier studies was the use of Pc derived from BAL fluid obtained from SIV-infected monkeys with fulminant PCP for infection initiation. Although inocula were enriched for Pc, introduction of alloantigen and SIV as a component of the Pc inocula could not be excluded and increased the likelihood of transient, non-specific inflammatory responses. Additionally, the inoculum used in previous studies was a high dose of Pc, which facilitated progression to PCP in these monkeys.

For these reasons and to investigate the earliest points following Pc colonization, we examined natural acquisition of Pc during SIV infection. Pc colonization status was evaluated prior to SIV infection, and although nested PCR of BAL was negative for all monkeys, 4 of 5 monkeys had detectable anti-KEX1 IgG titers ranging from 600 to 1600 (Fig. 2 and 3). Detection of anti-KEX1 antibodies in monkeys without detectable PCR products in BAL may reflect prior Pc exposure or current colonization below the detectable limit of nested PCR. To clear potential Pc colonization at the start of SIV infection, all monkeys received treatment doses of TMP-SMX for 4 months, which resulted in undetectable antibody titers and negative nested PCR of BAL for Pc DNA. Monkeys then continued on a prophylactic dose of TMP-SMX to prevent Pc colonization until peripheral blood CD4+ T cells declined to a level predicted to allow colonization. A rise in anti-KEX1 titers subsequently occurred in all SIV-infected monkeys

followed by detection of Pc DNA in BAL samples, indicative of newly acquired Pc colonization. Interestingly, we observed a serial rise in anti-Pc antibody titers and positive PCR results in some animals during the period of TMP-SMX prophylaxis, indicating this regimen did not adequately prevent Pc colonization. These results support human epidemiologic studies that showed the risk of Pc colonization was not related to prophylaxis (Huang et al., 2003; Morris et al., 2004a). While Pc prophylaxis may reduce organism burden sufficiently to prevent active PCP, the results suggest Pc colonization may persist or recur during prophylaxis. In addition, the serial rise in anti-KEX1 titers occurred prior to detection of Pc DNA in BAL, suggesting the serial KEX1 ELISA is a more sensitive indicator of early Pc colonization in this model.

These results indicate co-housing of SIV-infected primates is an efficient method to establish colonization and will allow us to examine long-term consequences of Pc colonization in a model of AIDS. Development of this model is important because a large number of HIV-infected persons appear to be colonized with Pc and effects of such colonization are unknown (Huang et al., 2003; Morris et al., 2004a). Given the high rate of seropositivity of normal monkeys in this study, it is likely Pc colonization of SIV-infected macaques represents secondary exposure and the antibody response is a secondary response rather than a primary response. This is likely the case in HIV-infected subjects, thus this model may be used to address questions regarding the effect of HIV on the development and strength of memory responses to opportunistic pathogens. Although antibody responses were predominantly IgG, one monkey had an IgM response (Fig. 3-5). Since this monkey had detectable anti-KEX1 IgG titers at baseline, the IgM production may reflect exposure and response to a new Pc strain.

While all SIV-infected monkeys became colonized with Pc and colonization appeared to persist, none developed PCP during the course of this study (>15 months) despite the fact that

none of the monkeys received treatment or prophylaxis for Pc after becoming colonized. These results contrast to our previous studies using intrabronchial inoculation of Pc in SIV-infected macaques, where most monkeys developed PCP (Board et al., 2003). This discrepancy may be due to the larger inoculum used in previous studies compared to the low level of Pc exposure that likely occurs during co-housing. In addition, it may be that the duration of the study was insufficient to allow for development of PCP from low-level colonization. Others have shown development of PCP in SIV-infected, co-housed macaques was associated with duration of SIV infection (Vogel et al., 1993). While Pc colonization was detected at necropsy in monkeys SIV-infected for less than 2 years, development of PCP was significantly higher in monkeys infected between 2 and 4 years (Vogel et al., 1993). Likewise, our laboratory and others have reported development of active PCP in an SIV model (Baskin et al., 1988; Baskerville et al., 1991; Furuta et al., 1993; Yanai et al., 1999; Croix et al., 2002; Board et al., 2003), though development of Pc colonization in SIV-infected macaques has not been addressed.

Apparent control of Pc infection in these monkeys might also be due to development of a strong IgG response in the majority of monkeys, despite progressive immunosuppression due to SIV. We have previously reported development of an anti-Pc humoral response in SIV-infected macaques and ability to undergo immunoglobulin class switching may contribute to control of Pc and conversely, a diminished secondary humoral response may be associated with progression to PCP (Board et al., 2003). Although the precise role of antibodies in Pc infection is not well-defined, experimental immunization studies in rodent models of Pc infection indicate the humoral response plays an important role in prevention of PCP (Harmsen et al., 1995; Garvy et al., 1997; Gigliotti et al., 1998a; Gigliotti et al., 1998b; Gigliotti et al., 2002; Empey et al., 2004; Wells et al., 2006a). Control of experimental Pc infection can be enhanced by either

establishment of a Pc-specific antibody response prior to CD4+ T cell depletion sufficient to resolve infection (Harmsen et al., 1995; Garvy et al., 1997; Theus et al., 1998; Pascale et al., 1999; Zheng et al., 2005; Wells et al., 2006a) or by passive transfer of polyclonal immune sera or monoclonal Pc-specific antibodies (Gigliotti et al., 2002; Empey et al., 2004). The immune responsiveness of SIV-infected monkeys to Pc colonization is an important finding since it suggests that although monkeys are susceptible to Pc colonization during SIV infection, they are capable of mounting a strong humoral response even with diminished CD4+ T cell help. The KEX1 ELISA is also useful in distinguishing primary and secondary humoral responses and evaluating the role of a secondary response in preventing PCP in SIV-immunosuppressed monkeys.

In summary, we have developed an antibody test for Pc colonization that appears to be more sensitive than PCR. Using this test, we found the majority of immunocompetent monkeys have detectable anti-Pc antibody titers and transient Pc colonization occurs in these animals. Further, we have established a reliable, efficient model of Pc colonization induced by co-housing of monkeys in the setting of AIDS-like immunodeficiency. Although PCP treatment was able to clear colonization, colonization still developed in these animals despite administration of PCP prophylaxis. These studies have direct clinical relevance to HIV-infected patients as Pc-colonization may occur despite use of Pc prophylaxis and HAART (Morris et al., 2004a).

While active PCP has been described in SIV-immunosuppressed monkeys by our laboratory (Croix et al., 2002; Board et al., 2003) and others (Baskerville et al., 1991; Furuta et al., 1993; Vogel et al., 1993; Yanai et al., 1999), the present model of Pc colonization will be useful in studies examining the role of the immune response in development and progression of colonization and in examining long-term effects of colonization on the lung. By defining

circumstances associated with early Pc colonization we will be able to address specific questions regarding transmission, clearance, susceptibility and clinical consequences of persistence of the organisms in an AIDS model.

2.6 AUTHOR CONRIBUTIONS AND ACKNOWLEDGEMENTS

Heather M. Kling (Molecular Virology and Microbiology Graduate Program, University of Pittsburgh School of Medicine) generated the majority of the data and prepared the manuscript. Timothy W. Shipley (Immunology Graduate Program, University of Pittsburgh School of Medicine) provided assistance with macaque blood and bronchoalveolar lavage fluid processing and sample analysis. Sangita Patil (Department of Immunology, University of Pittsburgh School of Medicine) provided assistance with sample collection and analysis, and generated PCR and flow cytometry data. Alison Morris (Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh) assisted in providing critique of the project and the manuscript. Karen A. Norris (Department of Immunology, University of Pittsburgh School of Medicine), as the mentor and principal investigator on the project, provided extensive scientific knowledge, insight and critique of the project and the manuscript. All authors, especially Karen A. Norris, contributed to scientific discussion regarding the project and critical reading and editing of the manuscript.

The authors acknowledge Anita Trichel, D.V.M, Ph.D. and Nicole Banichar, C.V.T. for excellent veterinary care, Dr. Michael Murphey-Corb for assistance in SIV inoculations and Dr. Margaret Beucher for critically reviewing this manuscript.

3.0 PNEUMOCYSTIS HUMORAL IMMUNITY PROTECTS AGAINST COLONIZATION AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE IN A PRIMATE MODEL OF HIV INFECTION

This chapter is being prepared for submission to *Infection and Immunity*. The authors are Heather M. Kling, Timothy W. Shipley, Sangita Patil, Jan Kristoff, Marianne Bryan, Ronald C. Montelaro, Alison Morris, and Karen A. Norris.

3.1 ABSTRACT

Pulmonary colonization by the opportunistic pathogen *Pneumocystis* (Pc) is common in HIV+ subjects and has been associated with the development of chronic obstructive pulmonary disease (COPD). Host and environmental factors associated with susceptibility to colonization are undefined. Using a simian-human immunodeficiency virus (SHIV) model of HIV infection, immunologic parameters associated with natural transmission of Pc colonization were evaluated. Two independent experiments were conducted in which SHIV infected macaques were exposed to Pc transmission by co-housing with immunosuppressed, Pc colonized macaques. Serial plasma and bronchoalveolar lavage (BAL) fluid samples were examined for changes in anti-Pc antibodies titers to recombinant Pc kexin protein (KEX1) and evidence of Pc colonization by nested PCR of BAL fluid samples. In Experiment 1, ten of fourteen monkeys became colonized with Pc (Pc+) by 8 weeks post-SHIV infection, while four animals remained Pc-negative (Pc-) throughout the duration of the study. In Experiment 2, eleven of seventeen animals became colonized with Pc by 16 weeks post-SHIV infection, while six monkeys remained Pc-. Baseline plasma anti-KEX1 IgG titers were significantly higher in monkeys that did not become Pc-colonized, compared with Pc-colonized monkeys, in Experiment 1 ($p=0.013$) and in Experiment 2 ($p=0.022$). Pc-negative monkeys had greater percentages of Pc-specific memory B cells following SHIV-infection, compared with Pc-colonized monkeys ($p=0.037$). SHIV-infected, Pc-colonized monkeys developed progressive obstructive pulmonary disease, while monkeys infected with SHIV alone maintained normal lung function throughout the study. These results support the concept of a protective role for anti-KEX1 humoral response in preventing Pc

colonization and the development of obstructive lung disease in the SHIV model. In addition, these results indicate that an effective Pc-specific memory B cell response is maintained despite progressive loss of CD4+ T cells during SHIV infection.

3.2 INTRODUCTION

Despite the introduction of anti-retroviral therapy (ART) and advances in treatment strategies, pulmonary diseases remain a leading cause of morbidity and mortality in HIV infected patients (Louie et al., 2002). Both emphysema and chronic obstructive pulmonary disease (COPD) have been reported at an increased frequency in HIV-infected patients (Diaz et al., 2000b; Crothers et al., 2006), and unlike many AIDS-associated opportunistic infections, HIV-associated COPD may be increasing due to prolonged life expectancy of the HIV+ population with ART, and the high smoking rate in this population (Collins et al., 2001; Gritz et al., 2004). While cigarette smoking is a primary risk factor for the development of COPD, it is interesting to note that HIV+ non-smokers may also be at increased risk of disease (Diaz et al., 1992). In addition, the observation that most smokers do not develop the COPD (Lokke et al., 2006), indicates that other factors may play a role in disease development.

Evidence has accumulated suggesting a role for infectious agents as co-factors in the pathogenesis and exacerbation of COPD (Sethi and Murphy, 2008), where pulmonary inflammation due to cigarette smoke may be amplified by the presence of persistent infectious agents. The chronic inflammation associated with infectious agents is also thought to contribute to the development of COPD in HIV+ patients (Morris et al., 2008b; Sethi and Murphy, 2008).

This possibility is highlighted by the observation that HIV infected patients may be more prone to develop subclinical lung infections even if successfully treated with ART (Diaz et al., 2003).

Our laboratory and others have accumulated evidence in humans and in animal models that the fungal opportunistic pathogen, *Pneumocystis jirovecii* (formerly *Pneumocystis carinii* f. sp. *hominis*) is an important pathogen in COPD in both HIV+ and HIV- populations. COPD-like changes have been reported in HIV+ patients with *Pneumocystis* pneumonia (PcP) (Morris et al., 2000), and recent studies suggest that low level asymptomatic carriage of Pc may be associated with lung damage. An increased frequency of Pc colonization has been reported in HIV+ patients, including those on ART (Morris et al., 2004a), and we have recently shown that HIV+ subjects that were Pc colonized have worse airway obstruction compared to HIV+ subjects who were Pc-negative (Morris et al., 2009). An association between Pc colonization and COPD has also been shown in HIV- subjects (Calderon et al., 1996; Probst et al., 2000; Morris et al., 2004c).

Animal models also support the role of Pc colonization in the pathogenesis of COPD. In a model of immunocompetent mice, cigarette exposure and Pc colonization resulted in greater pulmonary function deficits compared to cigarette exposure alone (Christensen et al., 2008). Our laboratory has reported that in a simian immunodeficiency virus (SIV)- primate model of HIV infection, Pc colonization results in pulmonary inflammation, pulmonary function deficits, and anatomic emphysema (Croix et al., 2002; Board et al., 2003; Norris et al., 2006; Shipley, et al., in press). Despite the clear evidence that Pc colonization is associated with development of obstructive lung disease, particularly among HIV+ patients, factors the influence susceptibility to Pc colonization are not clearly understood.

Immunologic control of Pc infection is strongly correlated with CD4+ T cell responses, although B cells and antibodies also play a role in prevention of PcP (Harmsen et al., 1995; Marcotte et al., 1996; Garvy et al., 1997; Gigliotti et al., 1998a; Gigliotti et al., 1998b; Gigliotti et al., 2002; Empey et al., 2004; Wells et al., 2006a). There is a high frequency of Pc-specific seroprevalence in immunocompetent adults (Daly et al., 2002; Bishop and Kovacs, 2003), as well as in non-human primates (Demanche et al., 2005; Kling et al., 2009), suggesting the persistence of serological memory or Pc-specific, long-lived plasma cells in response to natural Pc exposure. Antibodies to the Pc endoprotease kexin (KEX1) may be particularly important, because immune responses to Pc KEX1 have been associated with control of Pc infection in immunosuppressed murine models (Zheng et al., 2005; Wells et al., 2006a).

In the current study, we investigated the capacity of simian/human immunodeficiency virus (SHIV)-infected macaques to generate a humoral immune response to KEX1 in response to natural Pc exposure and examined the relationship between anti-Pc humoral immunity, the development of Pc-colonization, and the development of COPD.

3.3 MATERIALS AND METHODS

3.3.1 Animals.

Adult, Chinese origin cynomolgus macaques (*Macacca fasicularis*), weighing between 5-8 kg, were used in this study**.** All animals were purchased from National Primate Centers or vendors approved by the University of Pittsburgh, Department of Laboratory Animal Research. Prior to admission to the study, all animals underwent complete physical examination (pulmonary and

cardiac auscultation, thoracic radiographs, computer tomography scanning, tuberculin skin testing, complete blood count, chemistry panel, urinalysis, and flow cytometric analysis of peripheral blood mononuclear and BAL cells) and were screened for simian retroviruses (SIV, SRV, and STLV) to verify that they were free of any pre-existing disease. The animals were housed in an American Association for Accreditation of Laboratory Animal Care-accredited, biosafety level 2+ primate facility at the University of Pittsburgh. Animal husbandry and experimental procedures were conducted in accordance with standards set forth by the Guide for the Care and Use of Laboratory Animals (ref. National Research Council, 1996) and the Provisions of the Animal Welfare Act. Prior to the initiation of this study, all animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

3.3.2 Study Design.

Two independent experiments were performed using 14 monkeys (Experiment #1) and 17 monkeys (Experiment #2). Monkeys were intravenously inoculated with $1x10^{4.9}$ $TCID_{50}$ (50% tissue culture infectious doses) of $SHIV_{89.6P}$ (gift of Dr. Opendra Narayan, University of Kansas), which induces CD4+ T cell lymphopenia and AIDS-like disease with wasting and opportunistic infections (Reimann et al., 1996; Pawar et al., 2008). To facilitate natural transmission of Pc, SHIV-infected macaques were continuously exposed by co-housing in the same room with 10-20 SIV- or SHIV-immunosuppressed macaques which served as a Pc source (Kling et al., 2009). None of the macaques (source or recipients) contracted acute *Pneumocystis* pneumonia (PcP) during the study. Determination of Pc colonization status was performed by detection of Pc DNA in the BAL fluid samples by nested PCR and by anti-Pc KEX1 serology

(Board et al., 2003; Kling et al., 2009). Pc colonization was defined as a positive nested PCR of BAL fluid and at least a 3-fold change in plasma anti-Pc KEX1 titers (Board et al., 2003; Kling et al., 2009). Additionally, BAL samples were stained for organisms by modified Geimsa and silver staining (Board et al., 2003).

3.3.3 BAL and blood collection.

Peripheral blood and BAL samples were collected at baseline on all animals. Serial plasma and PBMC samples from SHIV-infected monkeys were collected weekly for the first 8 weeks following SHIV-infection and monthly thereafter. BAL fluid samples were collected monthly. Samples were collected and processed as described previously (Board et al., 2003; Kling et al., 2009). Briefly, plasma was isolated from 10mL of EDTA (ethylenediaminetetraacetic acid)-treated whole blood by centrifugation; PBMC were purified over a Percoll gradient (Amersham Bioscience, Piscataway, NJ) and washed with sterile phosphate buffered saline (PBS) (Shipley et al., in press). Plasma aliquots were stored at -80°C prior to assay. PBMC were counted, stained and fixed for analysis by flow cytometry. BAL fluid was processed for cell isolation as previously described (Croix et al., 2002; Kling et al., 2009). Unfractionated BALF aliquots were used for bacterial, fungal and viral culture (Antech Diagnostics, Pittsburgh, PA) and PCR detection of Pc DNA. Remaining fluid was filtered through a 40-micron cell strainer and manual cell counts were performed using a hemocytometer. 1×10^5 cells were removed and stained with modified Giemsa stain (Dade Behring, Newark, DE) and differential counts performed manually (Croix et al., 2002). Remaining cells were pelleted and

supernatant fluid was collected and stored at -80°C. Recovered cells were prepared for flow cytometry as described (Croix et al., 2002; Board et al., 2003).

3.3.4 Endpoint antibody titer determination.

A partial fragment of the macaque-derived, Pc kexin gene in the pBAD expression vector (gift from C.G. Haidaris, University of Rochester) (Kling et al., 2009) was used to produce recombinant kexin for ELISA. ELISA was performed as previously described (Kling et al., 2009). Plasma and BAL fluid supernatant were heat inactivated (56°C, 30 min) prior to use in ELISA for detection of Pc-specific antibodies. BAL fluid samples were normalized based on plasma urea concentration (Rennard et al., 1986). Microtiter plates (Immunolon 4HBX, Thermo Fisher Scientific, Inc., Waltham, MA) were coated with purified KEX1 at 5 μg/ml in Na_2CO_3, (pH 9.5). Heat-inactivated plasma samples were diluted 1:100 in blocking buffer (PBS with 5% non-fat milk). BAL fluid supernatant samples were diluted according to normalized concentrations in PBS. Fifty μL of diluted plasma or BAL fluid supernatant were plated into KEX1-coated wells and serial dilutions were made to determine endpoint titers. Goat anti-monkey immunoglobulin-conjugated horseradish peroxidase (1:10,000 for IgG, 1:2000 for IgM) (Nordic Immunology, Tilburg, the Netherlands) was used for detection and plates were developed by standard methods. Normal (uninfected, Pc-negative by antibody titer) macaque plasma or BAL fluid supernatant was used as negative control and sample from a monkey with PCP was used for positive control. The reciprocal endpoint titer was calculated as the highest dilution at which the optical density (O.D.) values for the test sample were the same or less than the normal sample O.D.

3.3.5 Nested PCR of BAL fluid.

BAL fluid cell lysate samples were analyzed for the presence of Pc DNA by nested PCR of the mitochondrial large subunit ribosomal RNA gene (mtLSU), as described (Board et al., 2003; Patil et al., 2003). Nested PCR was performed on 5 μl of the first round product using primers P1 and P2 (Savoia et al., 1997). PCR for β-globin was also performed on BAL samples to control for DNA quality (Croix et al., 2002).

3.3.6 Flow Cytometry.

Stained, fixed cells from whole blood and BAL fluid were analyzed by flow cytometry (Croix et al., 2002). The following antibodies were used: mouse anti-monkey CD3-fluorescein isothiocyanate (clone SP34), mouse anti-human CD8-Pacific Blue (clone RPA-T8) and mouse anti-monkey CD4-allophycocyanin (clone L200), all purchased from BD Pharmingen (San Diego, CA). Acquisition was performed on BD LSRII flow cytometer using BD FacsDiva software. Forward/side scatter dot plot was used to gate the live lymphocyte population. All analyses were performed using FlowJo flow cytometry analysis software (Tree Star Inc., Ashland, OR).

3.3.7 Plasma SHIV viral load determination.

Virus loads in plasma and BAL fluid supernatant were determined as described elsewhere (Pawar et al., 2008). Briefly, RNA was extracted from plasma and BAL fluid supernatant and

was quantified as RNA copies per ml using an adapted protocol for quantitative real-time reverse transcriptase PCR detecting the SIV *gag* sequence.

3.3.8 Determination of SHIV-antibody titers.

Anti-Gag antibody titers were measured by ELISA in serial plasma samples. Samples were assayed as described elsewhere (Pawar et al., 2008) for antibody response to SIV core Gag protein. Briefly, ELISA plates were coated with detergent-disrupted SIV-B7, washed and blocked. Serial dilutions of monkey plasma were made to determine endpoint titers. Anti-Gag IgG atibodies were detected using anti-monkey IgG-HRP and TM blue substrate. Reactions were stopped with 1N sulfuric acid.

3.3.9 Enzyme-linked Immunospot (ELISPOT) assay for quantification of IgG- and KEX1-specific antibody secreting cells (ASC).

For detection of plasma cells, freshly-isolated PBMC were assayed directly *ex vivo*. ASC ELISPOT assays were adapted from a protocol published elsewhere (Crotty et al., 2004). Briefly, ELISPOT plates (non-sterile MultiScreen IP HTS plates, Milllipore Billerica, MA) were coated with either purified recombinant kexin protein (KEX1) (5µg/mL), Keyhole limpet hemocyanin (Pierce Imject mcKLH, Thermo-Scientifc, Rockford, IL) (2.5µg/mL), or affinity purified anti-monkey IgG (5µg/mL, Rockland, Inc., Gilbertsville, PA) at 4°C overnight. Plates were washed with PBS + 0.05% Tween-20 (PBS-T) (1x) and PBS (3x) then blocked in complete R-10 for at least 2 hours at 37°C, 5% CO_2. Blocking media was removed and $1x10^6$ PBMC were added in duplicate wells of the coated plates, and serial 3-fold dilutions were made in the

plate. PBMC were incubated at 37°C, 5% CO_2 overnight, and then cells were washed away with PBS and PBS-T (4x each). For detection, biotin-conjugated secondary antibody (50µL per well, Rockland Inc.) was then added, diluted at 1:1000 in PBS-T + 10% FBS, and plates were incubated overnight at 4°C. Plates were removed, washed with PBS-T (5x), and 50µL of streptavidin-horseradish peroxidase (BD Biosciences, San Diego, CA) diluted 1:1000 in PBS-T + 10% FBS was added to each well, followed by incubation for 60 minutes at room temperature. Plates were washed and then developed with AEC substrate (BD Biosciences, San Diego, CA) (50µL per well) in the dark for 8-15 min at room temperature. Plates were washed with water and then air-dried overnight while protected from light exposure. Plate images were acquired using Immunospot CTL plate reader (CTL Technologies Limited, Shaker Heights, OH) and Image Acquisition 4.5 software (CTL Technologies Ltd.). Spots were enumerated using Immunospot 5.0 Professional software (CTL Technologies Ltd.). KEX1-specific plasma cells are reported as ASC (number of spots) per $1x10^6$ PBMC.

3.3.10 Memory B cell ELISPOT.

PBMC from animals in Experiment #2 were assayed following 6-7 day stimulation, as reported elsewhere (Crotty et al., 2004). Briefly, cells were freshly isolated from whole blood and washed with PBS. Cells were cultured in complete GIBCO RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and Pen/Strep (R-10) in 24-well plates at a concentration of 0.5×10^6 cells per well with the following: 1:10,000 *Staphylococcus aureus* protein A Cowan strain (SAC, Sigma-Aldrich, St. Louis, MO), *Phytolacca americana* pokeweed mitogen (PWM Emory Stock, gift from S. Crotty), and 2µg/mL CpG ODN 2006 (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'; Oligos, Etc., Wilsonville, OR). Cells were expanded

in culture for 6-7 days (37°C, 5% CO_2) to induce differentiation into plasma cells, washed with R-10, then assayed by ELISPOT for IgG- and KEX1-specific secreting cells, as described above. KEX1-specific memory B cells are expressed as a percentage of total IgG-secreting cells. PBMC were evaluated for KEX1-specific memory cells at approximately 9-12 months post-SHIV infection.

3.3.11 Pulmonary Function Testing.

To assess airflow obstruction, pulmonary function tests (PFT) were performed at baseline and every other month after SHIV infection using whole body plethysmography (Proskocil et al., 2005) and forced deflation technique (Shipley et al., in press). Intravenous propofol (7.5-12.5 mg/kg body weight) was used to anesthetize monkeys, and 2% lidocaine was given prior to intubation (3.5 mm endotracheal tube) to desensitize the oropharynx. Chest radiograph was performed to verify endotracheal tube placement, which was monitored using a disposable CO_2 detector (Nellcor Pedi-cap, Boulder, CO). Pulmonary function testing was performed using a Buxco whole body plethysmograph (Buxco Electronics, Inc., Sharon, CT), and data on flow rates and volumes were collected using the BioSystems for Maneuvers Software (Buxco Electronics, Inc.). Tests were considered valid when three measurements for forced vital capacity (FVC) were within 10% of each other.

For bronchodilator challenge, standard PFTs were performed, followed by administration of one pediatric dosette of nebulized albuterol (3 ml of 0.083% albuterol for a total dosage of 2.5 mg) (Nephron Pharmaceuticals Corp., Orlando, FL). Fifteen minutes after administration, PFTs were repeated and compared to baseline values.

3.3.12 Statistical Analyses.

Statistical analyses for both Experiments #1 and #2 were performed using Prism software or InStat software, both by GraphPad (La Jolla, CA). Unpaired two-tailed Student's *t*-test was used to compare Pc+ and Pc- monkeys, unless otherwise noted. Paired two-tailed student's *t*-test was used to compare different time-points with baseline values. When comparing Pc+ and Pc- monkeys over multiple time-points, two-way repeated measures ANOVA was used for comparison. A p value of less than 0.05 was considered significant.

3.4 RESULTS

3.4.1 Natural Pc Colonization of SHIV-infected macaques.

Prior to SHIV-infection, macaques were determined to be negative for Pc colonization by nested PCR of BAL fluid. Baseline anti-Pc KEX1 plasma IgG reciprocal endpoint titers ranged from undetectable (<1:100) to 1:12,800 in macaques in Experiment 1 and undetectable to 1:22,400 in macaques in Experiment 2. In Experiment #1, 10 of 14 monkeys became naturally colonized with Pc (Pc+) by 8 weeks post-SHIV infection, while 4 monkeys remained Pc-negative (Pc-) throughout the study (53 weeks). In Experiment #2, 11 of 17 SHIV-infected monkeys became naturally colonized with Pc by 16 weeks post-SHIV infection, while six monkeys remained Pc- for the duration of the experiment (58 weeks).

In both Experiments 1 and 2, rapid declines in peripheral blood CD4+ T cells were observed within 2 to 4 weeks following SHIV-infection (Fig 6A, D). There was no difference

between Pc+ and Pc- monkeys in mean CD4+ T cell numbers during SHIV infection in Experiment 1 ($p=0.488$, 2-way repeated measures ANOVA) or in Experiment 2 ($p=0.326$, 2-way repeated measures ANOVA). Additionally, peak plasma viral titers were similar between Pc+ and Pc- monkeys in both Experiment 1 ($p = 0.749$, Fig 6B) and Experiment 2 ($p=0.595$, Fig 6E). No significant differences in anti-Gag antibody responses were observed in Pc+ and Pc- animals ($p=0.419$, Fig 6C), indicating that susceptibility to Pc colonization was not a result of a generalized humoral defect in the monkeys that became Pc-colonized or more severe immunosuppression.

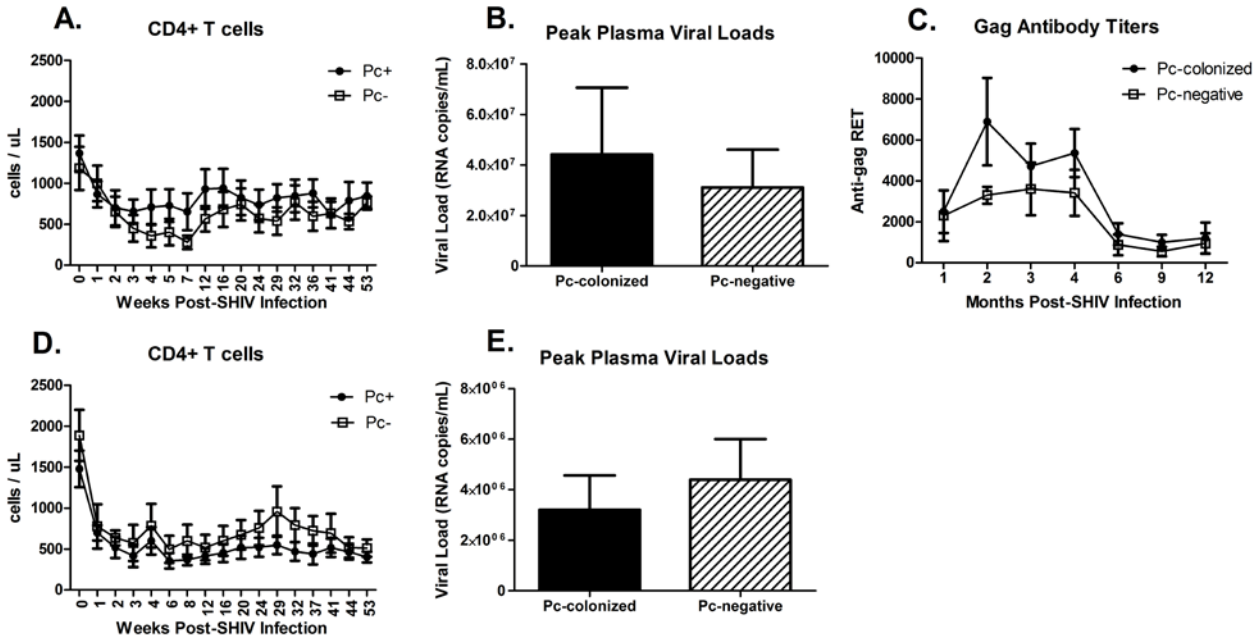

Figure 6. CD4+ T cell counts, peak plasma viral loads, and gag antibody responses were not significantly different between Pc-colonized and Pc-negative monkeys. No significant difference in mean CD4+ T cell counts in peripheral blood of Pc+ and Pc- monkeys in either Experiment 1 (p=0.488, 2-way ANOVA) (**A**) or Experiment 2 (p=0.326, **D**) were observed. Peak plasma viral titers were also similar between Pc+ and Pc- monkeys in both Experiment 1 (p = 0.749, **B**) and 2 (p=0.595, **E**). Pc+ and Pc- monkeys produced similar antibody responses to SHIV gag protein (p=0.419, 2-way ANOVA) (**C**).

3.4.2 Anti-KEX1 antibodies and KEX1-specific antibody secreting cells at baseline correlate with protection from *Pneumocystis* colonization.

Baseline anti-KEX1 endpoint titers were compared between macaques that became naturally Pc-colonized and those that remained Pc-negative post-SHIV infection. Baseline plasma KEX1 IgG

antibody titers were significantly higher in monkeys that did not become Pc-colonized, compared with Pc-colonized monkeys in both Experiments 1 ($p=0.013$, Fig 7A) and 2 ($p=0.022$, Fig 7B). These data suggest that a low KEX1-antibody titer prior to immunosuppression predicts susceptibility to Pc colonization, and that conversely, a high KEX1 titer at baseline correlates with protection. Baseline KEX1-IgG reciprocal endpoint titer (RET) of less than 10,000 was associated with Pc-colonization following immunosuppression ($p=0.011$, Fisher's exact test, Fig 7C). PBMC were examined for KEX1-specific antibody secreting cells by plasma cell ELISPOT, and a similar trend was observed. Pc- animals had significantly greater numbers of KEX1-specific antibody secreting cells at baseline than did animals that became Pc-colonized following SHIV infection ($p=0.018$, Fig 7D).

Representative longitudinal profiles of Pc anti-KEX1 antibody production, peripheral blood CD4+ T cells numbers, and nested PCR results are shown in Figure 8. Macaques with a low baseline anti-KEX1 titer (<1:4000) exhibited evidence of Pc colonization generally by 8-12 weeks post-SHIV inoculation (Fig 8A). These animals had increases in anti-KEX1 titers of at least three-fold over baseline, generally by 3-4 weeks post-SHIV infection. The rise in anti-KEX1 titers was followed by nested-PCR detection of Pc DNA in the BAL fluid, approximately 5-12 weeks later (Fig 8A, and data not shown). Macaques with high baseline anti-KEX1 titers (≥1:10,691), however, maintained a high titer throughout infection, but did not exhibit either a 3-fold increase over baseline titer or a positive nested PCR for Pc, which would indicate active colonization (Fig 8B).

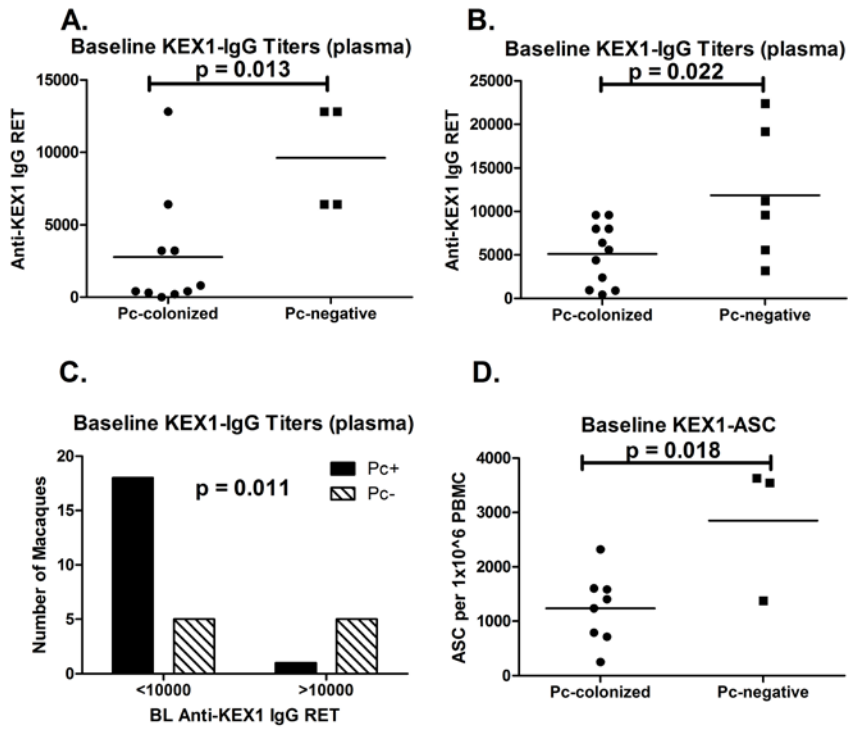

Figure 7. Baseline plasma anti-KEX1 IgG reciprocal endpoint titer and numbers of KEX-specific antibody secreting cells (ASC) predict *Pneumocystis* colonization following SHIV-immunosuppression. Comparative analysis of baseline anti-KEX1 IgG titers between monkeys that became colonized following SHIV-infection and monkeys that remained Pc-negative. Monkeys that remained Pc-negative had significantly higher baseline KEX-titers than monkeys that remained Pc-colonized in Experiment 1 (**A**), with similar results in Experiment 2 (**B**). Panel **C** indicates the numbers of macaques in the combined set of both experimental groups with baseline KEX1-IgG titers of less than and greater than 10,000 that became Pc-colonized following SHIV-infection. These data demonstrate that monkeys with a baseline KEX1-IgG reciprocal endpoint titer of less than 10,000 are significantly more likely to become Pc-colonized following immunosuppression than those with a titer of greater than 10,000 (C, p=0.011, Fisher's Exact Test). A subset of baseline samples from Experiment 1 was examined for KEX1-specific

ASC (**D**). Monkeys that that remained Pc-negative also had higher numbers of KEX1-specific ASC at baseline than monkeys that became Pc-colonized (p=0.018).

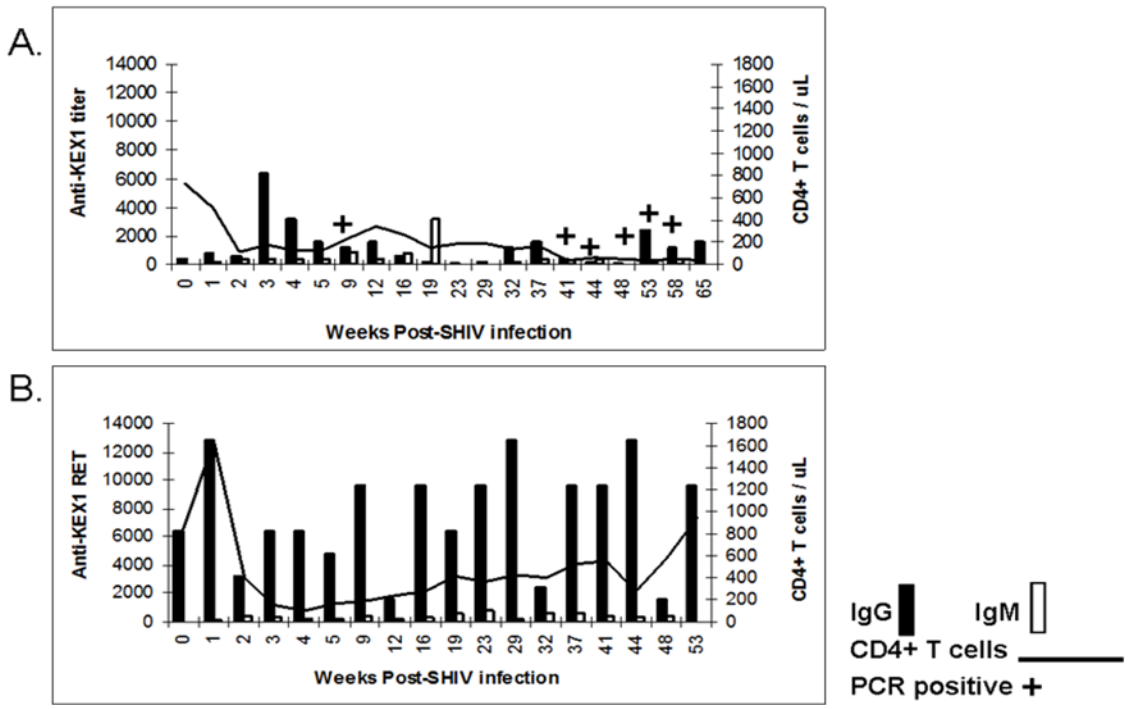

Figure 8. Representative KEX1-antibody production and CD4+ T cell profiles of a macaque with low baseline KEX1 titer (A) and a macaque with a comparably higher baseline KEX1-titer (B). Macaques with a low baseline anti-KEX1 titer exhibited evidence of Pc colonization quickly after SHIV-infection (A). These animals demonstrated increases of at least 3-fold over baseline anti-KEX1 titer, by 3-4 weeks post-SHIV infection, which was followed by nested PCR detection of Pc DNA in the BAL fluid, approximately 5-12 weeks later (**A**, and data not shown).Macaques with high baseline anti-KEX1 titers, however, maintained a high titer throughout infection, suggesting exposure to Pc, but did not exhibit either a 3-fold

increase over baseline titer or a positive nested PCR for Pc (**B**), and were therefore considered Pc-negative

3.4.3 Earlier detection of anti-KEX1 antibodies in the BAL fluid supernatant correlates with protection from Pc colonization.

To determine whether KEX1-specific antibodies could be detected in the BAL fluid supernatant and to evaluate the possible role of KEX1-specific antibodies in promoting protection from Pc colonization, BAL fluid supernatant anti-KEX1 titers were determined. (Fig 9). There was a significant positive correlation between baseline plasma anti-KEX1 IgG and peak BAL fluid IgA titer following SHIV-infection (Fig 9A, $p=0.043$, $R^2=0.279$). In addition, the kinetics of a class-switched anti-KEX1 response was examined. Earlier production of KEX1-specific IgA was observed in monkeys that resisted Pc-colonization (Fig. 9B, $p=0.041$, Fisher's exact test). Monkeys in which Pc KEX1-specific IgA antibodies were detectable in the BAL fluid by 4 weeks post-SHIV infection were less likely to become Pc colonized than monkeys that were not making Pc-specific IgA by this time-point (Fig 9B). The timing (detection post-SHIV infection) and levels of KEX1-specific IgG or IgM production in BAL was not significantly different between Pc+ and Pc- monkeys (data not shown).

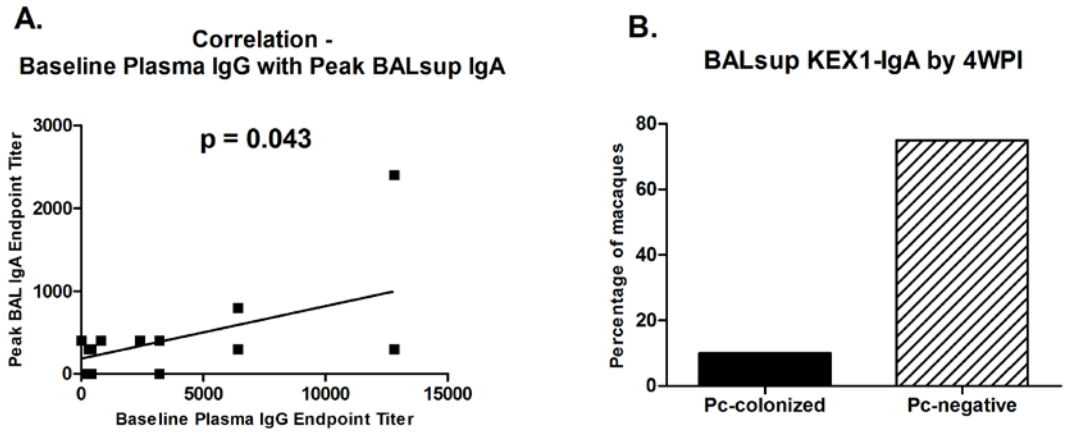

Figure 9. KEX1-IgA production in the BAL fluid. Baseline plasma KEX1-IgG titers positively correlate with peak KEX1-IgA titers in the BAL fluid (**A**, p=0.043). Seventy-five percent of Pc- macaques were producing KEX1-IgA in the BAL fluid by week 4 post-SHIV infection, compared with only 10 percent of Pc-colonized macaques that were production KEX1-IgA by this time-point (**B**, p=0.041, Fisher's Exact Test).

3.4.4 KEX1-specific memory response correlates with protection from Pc-colonization.

In Experiment 2, PBMC were evaluated to assess the Pc-specific memory response in SHIV-infected monkeys. PBMC were evaluated at approximately 9-12 months post-SHIV infection; Pc- monkeys had significantly higher percentages of KEX1-specific memory B cells than Pc+ monkeys (Fig 10). These results suggest that a stronger Pc-specific memory response persists during SHIV infection and correlates with protection from natural Pc-colonization in immunosuppressed macaques.

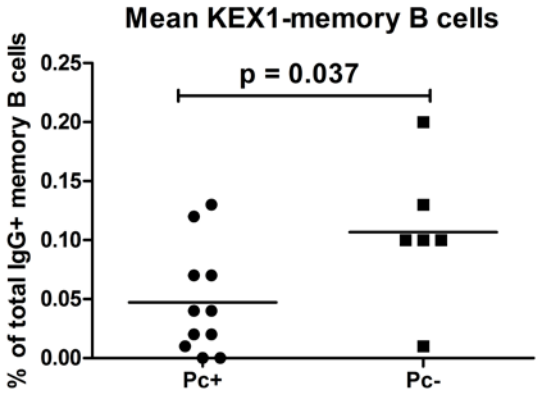

Figure 10. Percentages of peripheral blood KEX1-specific memory B cells were significantly higher in Pc- monkeys than in Pc+ monkeys (p=0.037)**.** PBMC from macaques in Experimental group #2 were isolated at 9-12 months post-SHIV infection and stimulated in culture for 6-7 days and assayed by B cell ELISpots for total IgG and KEX1-specific IgG-secreting cells. KEX1-specific cells are expressed as a percentage of total IgG-secreting cells. Mean percentages of KEX1-specific memory cells for Pc- monkeys were significantly higher than for Pc+ monkeys (p=0.037, Student's *t*-test).

3.4.5 Pc-colonized monkeys exhibit evidence of pulmonary obstruction.

Previous studies have shown an association between persistent Pc colonization and COPD in HIV and non-HIV infected human subjects and in animal models (Morris et al., 2004c; Morris et al., 2008a; Morris et al., 2009); Shipley et al, in press). We therefore investigated whether Pc colonization correlated with preserved lung function in long-term SHIV-infected macaques. Pulmonary function tests were performed at baseline and at 1-2 month intervals, up to 50 weeks post-SHIV infection. Declines in peak expiratory flow

(PEF) and forced expiratory volume in 0.4 seconds ($FEV_{0.4}$) were evaluated to assess progression of airway obstruction. Declines in these parameters are indicative of obstructive disease.

In Experiment 1, 6 of 8 Pc+ monkeys demonstrated significant decreases in PEF and $FEV_{0.4}$ from baseline, whereas no significant declines from baseline were observed in Pc- monkeys (Fig. 11A, B). PEF in Pc+ monkeys declined from 526.8 ml/s at baseline to 452.9ml/s at study endpoint (p=0.020), compared with Pc- monkeys who did not exhibit a significant change from baseline (p=0.854). Pc+ monkeys declined significantly in $FEV_{0.4}$, from a mean of 188.6mL at baseline to 165.8mL at 10 months post-SHIV infection (p=0.023), whereas Pc- monkeys did not exhibit significant declines in $FEV_{0.4}$ (Fig 11B).

In Experiment 2, Pc+ monkeys also showed significant declines in pulmonary function parameters from baseline levels. From baseline to 10 months post-SHIV infection, Pc+ monkeys declined from a mean PEF of 555.3mL/s to 484.4mL/s (p=0.002), while Pc- monkeys did not decline significantly from baseline (p=0.320, Fig 11C). $FEV_{0.4}$ values exhibited a similar trend, with a mean of 198.1mL at baseline to 173.0mL at 10 months post-SHIV infection for the Pc+ monkeys (p=0.002, Fig 11D). Again, no significant changes were observed for the Pc- group of animals (p=0.122, Fig 11D).

We also examined whether administration of the bronchodilator, albuterol, affected pulmonary function because airflow limitation associated with COPD is poorly reversible in response to bronchodilator treatment. No significant differences were

observed for either group in any pulmonary function measurement post-treatment (data not shown).

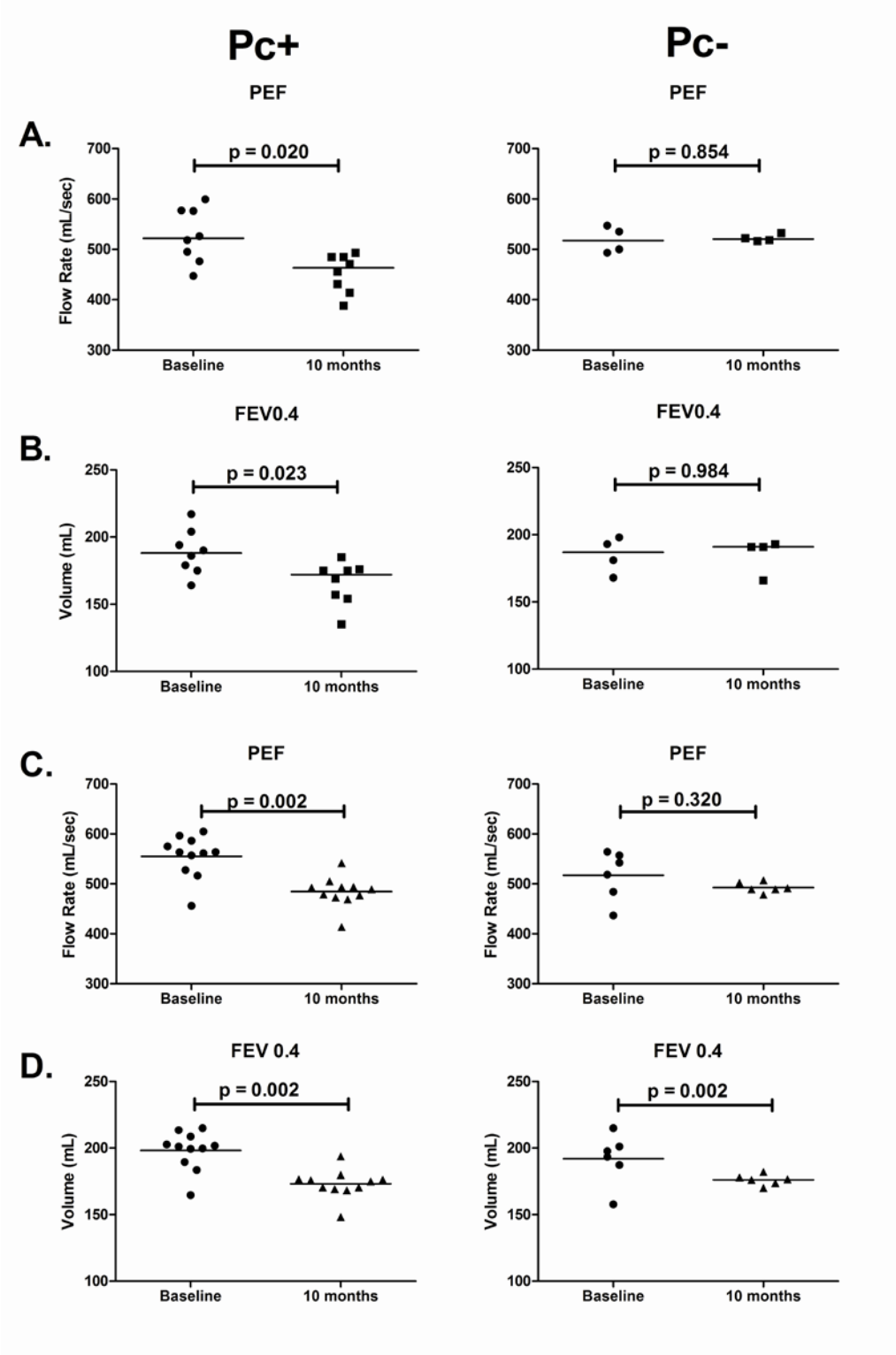

Figure 11. Pc-colonization of immunosuppressed monkeys results in pulmonary obstruction, as measured by pulmonary function testing. Pulmonary function parameters were measured using whole body plethysmography (Proskocil et al., 2005) at baseline and serially every one (Experiment 2) or two (Experiment 1) months following SHIV-infection, and changes were analyzed according to Pc colonization status. PEF (peak expiratory flow) and $FEV_{0.4}$ (forced expiratory volume in 0.4 seconds) are measures used to evaluate airway obstruction. Pulmonary function test values for Pc+ and Pc- animals in both Experiment 1 (**A, B**) and Experiment 2 (**D**, **E**) were compared by paired Student's T-test from baseline to 10 months post-SHIV infection. Significant declines from baseline occurred in the Pc+ group, but not in the Pc- group of animals. Corresponding *p*-values for each comparison are given in each panel.

3.4.6 KEX1-specific antibody production is associated with protection from pulmonary function decline.

To investigate whether there was an association between KEX1 titers at baseline and protection from pulmonary function decline, we examined baseline KEX1-IgG RET in the combined data set from animals in Experiments 1 and 2. We found animals that exhibited at least a 12 percent decline in PEF had a significantly lower baseline KEX1-IgG titer than animals that did not exhibit this decline (p=0.021). The timing of the KEX1-IgA response in the BAL fluid was also examined for correlation with protection from pulmonary function decline. Monkeys that

produced KEX1-IgA later than 4 weeks post-SHIV infection exhibited a greater decline in PEF (average decline of 12 +/- 4.3 percent), compared with monkeys that were producing KEX1-IgA by week 4 (on average, no significant decline, Fig 12B). Although this association did not reach statistical significance ($p=0.062$), the trend suggests that earlier production of KEX1-IgA in the BAL fluid may be associated with protection from pulmonary damage.

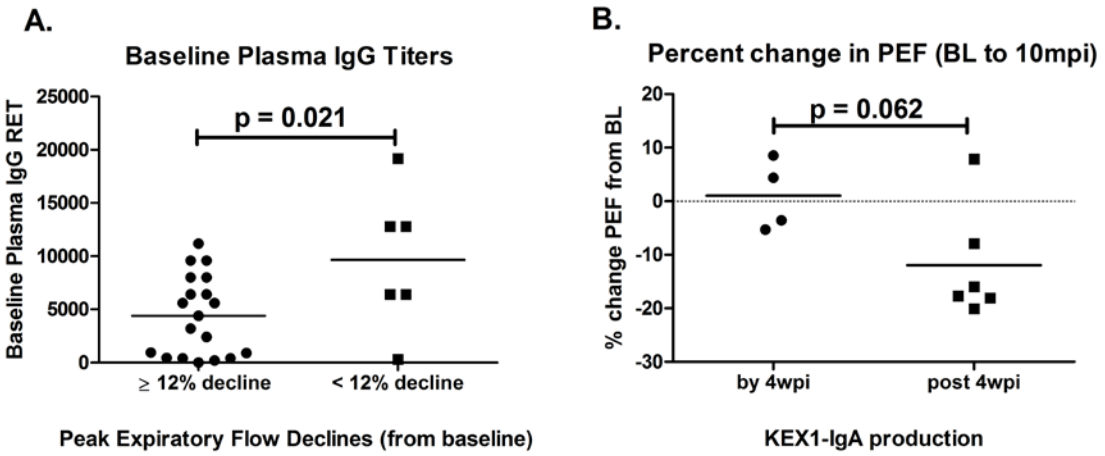

Figure 12. KEX1-specific antibody production is associated with protection from pulmonary function decline. In the combined data set from animals in Experiments 1 and 2, animals that exhibited at least a 12% decline (clinically significant decline in pulmonary function (ref) in peak expiratory flow (PEF), following immunosuppression and subsequent Pc-exposure, had a mean baseline KEX1-IgG titer of 4,405, while animals that did not exhibit this decline exhibited a mean baseline KEX1-IgG RET of 9,650 (**A**, p=0.021). In Experiment 1, timing of the KEX1-IgA response in the BAL fluid was examined for correlation with pulmonary function decline. Monkeys that produced KEX1-IgA later than 4 weeks post-SHIV infection exhibited a greater decline in PEF (mean change in PEF = 12% decrease from baseline), compared with monkeys that were producing KEX1-IgA by week 4 (mean change in PEF = 1% increase, **B**). This association did not reach statistical significance (p=0.062); however, the trend suggests that earlier production of KEX1-IgA in the BAL fluid may be associated with protection from pulmonary damage.

3.5 DISCUSSION

SIV and SHIV infection of macaques are valuable models of HIV infection (Li et al., 1992; Dunn et al., 1996; Reimann et al., 1996; Joag, 2000) and of HIV-associated opportunistic infection (Croix et al., 2002; Board et al., 2003; Pawar et al., 2008; Kling et al., 2009). We have previously used SIV and SHIV models to characterize natural transmission and persistence of Pc colonization (Kling et al., 2009; Shipley et al., in press). In previous studies, persistent Pc colonization of SHIV-infected macaques was associated with inflammatory responses in the lungs and the development of COPD-like changes in lung function (Norris et al., 2006; Shipley et al., in press). In the present study, we used a SHIV model to 1) determine the relationship between baseline anti-KEX1 antibody titers, susceptibility to Pc colonization and the development of COPD and, 2) to test the hypothesis that an effective, Pc-KEX1-specific, B cell response is maintained despite persistent SHIV-induced immunosuppression.

In this study, we showed that higher baseline anti-KEX1 titers and higher numbers of KEX1-specific ASC prior to immunosuppression correlated with prevention or delay of Pc colonization following SHIV-immunosuppression. Furthermore, high plasma anti-KEX1 titers at the time of SHIV infection correlated with improved kinetics and increased magnitude of a KEX1-specific IgA levels in the lung, upon Pc exposure. Macaques with a high baseline anti-KEX1 IgG response maintained this response throughout SHIV infection and remained free from detectable Pc colonization. Differences in susceptibility to Pc colonization were not due to lack of environmental exposure to Pc, as all monkeys were housed together for the duration of the experiments, but only monkeys with lower baseline anti-KEX1 titers became Pc colonized. The failure of monkeys with low anti-KEX1 baseline titers to prevent Pc colonization was not due to a more severe SHIV infection or greater loss of CD4+ T cells as indicated by the finding that

both groups (Pc+ and Pc-) had similar viral loads, CD4+ T cell counts and antibody titers to the SHIV protein, Gag throughout the study.

These results support the findings of previous studies that have shown the importance of Pc-specific humoral responses in murine models of PcP (Harmsen et al., 1995; Garvy et al., 1997; Gigliotti et al., 1998a; Gigliotti et al., 2002; Empey et al., 2004; Wells et al., 2006a). In the experimental mouse model of infection, passive transfer of immune sera or monoclonal Pc-specific antibodies affords protection (Gigliotti et al., 2002; Empey et al., 2004). Additionally, experimental immunization with Pc organisms/antigens of mice prior to T-cell depletion results in high levels of specific antibody production and clearance of Pc organisms following challenge (Garvy et al., 1997; Theus et al., 1998; Pascale et al., 1999). Murine studies have also provided evidence of the protective capacity of Pc-kexin (Gigliotti et al., 1998a; Zheng et al., 2005; Wells et al., 2006a).

The current study extends this knowledge by demonstrating that Pc-KEX1 antibody responses were important in preventing or resolving Pc colonization in a highly-relevant non-human primate model of Pc transmission and HIV infection (Li et al., 1992; Dunn et al., 1996; Reimann et al., 1996; Pawar et al., 2008). These results are consistent with previous findings that SIV-infected macaques with high Pc-specific antibody titers prior to intrabronchial inoculation with Pc organisms were less likely to develop PcP compared to animals with lower antibody responses prior to Pc inoculation (Board et al., 2003).

In addition to predicting resistance to Pc colonization, high baseline anti-KEX1 plasma IgG titers were associated with higher levels and earlier detection of specific IgA in the lungs of Pc- monkeys.. These results support the concept of a role for IgA-mediated protection from Pc colonization, and are consistent with clinical studies that showed mucosal antibodies to Pc are

decreased in patients with PCP, compared with HIV+ patients without PCP or with HIV-negative controls (Laursen et al., 1994; Jalil et al., 2000).

In addition to the association of baseline KEX1 antibodies and ASC with protection from Pc colonization, we also determined that Pc- animals maintained a significantly higher KEX1-specific B cell memory response compared with animals that became Pc colonized. Murine models of Pc infection have demonstrated the importance of B cells in controlling Pc infection, as it has been shown that B cell-deficient mice are highly susceptible to PcP (Marcotte et al., 1996), and other studies have demonstrated that antibody-independent B cell effector functions may be important in control of Pc infection (Lund et al., 2003; Lund et al., 2006). Our results correlating baseline KEX1-specific antibody titers, ASC and persistence of antigen-specific memory cells to prevention of Pc colonization highlight the importance of a robust memory response to KEX1 during SHIV infection and identify low baseline KEX1 B cell response as a predictor of susceptibility to Pc infection during SHIV infection.

Several studies of HIV+ patients report B cell functional deficits and abnormalities that may contribute to poor responses to antigenic stimulation, and result in their diminished vaccine responsiveness and increased susceptibility of opportunistic infections (De Milito, 2004; Malaspina et al., 2005; Titanji et al., 2006; Jiang et al., 2008; Moir and Fauci, 2009). The diminished humoral responses in HIV-infected individuals upon vaccination with carbohydrate antigens and T-independent antigens, such as the pneumococcal vaccine, may be due to a deficit in a particular subset of memory B cells, such as IgM memory cells (Hart et al., 2007) or splenic marginal zone-like peripheral blood populations (Morrow et al., 2008), whereas memory responses to other types of antigens have not been thoroughly investigated. Recent studies from our laboratory suggest similar phenotypic alterations occur during SHIV-infection as have been

reported in HIV+ patients, such as a decrease in total and memory B cells, significantly increased percentages of activated B cells, reduced percentages of CD21+ B cells, and a significant reduction in the percentages of IgM memory B cells (Kling et al., in preparation; see Chapter 4). These results support the use of the SHIV-macaque model to investigate HIV-related B cell dysfunctions.

Several studies of HIV+ patients focus on recall responses to antigens or pathogens against which study participants have been vaccinated (Malaspina et al., 2005; Titanji et al., 2006), or to antigens that they are not likely to be naturally exposed (i.e., tetanus toxoid) (De Milito et al., 2004; Hart et al., 2007). The current study explores humoral responses to a naturally-acquired, ubiquitous organism to which most humans (Peglow et al., 1990; Daly et al., 2002; Morris et al., 2008a) and non-human primates (Dei-Cas et al., 1998; Kling et al., 2009) have been continuously exposed before and after HIV or SHIV-infection. The maintenance of the Pc-KEX1-specific antibody responses and B cell memory during SHIV-induced immunosuppression in monkeys that had high Pc humoral immunity pre-infection, suggests that responses to this antigen were not significantly affected by SHIV-infection. Thus, Pc-KEX1 is a potential model antigen for longitudinal analysis of the preservation of functional humoral responses in patients with defects in CD4+ T cell numbers, due to lentivirus infection or other immunosuppressive states (Duchini et al., 2003).

We further investigated the potential clinical consequence of persistent Pc colonization and the role of a humoral response to Pc in prevention of pulmonary damage. It has been postulated that persistent microbial colonization may be involved in perpetuating the inflammatory response, eventually leading to tissue destruction, airway thickening and clinical COPD (Sethi and Murphy, 2008). HIV+ patients are at increased risk for Pc colonization

(Morris et al., 2004a) as well as an accelerated form of emphysema (Diaz et al., 2000b), and additional evidence suggests a role for Pc colonization in development of COPD in HIV-negative patients (Calderon et al., 1996; Probst et al., 2000). In HIV+ subjects, the prevalence of Pc colonization is high and occurs even in patients with high CD4+ T cells counts on ART (Morris et al., 2009). Recently, Morris et al. demonstrated a link between Pc colonization and airway obstruction in HIV+ patients, and those who were colonized had a significantly lower spirometric values compared to non-colonized subjects (Morris et al., 2009). These results demonstrate a link between Pc colonization and airway obstruction in HIV. The longitudinal study of pulmonary function in SHIV-infected macaques presented here support these findings as well as confirm our previous studies proposing a role for Pc colonization and COPD development in the SHIV model (Norris et al., 2006; Shipley et al., in press). The current study extends these findings by identifying an association between baseline anti-KEX1 antibody titers and susceptibility to Pc colonization and development of COPD. These results are also consistent with our previous findings that showed a correlation between low KEX1 titers and COPD in non-HIV infected smokers (Morris et al., 2008a). In addition, preliminary studies from our group also show that low KEX1 antibody levels are associated with subsequent development PcP in HIV+ subjects (Gingo, M. et al., submitted). Results from the current study in combination with previously published reports suggest that vaccination for the prevention of Pc colonization and development of obstructive lung disease may be feasible in humans.

In summary, these results support the concept that Pc-specific humoral immunity established prior to immunosuppression is associated with improved resistance to subsequent Pc colonization and pulmonary obstruction, despite declining CD4+ T cell numbers. These data further suggest that the humoral effector mechanisms responsible for this protection include high

levels of circulating KEX1-specific IgG, KEX1-specific IgA present in the lung, and the maintenance of a KEX1-specific memory B cell pool following immunosuppression,. These results underscore the importance of a Pc-specific humoral response resistance to Pc colonization and prevention of pulmonary damage and support the feasibility of a Pc-KEX1 vaccine strategy for protection of high risk populations.

3.6 AUTHOR CONTRIBUTIONS AND ACKNOWLEDGEMENTS

Heather M. Kling (Molecular Virology and Microbiology Graduate Program, University of Pittsburgh School of Medicine) generated the majority of the data and prepared the manuscript. Timothy W. Shipley (Immunology Graduate Program, University of Pittsburgh School of Medicine) provided assistance with sample processing and analysis, especially with regard to pulmonary function testing and data analysis. Sangita Patil (Department of Immunology, University of Pittsburgh School of Medicine) provided assistance with sample processing and analysis, and assisted with generation of PCR and flow cytometry data. Jan Kristoff (Department of Immunology, University of Pittsburgh School of Medicine) provided assistance with sample processing, collection of flow cytometry data, and generation of PCR data. Marianne Bryan (Immunology Graduate Program, University of Pittsburgh School of Medicine) and Alison Morris (Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh) assisted with providing insight and critique of the project and the manuscript. Karen A. Norris (Department of Immunology, University of Pittsburgh School of Medicine), as the mentor and principal investigator on the project, provided extensive scientific knowledge, insight and critique of the project and the manuscript. Ronald C. Montelaro (Department of

Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine) provided the virus used for experimental inoculation of animals. All authors, especially Karen A. Norris, contributed to scientific discussion regarding the project and critical reading and editing of the manuscript.

The authors would like to thank Chris Janssen, D.V.M, Ph.D. and Nicole Banichar, C.V.T. for excellent veterinary care. Funding for these experiments was provided by the National Institutes of Health grants HL077095-01A1 and HL077914-01 (KAN), and National Institutes of Health training grant T32 AI49820 (HMK).

4.0 SHIV-INFECTED CYNOMOLGUS MACAQUES EXHIBIT ABNORMALITIES IN PERIPHERAL BLOOD B-CELL POPULATIONS

Portions of this chapter are being prepared for submission to an academic journal. The authors are Heather M. Kling, Timothy W. Shipley, and Karen A. Norris.

4.1 ABSTRACT

In addition to CD4+ T cell depletion, abnormalities in the B cell compartment of HIV-infected individuals are also reported, including deficits and diminished responses to *ex vivo* antigenic stimulation and *in vivo* vaccination. In the current study we used chimeric Simian-Human Immunodeficiency Virus (SHIV)-infection of cynomolgus macaques to investigate phenotypic changes in peripheral blood B cell populations. We observed similar alterations in B cell populations of SHIV-infected macaques as have been reported in HIV-infected patients. Compared with pre-SHIV infection values, we observed significant declines in total (CD20+), memory (CD20+CD27+), CD21+, and naïve (CD20+CD27-) B cells in the peripheral blood of cynomolgus macaques following SHIV-infection. Additionally, we also noted declines in subsets of memory B cells, including both IgM+ and class-switched (IgM-IgD-) memory cells, with sustained deficits in the IgM+ memory population. Compared with baseline, we also report significantly elevated levels of B cell activation, as measured by CD95 (Fas) expression, as well as hypergammaglobulinemia. We also investigated whether phenotypic or activation abnormalities in B cell populations correlated with natural colonization of SHIV-infected macaques by the opportunistic pathogen, *Pneumocystis*. We found similar perturbations in B cell populations of both *Pneumocystis*-positive and *Pneumocystis*-negative animals; therefore, although the B cell compartment of macaques appears to be significantly affected by SHIV-infection, these abnormalities do not contribute to susceptibility to infection by this opportunistic pathogen. The similarity of B cell alterations observed in these studies to those observed in

HIV+ subjects support the utility of the SHIV-macaque model for examination of HIV-related B cell dysfunction.

4.2 INTRODUCTION

In addition to severe depletion of CD4+ T cells, major immune abnormalities occur within the B cell compartment during HIV-infection (De Milito, 2004). HIV infection has been associated with reduced total and memory B cells (De Milito et al., 2001; Nagase et al., 2001), increased B cell activation (Mizuma et al., 1988; Shirai et al., 1992; Titanji et al., 2005; Moir et al., 2008), decreased circulating antigen-specific Ig, impaired responsiveness to stimulation (Jiang et al., 2008), hypergammaglobulinemia and spontaneous Ig secretion in vitro (Nagase et al., 2001; De Milito et al., 2004; Titanji et al., 2005), and germinal center abnormalities (Guarda et al., 1983; Chalifoux et al., 1984; Chalifoux et al., 1987; Margolin et al., 2002; Margolin et al., 2006).

In vivo polyclonal activation of B cells in the peripheral blood of HIV-infected persons is evidenced by abnormal levels of B cell activation in HIV-infected individuals (Lane et al., 1983), resulting in spontaneous secretion of immunoglobulins (Mizuma et al., 1987; Mizuma et al., 1988). Symptomatic HIV-infected patients tend to have higher levels of circulating IgG than asymptomatic patients (De Milito, 2004); thus, IgG levels have been reported as a prognostic marker of infection. Decreased proliferative responses to T-cell independent B cell mitogens *ex vivo* (Lane et al., 1983; Jiang et al., 2008), in both the naïve and memory B cell populations (Jiang et al., 2008), accompany the chronic immune activation reported in HIV+ patients. Thus, B cell function, measured by the B cell response to specific stimuli or by the capacity to provide co-stimulatory signals to CD4+ T cells, appears impaired during HIV-infection (Malaspina et al.,

2003; Moir et al., 2003). Elevated levels of CD21-low/negative B cells in HIV-infected subjects is hypothesized to account for poor proliferative responses of B cells (Moir et al., 2001; Jiang et al., 2008; Moir and Fauci 2009). CD21+ B cells are thought to be terminally differentiated and thus, proliferate poorly in response to mitogenic stimulation. Reports indicate that the frequency of CD21+ memory B cells is directly related to the capacity of these cells to proliferate in response to stimulation (Jiang et al., 2008). However, abnormal levels of B cell hyperactivation and hypersecretion of Ig appear to be closely related to viremia levels, thus, appear to be reversible by effective anti-retroviral therapy (ART) (Moir et al., 2001; Fournier et al., 2002; De Milito, 2004; Moir and Fauci, 2009).

In contrast to what has been reported for activation level abnormalities, HIV- associated loss of memory B cells and decreased memory B cell function (Titanji et al., 2006) does not seem to be restored by ART. Loss of memory B cells may be due to increased expression of particular surface makers, as a result of HIV-infection, which contributes to increased apoptosis rates of B cells, in particular, memory B cells (De Milito et al., 2001). Upregulated expression of activation markers may be responsible for the elevated rates of differentiation from memory cells to immunoglobulin-secreting cells, which, along with increased activation of naïve B cells, results in the observed hypergammaglobulinemia associated with HIV-infection (Nagase et al., 2001; De Milito et al., 2004; Cagigi et al., 2008; Moir and Fauci, 2009).

Impaired B cell memory may contribute to the observed reduced capacity of HIV-infected individuals to respond effectively to pathogens, diminished responses to vaccination (Malaspina et al., 2005), and may also contribute to the increased susceptibility of HIV-infected patients to opportunistic pathogens. HIV-infected patients have deficits in circulating antibodies to immunizing antigens, such as measles and influenza, as well as antibodies to opportunistic

pathogens, such as *Streptococcus pneumonia* and *Cryptococcus neoformans*, deficits which are not restored by ART (Titanji et al., 2006). Specific subsets of memory B cells have also been reported to be diminished, such as IgM memory B cells (Titanji et al., 2005; Hart et al., 2007) and peripheral blood marginal zone-like populations (Morrow et al., 2008), both of which are hypothesized to contribute to defective immune responses to T-independent pathogens, such as *Pneumococcus*, as a consequence of HIV infection.

The fungal opportunistic pathogen, *Pneumocystis jirovecii* (formerly *Pneumocystis carinii* f. sp. *hominis*) (Pc) is an important pathogen in HIV+ populations. Despite advances in treatment strategies, Pneumocystis pneumonia (PcP) remains one of the most common serious infections associated with HIV infection (Kaplan et al., 2000; Morris et al., 2004b). Several studies have shown that subpopulations of HIV-infected individuals remain at risk for development of PcP despite anti-retroviral therapy (Connors et al., 1997; Morris et al., 2004b), although the basis of susceptibility is poorly understood.

Pc colonization is defined as the presence of Pc in respiratory samples from subjects without overt signs of clinical disease, and is uncommon in immunocompetant subjects (Wakefield et al., 1990c; Peters et al., 1992; Leigh et al., 1993; Tamburrini et al., 1997). An increased frequency of Pc colonization has been reported in HIV+ patients, including those on anti-retroviral therapy (ART) (Morris et al., 2004a). Pc colonization is important as it not only may increase risk for progression to PcP in susceptible individuals, but even at low levels may cause pulmonary damage, and has been shown to be associated with chronic obstructive pulmonary disease in both human subjects (Morris, Huang et al. 2000, Calderon 1996, Probst 2000, Helweg-Larsen 2002, Morris 2009) and in animal models (Shipley et al., in press).

Although effective CD4+ T cell responses are important in immunologic control of Pc infection, B cells and antibodies also play a role in prevention of PcP (Harmsen et al., 1995; Marcotte et al., 1996; Garvy et al., 1997; Gigliotti et al., 1998a; Gigliotti et al., 1998b; Gigliotti et al., 2002; Empey et al., 2004; Wells et al., 2006a). There is a high frequency of Pc-specific seropositivity in immunocompetent adults (Daly et al., 2002; Bishop and Kovacs, 2003), as well as in non-human primates (Demanche et al., 2005; Kling et al., 2009), suggesting the persistence of serological memory in response to natural Pc exposure. The experimental murine model demonstrates the importance of B cells in controlling Pc infection; B cell-deficient mice are highly susceptible to Pc (Marcotte et al., 1996), and B cells may play other roles aside from antibody production in control of Pc infection (Lund et al., 2003; Lund et al., 2006).

Limited studies using non-human primate (NHP) models of HIV infection have been used to investigate virus-induced B cell dysfunction (Margolin et al., 2002; Margolin et al., 2006). Infection of macaques with SHIV induces follicular hyperplasia and germinal center abnormalities similar to those associated with HIV infection, although a comprehensive study of B cell abnormalities in this model has not been reported. In the current study we investigated phenotypic changes to B cell populations commonly reported to be affected by HIV to ascertain the strength of the SHIV-NHP model for examining AIDS/HIV-associated B cell dysfunction. Because of the importance of B cells in controlling Pc infection, we also used our model of natural Pc-colonization (Kling et al, 2009) to investigate whether B cell abnormalities induced by SHIV-infection affected the animals' susceptibility to colonization by this opportunistic pathogen.

4.3 MATERIALS AND METHODS

4.3.1 Animals.

Adult, Chinese origin cynomolgus macaques (*Macacca fasicularis*), weighing between 5-8 kg, were used in this study**.** All animals were purchased from National Primate Centers or vendors approved by the University of Pittsburgh, Department of Laboratory Animal Research. Prior to admission to the study, all animals underwent complete physical examination (pulmonary and cardiac auscultation, thoracic radiographs, computer tomography scanning, tuberculin skin testing, complete blood count, chemistry panel, urinalysis, and flow cytometric analysis of peripheral blood mononuclear and BAL cells) and were screened for simian retroviruses; SIV, SRV, and STLV to verify that they are free of any pre-existing disease that may confound the study. The animals were housed in an American Association for Accreditation of Laboratory Animal Care-accredited, biosafety level 2+ primate facility at the University of Pittsburgh. Animal husbandry and experimental procedures were conducted in accordance with standards set forth by the Guide for the Care and Use of Laboratory Animals (ref. National Research Council, 1996) and the Provisions of the Animal Welfare Act. Prior to the initiation of this study, all animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

4.3.2 Study Design.

Twenty-nine macaques were intravenously inoculated with $1x10^{4.9}$ $TCID_{50}$ (50% tissue culture infectious doses) of $SHIV_{89.6P}$ (gift of Dr. Opendra Narayan, University of Kansas), which

induces CD4+ T cell lymphopenia and AIDS-like disease with wasting and opportunistic infections (Reimann et al., 1996; Pawar et al., 2008). To promote natural transmission of Pc, SHIV-infected macaques were continuously exposed by co-housing in the same room with 10-20 SIV- or SHIV-immunosuppressed macaques which served as a Pc source (Kling et al., 2009). None of the macaques (source or recipients) contracted fulminate *Pneumocystis* pneumonia (PcP) during the study. Determination of Pc colonization status was performed by detection of Pc DNA in the BAL fluid samples by nested PCR and by anti-Pc KEX1 serology (Board et al., 2003; Kling et al., 2009). Pc colonization was defined as a positive nested PCR of BAL fluid and at least a 3-fold change in plasma anti-Pc KEX1 titers (Board et al., 2003; Kling et al., 2009). Additionally, BAL samples were stained for organisms by modified Giemsa and silver staining (Board et al., 2003).

4.3.3 BAL and blood collection.

Peripheral blood and BAL samples were collected at baseline on all animals. Serial plasma and PBMC samples from SHIV-infected monkeys were collected weekly for the first 8 weeks following SHIV-infection and monthly thereafter. BAL fluid samples were collected monthly. Samples were collected and processed as described previously (Board et al., 2003; Kling et al., 2009). Briefly, plasma was isolated from 10mL of EDTA (ethylenediaminetetraacetic acid)-treated whole blood by centrifugation; PBMC were purified over a Percoll gradient (Amersham Bioscience, Piscataway, NJ) and washed with sterile phosphate buffered saline (PBS) (Shipley et al. in press). Plasma aliquots were stored at -80°C prior to assay. PBMC were counted, stained and fixed for analysis by flow cytometry. BAL fluid was processed for cell isolation as previously described

(Croix et al., 2002; Kling et al., 2009). Unfractionated BALF aliquots were used for bacterial, fungal and viral culture (Antech Diagnostics, Pittsburgh, PA) and PCR detection of Pc DNA. Remaining fluid was filtered through a 40-micron cell strainer and manual cell counts were performed using a hemocytometer. 1 x 10^5 cells were removed and stained with modified Giemsa stain (Dade Behring, Newark, DE) and differential counts performed manually (Croix et al., 2002). Remaining cells were pelleted and supernatant fluid was collected and stored at -80°C. Recovered cells were prepared for flow cytometry as described (Croix et al., 2002; Board et al., 2003).

4.3.4 Flow cytometry.

Stained, fixed cells from whole blood were analyzed by flow cytometry (Croix et al., 2002). Antibodies used were: mouse anti-monkey CD3-fluorescein isothiocyanate (clone SP34), mouse anti-human CD8-Pacific Blue (clone RPA-T8) and mouse anti-monkey CD4-allophycocyanin (clone L200), mouse anti-human CD21 (clone B-ly4)- phycoerythrin, mouse anti-human CD95 (clone DX2)- fluorescein isothiocyanate, anti-human IgM-fluorescein isothiocyanate, all purchased from BD Pharmingen (San Diego, CA); mouse anti-human CD20 (clone 2H7)-Pacific Blue, mouse anti-human CD27 (clone O323)-allophycocyanin, purchased from eBioscience (San Diego, CA), and anti-human IgD-biotin, purchased from Southern Biotech (Birmingham, AL). A streptavidin-Pacific Orange conjugate (Invitrogen, Carlsbad, CA) was used to detect biotin-conjugated antibodies. Acquisition was performed on BD LSRII flow cytometer using BD FacsDiva software. Forward/side scatter dot plot was used to gate the live lymphocyte population. All analyses were performed using FlowJo flow cytometry analysis software (Tree Star Inc., Ashland, OR).

4.3.5 Total immunoglobulin quantification.

Plasma samples were heat-inactivated at 56°C for 30 minutes, and then diluted in blocking buffer (PBS, 5% non-fat milk) prior to assay. Microtiter plates (Immunolon 4HBX, Thermo Fisher Scientific, Inc., Waltham, MA) were coated with affinity purified anti-monkey IgG (1μg/mL, Rockland, Inc) at 4°C overnight. One-hundred μL of plasma were plated in triplicate into anti-monkey IgG-coated wells. Goat anti-monkey immunoglobulin-conjugated horseradish peroxidase (1:10,000 for IgG, 1:2000 for IgM) (Nordic Immunology, Tilburg, Netherlands) was used for detection and plates were developed by standard methods. Normal (uninfected) macaque plasma was used as a plate control. Serial dilutions of whole molecule monkey IgG (Rockland, Inc) were used to generate a standard curve.

4.3.6 Statistical Analyses.

Statistical analyses were performed using Prism software, by Graphpad (La Jolla, CA). Comparisons among multiple time-points were made using repeated measures ANOVA, with Bonferroni post-tests. Additionally, paired two-tailed student's *t*-test was used to compare post-SHIV-infection time-points with baseline values. When comparing Pc+ and Pc- monkeys over multiple time-points, two-way ANOVA was used for comparison. A p value of less than 0.05 was considered significant.

4.4 RESULTS

4.4.1 Natural Pc Colonization of SHIV-infected macaques.

Prior to SHIV-infection, macaques were determined to be negative for Pc colonization by nested PCR of BAL fluid. Plasma antibodies to the recombinant Pc protein, kexin (KEX1), were examined by ELISA, as described in Chapter 3. Baseline anti-Pc KEX1 plasma IgG reciprocal endpoint titers ranged from undetectable (<1:100) to 1:22,400. Following SHIV-inoculation, nineteen of the animals became naturally colonized with Pc (Pc+) by 16 weeks post-SHIV infection, while ten animals remained Pc-negative (Pc-) throughout the duration of the experiment (53 weeks), as determined by nested PCR of BAL fluid and anti-KEX1 antibodies. Rapid declines in peripheral blood CD4+ T cells were observed within 2 to 4 weeks following SHIV-infection (see Fig 8 in Section 3.4.2), and CD4+ T cell numbers remained depressed throughout the experiment (see Fig 6 and 8 in Section 3.4). Additionally, SHIV viral loads and antibody responses are given in Section 3.4.2, Figure 7. There was no difference between Pc+ and Pc- monkeys in mean CD4+ T cell numbers or in SHIV viral loads during SHIV infection (see Fig 6, Section 3.4.1). Representative profiles of Pc-antibody production, CD4+ T cell decline, and nested PCR results are shown in Figure 8 in Section 3.4.2.

4.4.2 Significant declines in total B cell populations and increased percentages of CD95+ B cells in peripheral blood following SHIV-infection.

In order to examine whether similar B cell deficits or dysfunctions occur during $SHIV_{89.6}$-infection of cynomolgus macaques as have been reported in HIV-infected patients, and whether these dysfunctions may correlate with the animals' susceptibility to Pc colonization, we examined B cell populations and subsets in these animals. Baseline and serial time-points following SHIV-infection were examined by flow cytometry of PBMC for changes in B (CD20+) cell populations. PBMC from all animals (n=29) were assessed at serial time-points for approximately one year post-SHIV infection. Percentages (Fig 13A) and numbers (Fig 13B) of CD20+ cells from Pc+ (n=19) and Pc- (n=10) monkeys were then compared (2-way repeated measures ANOVA). Significant changes in proportions ($p=0.0004$, repeated measures ANOVA, Fig 13A) (CD20+) and numbers ($p<0.0001$, repeated measures ANOVA, Fig 13B) of B lymphocytes, compared with baseline values, were observed following SHIV-infection. When comparing individual time-points, significant declines in percentages (week 2, $p=0.026$, Fig 13A) and numbers (week 2, $p=0.0008$, Fig 13B) of CD20+ cells were observed early following SHIV-infection. However, CD20+ cells as a percentage of total lymphocytes had rebounded somewhat by approximately 7 months post-SHIV infection (week 28, $p=0.17$, paired Student's *t*-test) and remained near baseline levels by one-year following SHIV-infection (week 53, $p=0.23$, paired Student's *t*-test). Absolute numbers of CD20+ cells remained depressed throughout the experiment (Fig 13B, $p<0.0001$, repeated measures ANOVA), and by week 53 remained significantly declined from baseline ($p=0.0019$, Fig 13B). CD20+ B cell percentages and numbers of Pc+ and Pc- monkeys were compared (Fig 13A, B), and there was no significant

difference between the percentages (p=0.6, 2-way ANOVA) or numbers (p=0.763, 2-way repeated measures ANOVA) of CD20+ cells between these two groups of animals (Fig 13A, B).

Activation of B cells was measured via CD95 (Fas) surface expression. PBMC in a subset of SHIV-infected macaques (n=17) were examined at serial time-points for CD95+ B (CD20+CD95+) cells. Percentages and numbers of CD95+ B cells from Pc+ (n=11) and Pc- (n=6) monkeys were then compared (Fig 14). Percentages of CD95+ B cells were markedly increased early following SHIV-infection (Fig 14A, weeks 1-4 post-SHIV infection, $p<0.0001$, paired Student's *t*-test), increasing from 12.7±8.1% at baseline to 52.4±12.9% at week 1 post-infection, and percentages of activated B cells remained significantly increased at 51.1±10.0% of total B cells by 58 weeks post-SHIV infection ($p<0.0001$, Fig 14A). Absolute numbers of CD95+ B cells declined with total B cells following SHIV-infection (Fig 14B); however, by approximately 7 months post-SHIV infection, had returned to baseline levels (week 28, p=0.96, paired Student's *t*-test) and remained near baseline levels at around one-year post-infection (week 58, p=0.38, paired Student's *t*-test), despite the fact that total B cell numbers remained depressed at this point (Fig 13). Activation levels were similar between Pc+ and Pc- monkeys pre- (week 0, p=0.29, Student's *t*-test) and post-SHIV infection ($p>0.05$ at all time-points, 2-way repeated measures ANOVA with Bonferroni post-tests, Fig 14A). Numbers of CD95+ B cells were also similar between Pc+ and Pc- monkeys (Fig 14B, p=0.45, 2-way repeated measures ANOVA), indicating similar levels of expression of this activation marker regardless of Pc-status in immunosuppressed animals.

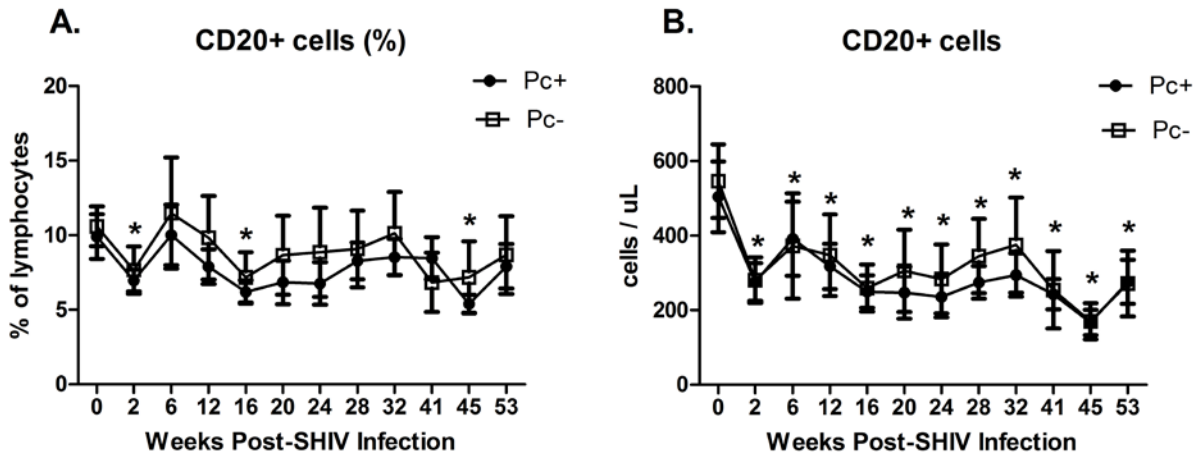

Figure 13. Peripheral blood total B (CD20+) cells declined following SHIV-infection. B cells were assessed by flow cytometry of PBMC, using the surface marker CD20 to distinguish B cells from other cells in the lymphocyte gate. PBMC from all animals (n=29) were assessed at serial time-points for approximately one year post-SHIV infection, by repeated measures ANOVA and paired Student's *t*-tests. Shown here, percentages (**A**) and numbers (**B**) of CD20+ cells from Pc+ (n=19) and Pc- (n=10) monkeys were then compared by 2-way repeated measures ANOVA. Individual time-points in which values were significantly different from baseline values are indicated by * ($p<0.05$, paired Student's *t*-test). There was no significant difference between the percentages ($p=0.6$, 2-way repeated measures ANOVA, **A**) or numbers ($p=0.763$, 2-way repeated measures ANOVA) of CD20+ cells between Pc+ and Pc- monkeys.

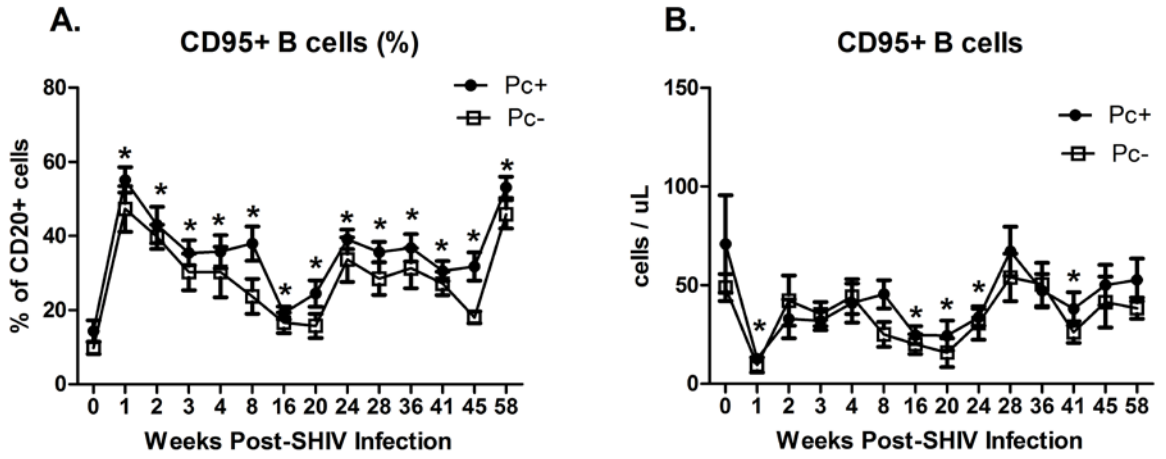

Figure 14. Significant B cell activation was observed following SHIV-infection. Activation of B cells was determined by flow cytometry for the surface marker CD95 (fas) in a subset of SHIV-infected macaques (n=17), and serial time-points were evaluated by repeated measures ANOVA and paired Student's *t*-tests. Shown here, percentages (A) and numbers (B) of CD95+ B cells (CD20+CD95+) from Pc+ (n=11) and Pc- (n=6) monkeys were then compared by 2-way repeated measures ANOVA. Individual time-points in which values were significantly different from baseline values are indicated by * ($p<0.05$, paired Student's *t*-test).

4.4.3 Decreased surface expression of CD21 on peripheral blood B cells in SHIV-infected macaques.

The surface marker CD21 is part of the B cell co-receptor (BCR) complex, which serves to strengthen the signal resulting from antigen recognition. Signaling pathways activated by CD21 amplify antibody responses and induce costimulatory molecules on the B cell, thereby increasing effectiveness at eliciting T-cell help. HIV+ patients demonstrate diminished CD21 expression on B cells (Moir et al., 2001; Jiang et al., 2008), which may result in reduced B cell

responsiveness to antigen and subsequent proliferation. To examine this phenotype as a correlate of antigen responsiveness capacity in SHIV-infected monkeys, we evaluated expression of the CD21 surface marker on peripheral blood B cells (CD20+CD21+) of macaques (n=29). The absolute number of CD21+ B cells in the peripheral blood was significantly declined following SHIV-infection ($p<0.0001$, repeated measures ANOVA, Fig 15A, B). Early significant declines in the percentages (week 2, $p=0.0012$, paired Student's t-test, Fig 15A) and absolute numbers (week 2, $p=0.0007$, paired Student's *t*-test, Fig 15B) of these cells were observed following SHIV-infection. Neither percentages nor numbers of CD21+ B cells returned to baseline levels by approximately one year post-SHIV infection ($p<0.0001$, percentages; $p=0.0003$, numbers; paired Student's *t*-test). However, Pc+ and Pc- animals exhibited similar declines in percentages ($p=0.73$, 2-way repeated measures ANOVA) and numbers ($p=0.52$, 2-way repeated measures ANOVA) of CD21+ B cells, demonstrating that colonized and non-colonized monkeys were similarly affected by SHIV-infection with regard to decreased percentages and numbers of CD21+ B cells.

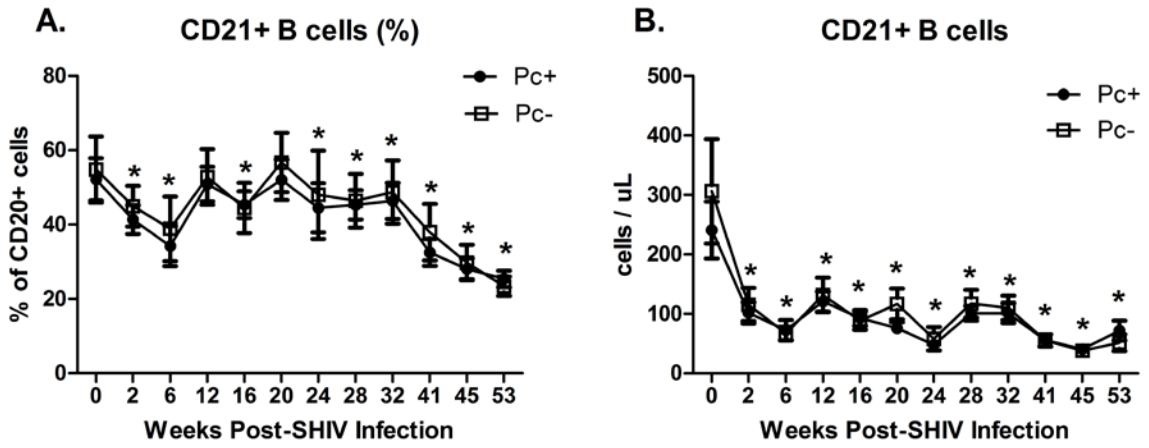

Figure 15. B cells expressing CD21 were significantly decreased following SHIV-infection. Percentages of peripheral blood B cells expressing the surface marker CD21 (CD20+CD21+) were determined by flow cytometry of PBMC of macaques (n=29) at serial time-points for approximately one year post-SHIV infection. Changes from baseline in the CD21+ B cell population were evaluated by repeated measures ANOVA and paired Student's *t*-tests. Shown here, percentages (A) and numbers (B) of CD21+ B cells from Pc+ (n=19) and Pc- (n=10) monkeys were then compared by 2-way repeated measures ANOVA. Individual time-points in which values were significantly different from baseline values are indicated by * ($p<0.05$, paired Student's *t*-test).

4.4.4 Peripheral blood memory and naïve B cells are affected in SHIV-infected macaques.

Memory B cells were assessed by CD27 surface marker expression (Vugmeyster et al., 2004). Flow cytometry of PBMC revealed significant changes in numbers ($p<0.0001$, repeated measures ANOVA, Fig 16A) of CD27+ B cells. CD27+ B cells declined significantly during acute SHIV-infection (week 2 post-SHIV infection, $p=0.037$, Fig 16A), but rebounded thereafter,

and by one-year post-SHIV infection remained near baseline levels (p=0.18, week 0 compared with week 53, Fig 16A). Similar to what was observed for other surface markers, there were no significant differences in numbers of CD27+ B cells (p=0.75, 2-way repeated measures ANOVA) between Pc+ and Pc- monkeys (Fig 16A). These results indicate that although there is an acute deficit in the memory B cell compartment following SHIV-infection, Pc+ and Pc- monkeys were equivalently affected.

It has been hypothesized that subsets of memory B cells may be differentially affected during HIV infection, especially with regard to class-switched (CD27+IgM-IgD- B cells) versus IgM+ memory (CD27+IgM+) B cells, with IgM+ memory cells reported to be significantly reduced in HIV+ patients (Hart et al 2007). Because we observed an early loss of total memory (CD27+) B cells, we further investigated whether SHIV infection induced alteration in subsets of memory B cells to examine whether differential effects of SHIV on those populations may exist between Pc+ and Pc- monkeys. Following SHIV-infection, percentages (p=0.0005, repeated measures ANOVA, Fig 16B) and numbers (p=0.0003, repeated measures ANOVA, Fig 16C) of class-switched (IgM-IgD-) memory (CD27+) B cells were significantly reduced. However, by 45 weeks post-SHIV infection, percentages (p=0.2) and numbers (p=0.16) of class-switched CD27+ B cells returned to baseline levels (Fig 16B, C). Additionally, percentages (p<0.0001, repeated measures ANOVA, Fig 16D) and numbers (p<0.0001, repeated measures ANOVA, Fig 16E) of IgM memory B cells (IgM+CD27+) were also significantly reduced following SHIV-infection. Percentages and numbers of IgM+ CD27+ B cells significantly declined by 16 weeks post-SHIV infection, and, in contrast to the rebound observed for class-switched memory B cells by 45 weeks post-SHIV infection, remained depressed for the duration of the experimental infection (week 0 vs. 58; p=0.014, percentages; p=0.007, numbers; Fig 16D, E). Pc+ and Pc- monkeys

had similar percentages (p=0.33, Fig 16B) and numbers (p=0.55, Fig 16C) of class-switched CD27+ B cells, as well as percentages (p=0.47, 2-way repeated measures ANOVA, Fig 16D) and numbers (0.89, 2-way repeated measures ANOVA, Fig 16E) of IgM+ memory B cells.

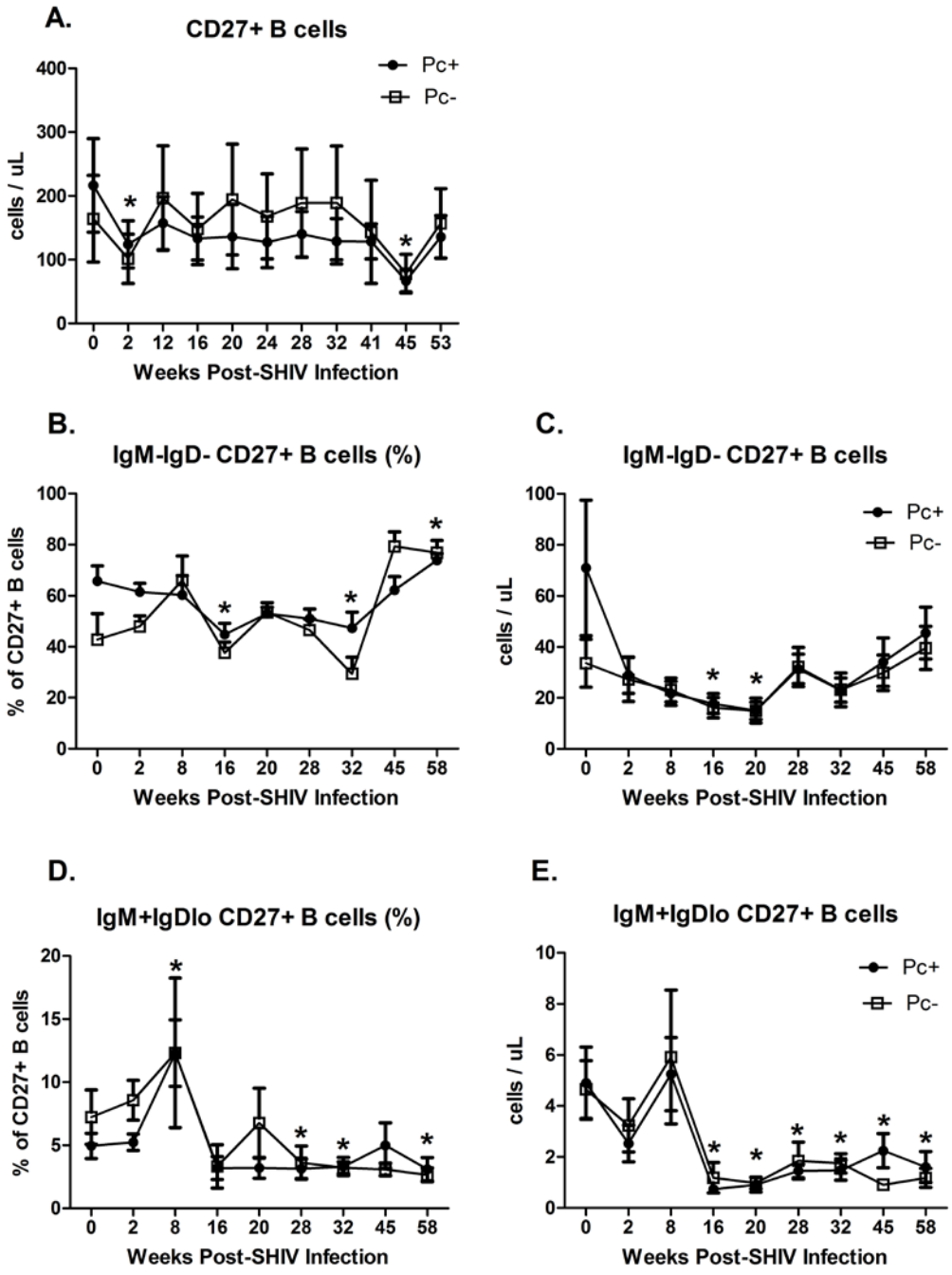

Figure 16. CD27+ B cells and subsets in peripheral blood of SHIV-infected macaques. Flow cytometry was used to evaluate memory B cells (CD27+CD20+ lymphocytes) of macaques (n=29) at serial time-points for approximately one year post-SHIV infection. Changes from baseline in the CD27+ B cell population were evaluated by repeated measures ANOVA and paired Student's *t*-tests. Shown here, numbers (**A**) of CD27+ B cells from Pc+ (n=19) and Pc- (n=10) monkeys were then compared by 2-way repeated measures ANOVA. Percentages (**B**) and numbers (**C**) of class-switched (IgM-IgD-) and IgM (percentages, **D**; numbers, **E**) memory (IgM+CD27+) subsets of CD27+ B cell were also investigated by 2-way repeated measures ANOVA in a subset of macaques (n=17). Individual time-points in which values were significantly different from baseline values are indicated by * ($p<0.05$, paired Student's *t*-test).

We also examined the naïve (CD20+CD27-) B cell population in PBMC of SHIV-infected macaques (n=29). During HIV infection, naïve B cells are reported to be increased in peripheral blood (Chong et al., 2004); however, in a recent report of SIV-infected rhesus macaques, naïve B cells exhibited declines at early time-points following viral infection (Kuhrt et al., 2010). In the current examination of SHIV-infection of cynomolgus macaques, we also observed significant changes in percentages ($p<0.0001$, repeated measures ANOVA, Fig 17A) and numbers ($p<0.0001$, repeated measures ANOVA, Fig 17B) of CD27- B cells. Percentages of CD27- B cells (Fig 17A) declined significantly from baseline by week 12 ($p=0.0038$). Numbers of CD27- B cells (Fig 17B) declined significantly acutely following SHIV-infection (week 2, $p=0.0006$) and did not return to baseline levels for the duration of the experiment (week 53,

p=0.0011). Percentages (Pc+ vs Pc-, p=0.90 Fig 17A) and numbers (Pc+ vs Pc-, p=0.75, Fig 17B) of CD27- B cells were similarly affected in Pc+ and Pc- animals.

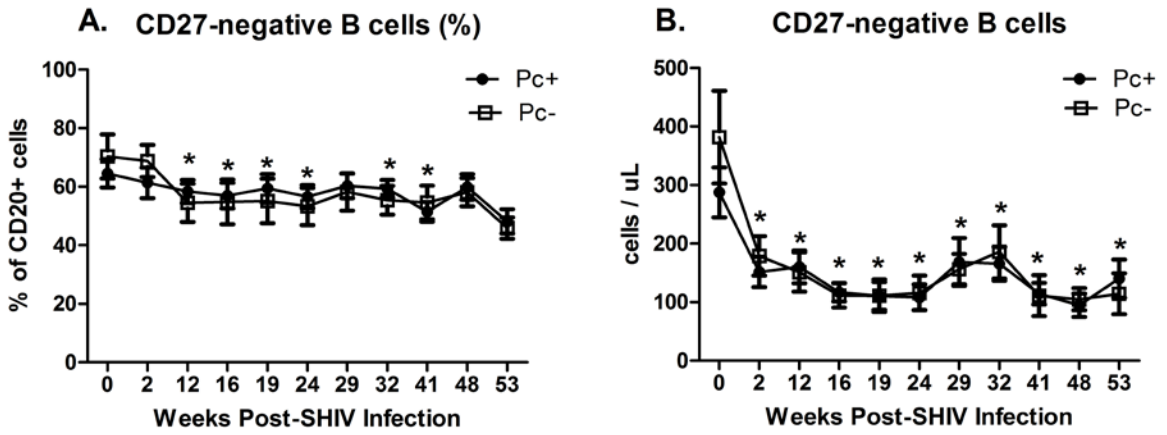

Figure 17. Naïve B cells (CD20+CD27- lymphocytes) were significantly declined from baseline following SHIV-infection. Flow cytometry was used to distinguish CD27- from CD27+ B cells in PBMC of macaques (n=29) at serial time-points for approximately one year post-SHIV infection. Changes from baseline in the CD27- B cell population were evaluated by repeated measures ANOVA and paired Student's *t*-tests. Shown here, percentages (A) and numbers (B) of CD27- B cells from Pc+ (n=19) and Pc- (n=10) monkeys were then compared by 2-way ANOVA. Individual time-points in which values were significantly different from baseline values are indicated by * ($p<0.05$, paired Student's *t*-test).

4.4.5 Evidence of hypergammaglobulinemia in the plasma of SHIV-infected macaques.

Chronic immune activation and hypergammaglobulinemia are well-documented in HIV-infection (Lane et al., 1983; Nagase et al., 2001), and is comprised of both virus specific and polyclonal antibodies. Several isotypes are increased, but IgG is predominantly affected, and serum IgG

levels may represent a prognostic marker of disease progression (De Milito, 2004; De Milito et al., 2004). To assess whether this abnormality occurs in SHIV-infected macaques, total plasma IgG levels were measured at baseline and at serial time-points following SHIV-infection (n=12). A significant change from baseline was observed over the course of SHIV-infection (Fig 18A, p=0.0041, repeated measures ANOVA), particularly at 7 months post-infection (Fig 18A, week 0 vs. week 29, p=0.0009, paired Student's *t*-test). Plasma IgG levels were not significantly different between Pc+ (n=8) and Pc- (n=4) monkeys (Fig 18B, p=0.54, 2-way repeated measures ANOVA). Thus, evidence of hypergammaglobulinemia was similar in both groups of animals and did not correlate with Pc colonization.

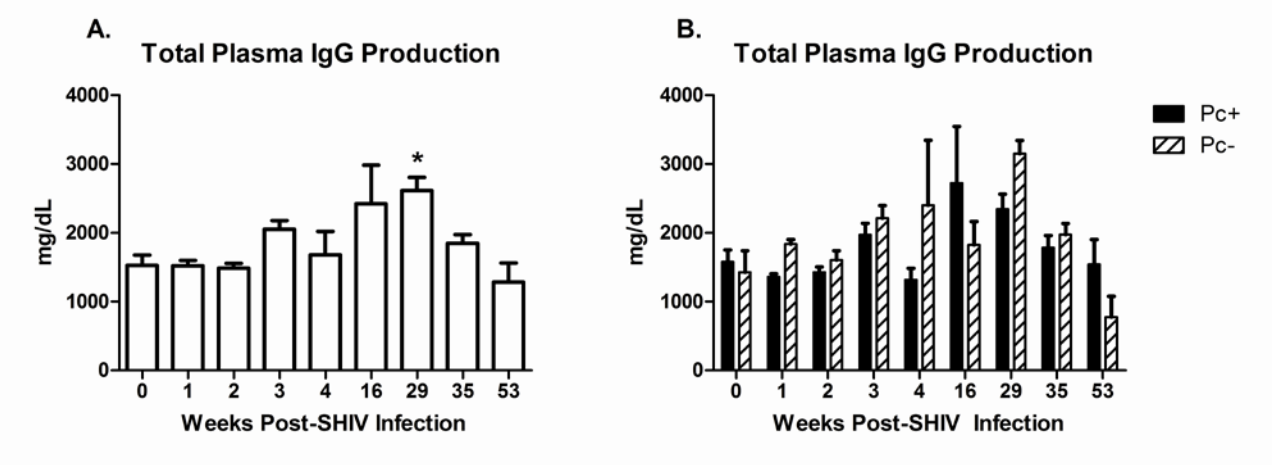

Figure 18. Evidence of hypergammaglobulinemia in SHIV-infected macaques. Levels of total IgG were measured by ELISA in plasma of a subset of macaques (n=12), at baseline and at serial time-points following SHIV-infection. Individual time-points in which values were significantly different from baseline values are indicated by * ($p<0.05$, paired Student's *t*-test).

4.5 DISCUSSION

The current study investigates the impact of SHIV-infection on peripheral blood B cell populations and subsets in cynomolgus macaques to examine whether similar abnormalities are observed in this model as have been reported in HIV-infected individuals. Compared with pre-SHIV infection values, we observed significant declines in total (CD20+), memory (CD20+CD27+), CD21+, and naïve (CD20+CD27-) B cells in the peripheral blood of cynomolgus macaques following SHIV-infection. Significant declines compared with pre-SHIV infection values were observed in both class-switched (IgD-IgM-) and IgM+ memory B cell subsets; however, class-switched memory cells appeared to recover later in infection, while IgM+ memory cells remained depressed. Compared with baseline, we also report significantly elevated levels of B cell activation as measured by CD95 (Fas) expression, as well as hypergammaglobulinemia. A subset of SHIV-infected macaques became naturally colonized with the opportunistic pathogen, *Pneumocystis*, upon immunosuppression and airborne exposure by co-housing (Kling et al., 2009; Shipley et al., in press). For each of the B cell populations we examined, we compared the Pc-colonized and Pc-negative groups of animals for any differential effects of SHIV-infection that may correlate with their colonization status. No significant differences were observed between the Pc-colonized and Pc-negative groups of animals with respect to B cell population abnormalities that were observed following SHIV-infection.

HIV+ patients are at increased risk for opportunistic infections, which may be attributed to generalized B cell deficits and dysfunctions, such as decreased numbers of memory B cells (De Milito et al., 2001), high levels of activation that result in hypergammaglobulinemia (Nagase et al., 2001; De Milito et al., 2004), and possibly the exhaustion of the memory B cell compartment (Moir et al., 2008). Studies that examine B cell subsets in HIV-infected patients

report a variety of reductions and dysfunctions in these populations (Moir et al., 2001; De Milito, 2004), including diminished total and memory (CD27+) B cells (De Milito et al., 2001; Titanji et al., 2006), increased activation (Mizuma et al., 1988; Amadori et al., 1989; Shirai et al., 1992; De Milito et al., 2004), reductions in subsets of memory B cells, such as IgM memory (Hart et al., 2007) and marginal-zone like B cells (Morrow et al., 2008), diminished responses to antigenic stimulation and immunization (Malaspina et al., 2005; Titanji et al., 2006; Moir et al., 2008), decreased expression of important co-stimulatory markers (Malaspina et al., 2003; Jiang et al., 2008), and hypergammaglobulinemia (Nagase et al., 2001; De Milito et al., 2004). Phenotypic B cell alterations induced by HIV may have functional consequences resulting in diminished host capacity to respond to invading pathogens, thereby increasing susceptibility to opportunistic infections. It is for these reasons that we examined B cells and B cell subsets by flow cytometry in SHIV-infected macaques.

Here, several aspects of HIV-induce B cell alterations were observed in the SHIV-macaque model. We observed significantly increased percentages of CD95+ B cells following SHIV-infection, indicating increased activation. Similar to what has been reported for activated T cells, B cells from HIV-infected subjects show elevated expression of Fas/Fas-ligand (De Milito et al., 2001; Titanji et al., 2003). Upregulation of this surface marker not only indicates high levels of activation, but also suggests a dysregulation of the Fas apoptotic pathway, and may contribute to increased susceptibility of B cells to CD95-mediated apoptosis (Moir and Fauci, 2009).

Percentages and absolute numbers of CD21+ B cells were significantly reduced in the peripheral blood of SHIV-infected macaques. Reports from studies of HIV-infected persons also report abnormalities in this population of B cells (Moir et al., 2001; Jiang et al., 2008; Moir and

Fauci, 2009). Evidence from the literature suggests that the frequency of CD21+ memory B cells is directly related to the capacity of these cells to proliferate in response to stimulation (Jiang et al., 2008). Thus, poor proliferative responses of B cells from HIV-infected subjects may be accounted for by the increased level of CD21-low/negative B cells in these subjects (Moir et al., 2001; Jiang et al., 2008; Moir and Fauci, 2009), as these cells are thought to be terminally differentiated and thus, proliferate poorly in response to mitogenic stimulation.

CD27 is a marker for memory B cells in humans, and CD27 has been shown to be a marker of B cell memory in macaques by evaluation of somatic hypermutation in CD27+ vs CD27- cells and by the lack of CD27+ cells in macaque cord blood samples (Kuhrt, et al., submitted). Several studies have reported a loss of CD27+ cells in HIV-infected subjects (De Milito et al., 2001; Nagase et al., 2001; Titanji et al., 2006) in addition to decreased levels of circulating antibodies to immunizing antigens, such as to measles or tetanus (De Milito et al., 2004). A suggested mechanism for this loss is the chronic immune activation and upregulation of CD70 on T cells, and subsequent increased CD70-CD27 interaction and activation (De Milito et al., 2001; Nagase et al., 2001), which results in terminal differentiation of CD27+ memory B cells into antibody-secreting plasma cells. This increased rate of terminal differentiation of memory cells into plasma cells may also then account for the hypergammaglobulinemia reported in HIV-infected patients.

Upon examination of IgM+ and class-switched (IgM-IgD-) memory B cell subsets, we found that both types of CD27+ B cells were affected by SHIV-infection. Although the class-switched CD27+ B cells experienced early deficits following SHIV-infection, this population appeared to recover in the later time-points of infection, and returned to baseline levels. In contrast, the IgM+ CD27+ B cell subset exhibited declines in both percentage and absolute

number around 4 months post-SHIV infection and remained depressed for the study duration, which was approximately one year. These results are comparable to what has been reported in HIV-infected patients, in which mean percentages of IgM+ memory, but not class-switched memory cells, are significantly reduced compared with healthy controls (Hart et al., 2007). IgM+ memory B cells are hypothesized to function in protection from T-independent-type antigens (Weller et al., 2004), such as encapsulated bacteria, and a reduction in this population in HIV-infected patients may account for their increased susceptibility to invasive pneumococcal disease (Hart et al., 2007).

We report significant declines in naïve (CD27-) B cells in SHIV-infected macaques. Recently it was reported that SIV-infected Rhesus macaques experience significant deficits in naïve B cells early following SIV-infection (Kuhrt et al., 2010). This study reported, similar to what we present here, that, compared with baseline levels, naïve B cell numbers remained depressed for longer after SIV-inoculation than did memory B cells (Kuhrt et al., 2010). Reports also indicate that naïve B cells in HIV-infected patients exhibit an activated and more differentiated phenotype, as well as increased spontaneous IgG-secretion (De Milito et al., 2004), and have been hypothesized to contribute to the hypergammaglobulinemia reported during HIV-infection. Further study is needed to determine whether naïve B cells in macaques during SHIV-infection play a role in the chronic immune activation state.

We observed statistically similar B cell declines and increased percentages of activated B cells in both the Pc-colonized and Pc-negative animals, suggesting that B cell declines in various subsets due to SHIV-infection did not correlate with susceptibility to Pc colonization. Previously, we reported that Pc-negative monkeys were capable of mounting a significantly higher Pc-specific memory response compared with Pc-colonized monkeys (Section 3.4.4,

Figure 10). Thus, it appears that Pc-protected monkeys may retain a Pc-specific memory B cell pool, despite an early loss of total CD27+ (memory) B cells. Data from these experiments suggest that exposure to Pc/Pc antigens, prior to immunosuppression, which results in high levels of circulating antibodies/plasma cells (Section 3.4.2), sufficient to prevent or delay Pc-colonization, contributes to the maintenance of a Pc-specific memory B cell pool following immunosuppression, higher levels of which also correlate with protection.

Previous studies of SHIV effects on B cells in macaques have been limited (Margolin et al., 2002; Margolin et al., 2006). Little has been reported, and to our knowledge, this is the first report of phenotype alterations of B cell subsets demonstrated in cynomolgus macaques as a result of SHIV-infection. The results presented here suggest similar phenotypic alterations occur during SHIV-infection as have been reported in HIV+ patients, such as a decrease in total and memory B cells, significantly increased percentages of activated B cells, reduced numbers of CD21+ B cells, and a significant reduction in IgM memory B cells. A significant advantage of this model for the study of the mechanisms responsible for altered humoral immune responses due to SHIV infection, is the ability to examine serial time points for multiple parameters in the same individual animals, which is often difficult to do in clinical studies. This model may be particularly useful for the examination of early disturbances in the B cell compartment early after infection, where changes were observed 2-4 weeks post-infection. Overall, these results provide a strong rationale for the use of the SHIV-macaque model to investigate HIV-related B cell dysfunctions, which may direct more efficient means of induction of protective, vaccine-induced HIV immunity.

4.6 AUTHOR CONTRIBUTIONS AND ACKNOWLEDGEMENTS

Heather M. Kling (Molecular Virology and Microbiology Graduate Program, University of Pittsburgh School of Medicine) generated the majority of the data and prepared the manuscript. Timothy W. Shipley (Immunology Graduate Program, University of Pittsburgh School of Medicine) provided assistance with sample processing and analysis. Karen A. Norris (Department of Immunology, University of Pittsburgh School of Medicine), as the mentor and principal investigator on the project, provided extensive scientific knowledge, insight and critique of the project and the manuscript. All authors, especially Karen A. Norris, contributed to scientific discussion regarding the project and critical reading and editing of the manuscript.

The authors acknowledge Chris Janssen, D.V.M, Ph.D. and Nicole Banichar, C.V.T. for excellent veterinary care. The authors also acknowledge Jan Kristoff and Xiuping Shao for assistance in collection of flow cytometry data. Funding for these experiments was provided by the National Institutes of Health grants HL077095-01A1 and HL077914-01 (KAN), and National Institutes of Health training grant T32 AI49820 (HMK).

5.0 SUMMARY AND CONCLUSIONS

Pneumocystis (Pc) infection remains a significant source of morbidity and mortality in immunocompromised populations, despite the increased availability and efficacy of anti-retroviral therapies as well increased use of Pc prophylaxis. Furthermore, despite abundant research, a general lack of understanding still exists regarding Pc transmission, biology and prevalence in both immunocompetent and immunocompromised hosts. Although Pc colonization in HIV patients is quite common, the clinical consequences of colonization with this pathogen are undefined. Pc contact with and/or attachment to host alveolar epithelial cells results in elevated levels of pro-inflammatory cytokines (Limper 1998, Pottratz 1998), and thus, even low-level persistent Pc colonization may elicit a potentially damaging inflammatory response. For these reasons, it is plausible that colonization of host lungs with Pc organisms may contribute to or exacerbate the development of chronic obstructive pulmonary disease (COPD). This hypothesis is supported by reports of high rates of Pc colonization in COPD patients (Calderon et al., 1996; Probst et al., 2000; Helweg-Larsen et al., 2002), as well as because similar inflammatory processes are described in both Pc infection and in COPD (Di Stefano et al., 1998; Saetta et al., 1998).

The central goal of this research was to establish immune correlates of protection from natural Pc infection in a macaque model of immunosuppression. The first aim of this research was to develop a model of natural Pc colonization in macaques immunosuppressed by infection

with SIV or SHIV. The macaque model of Pc infection possesses a number of advantages. Because this is a model of co-infection, we have the unique opportunity to assess the effects of prolonged Pc colonization in the context of AIDS immunosuppression. Because this is a longitudinal model of disease progression, we also have the opportunity to examine serial time-points and evaluate the host immune response at the earliest possible time-points of Pc colonization. Additionally, because our model uses natural airborne exposure, achieved via co-housing, as the route of infection, it emulates natural conditions of host exposure and Pc infection. This model of Pc colonization is useful for studies examining the role of the immune response in development and progression of colonization and in examining long-term effects of colonization on the lung. Development of this model is important because a large number of HIV-infected persons are colonized with Pc and effects of such colonization are unknown (Huang et al., 2003; Morris et al., 2004a).

Because Pc cannot be cultured *in vitro*, organisms used for inoculation must be derived from lung tissue or BAL fluid of another experimental animal. This is a major drawback of intratracheal inoculation with Pc because, although inocula have been enriched for Pc in previous experiments (Board et al., 2003), introduction of alloantigen and SIV as a component of the Pc inocula could not be avoided and increased the likelihood of transient, non-specific inflammatory responses. For these reasons, and because this model allowed us to investigate the earliest points following Pc colonization, the natural Pc colonization model was preferred over intratracheal Pc inoculation of macaques.

During this experiment monkeys received treatment doses of the anti-Pc medication, trimethoprim-sulfamethoxazole (TMP-SMX), in order to clear potential Pc colonization at the start of SIV infection. In order to prevent Pc colonization until peripheral blood CD4+ T cells

declined to a sufficient level to permit natural Pc colonization, monkeys then continued on a prophylactic dose of TMP-SMX. However, we observed a serial rise in anti-Pc antibody titers and positive PCR results in some animals during the period of TMP-SMX prophylaxis, indicating this regimen did not adequately prevent Pc colonization. While Pc prophylaxis may reduce organism burden sufficiently to prevent active PcP, the results suggest Pc colonization may persist or recur during prophylaxis. These studies have direct clinical relevance to HIV-infected patients as Pc-colonization may occur despite use of Pc prophylaxis and ART (Morris et al., 2004a).

For these studies, we developed an anti-Pc antibody ELISA to evaluate Pc colonization of SIV-infected macaques. Using this method, we were able to detect anti-Pc antibodies the plasma of both healthy and immunosuppressed macaques. Because we were also able to monitor Pc colonization by nested PCR of Pc DNA in the bronchoalveolar lavage (BAL) fluid, in this way we were able to validate changes in antibody responses detected by the ELISA, which indicated Pc colonization. The monkey model was useful to validate the reliability of the assay because, due to difficulties in obtaining serial samples of blood and BAL fluid from human subjects, studies of Pc prevalence in humans are limited in ability to correlate antibody levels with Pc colonization and exposure. In addition, the serial rise in anti-Pc antibody titers occurred prior to detection of Pc DNA in BAL, suggesting the serial ELISA is a more sensitive indicator of early Pc colonization in this model.

The target antigen for detection of anti-Pc antibodies is a recombinant fragment of Pc kexin (KEX1). Pc kexin shares sequence homology with a family of fungal serine endoproteases, and in *P. jirovecii* (human-derived Pc), kexin is encoded by a single copy gene (Lugli et al., 1997; Lee et al., 2000; Kutty and Kovacs, 2003). This makes KEX1 advantageous

over the use of the multicopy Pc MSG genes for detection of Pc-specific antibody responses (Kovacs et al., 1993; Smulian et al., 1997). Experimental mouse models of Pc infection have demonstrated that immune responses to Pc kexin are associated with control of infection and prevention of PcP (Zheng et al., 2005; Wells et al., 2006a), thus changes in anti-KEX1 titers and antibody isotypes during and after infection may serve as a useful correlate of protection.

The next aim of this research was then to evaluate the capacity of Pc KEX1 antibodies to protect immunosuppressed macaques from naturally acquired Pc infection and subsequent lung injury. Macaques were infected with chimeric $SHIV_{89.6P}$ to achieve immunosuppression (Li et al., 1992; Dunn et al., 1996; Reimann et al., 1996; Pawar et al., 2008), and allowed to become naturally Pc colonized using the cohousing model. However, not all animals became Pc colonized. A subset of animals remained Pc-protected (Pc-) for the duration of the experiment (approximately one year). SHIV viral loads and CD4+ T cell counts, standard measures of immunosuppression in lentiviral infections, were comparable in protected versus Pc-colonized animals, indicating that the observed differences in outcome were not due to variable immunosuppression levels.

However, when we evaluated baseline (pre-SHIV infection) KEX1 antibody titers and KEX1-specific antibody secreting cells (ASC), we found that higher baseline anti-KEX1 titers and higher numbers of KEX1-specific ASC prior to immunosuppression correlated with prevention or delay of Pc colonization following SHIV-immunosuppression. These data support what has been reported in mouse models of PcP, which demonstrate the protective capacity of Pc-specific antibody responses through both passive and active immunization (Harmsen et al., 1995; Garvy et al., 1997; Gigliotti et al., 1998a; Gigliotti et al., 2002; Empey et al., 2004; Wells et al., 2006a), especially with respect to Pc-kexin (Gigliotti et al., 1998a; Zheng et al., 2005;

Wells et al., 2006a). Here, we extend this knowledge by demonstrating that Pc-specific antibody responses were important in preventing or resolving Pc colonization in a highly-relevant non-human primate model of HIV infection.

Data from these experiments also suggest importance for IgA production in the lungs. Earlier production of KEX1-IgA in the lungs correlated with protection from Pc colonization in these animals. A plausible mechanism for this protection could be that IgA may block attachment of Pc organisms to alveolar epithelial cells. Human studies, which show that mucosal antibodies to Pc are decreased in patients with PcP, compared with HIV+ patients without PcP or with HIV-negative controls (Laursen et al., 1994; Jalil et al., 2000) support the concept of a protective role for mucosal IgA.

In these studies, we also showed that Pc-protected monkeys demonstrated a stronger KEX1-specific memory B cell response than Pc-colonized monkeys following SHIV-immunosuppression. B cells are known to be important in controlling infection in murine models of PcP as it is reported that B cell-deficient mice are highly susceptible to Pc (Marcotte et al., 1996), and others have suggested the importance of antibody-independent B cell functions in control of infection (Lund et al., 2003; Lund et al., 2006). Our results suggest the importance of a robust memory response in control of Pc infection and that susceptibility to Pc can be predicted by a low baseline KEX1-antibody/B cell response.

The implication of the importance of a strong B cell response needed for a secondary infection acquired during SHIV-immunosuppression is significant in light of reports of HIV-associated B cell deficits and abnormalities that may contribute to poor responses to antigenic stimulation, diminished vaccine responsiveness and increased susceptibility of opportunistic infections (De Milito, 2004; Malaspina et al., 2005; Titanji et al., 2006; Jiang et al., 2008; Moir

and Fauci, 2009). Our results suggest similar declines in B cell populations occur in SHIV-infected macaques as have been reported in HIV-infected individuals. Following SHIV-infection, we observed significant declines in numbers of total (CD20+), memory (CD20+CD27+), and naïve (CD20+CD27-) B cells in the peripheral blood of cynomolgus macaques following SHIV-infection. Numbers of CD21+ B cells were also significantly diminished, similar to what has been reported in HIV+ patients, which may contribute to reduced antigen responsiveness and proliferation (Jiang et al., 2008). Additionally, there was evidence of chronic immune activation, exhibited by sustained increased levels of CD95 (fas) expression on B cells following SHIV-infection and hypergammaglobulinemia.

HIV-infection is also associated with deficits in specific subsets, like IgM memory cells (Hart et al., 2007) or splenic marginal zone-like peripheral blood populations (Morrow et al., 2008), which may contribute to diminished responses of HIV-infected patients to vaccination with carbohydrate antigens and T-independent antigens, such as the pneumococcal vaccine. We also observed significant declines compared with baseline in numbers of both class-switched (IgD-IgM-) and IgM+ memory B cell subsets; however, IgM+ memory cells remained depressed for the duration of the experiment, whereas class-switched memory cells appeared to recover later in infection.

Previous studies of SHIV effects on B cells in macaques have been limited (Margolin et al., 2002; Margolin et al., 2006), and to our knowledge, this is the first report of phenotype alterations of B cell subsets demonstrated in cynomolgus macaques as a result of SHIV-infection. This model is advantageous for the study of the mechanisms responsible for altered humoral immune responses due to SHIV infection because it allows investigators to examine serial time points for multiple parameters in the same individual animals, as well as to investigate

perturbations in the B cell compartment that occur early after infection, which would be difficult, if not impossible, in clinical studies. Overall, the similarities in B cell phenotypic alterations and activation levels in SHIV-infected macaques to B cell abnormalities reported in HIV+ patients (De Milito et al., 2001; De Milito, 2004; Hart et al., 2007; Cagigi et al., 2008; Jiang et al., 2008; Moir and Fauci, 2009), as well as to those recently reported in SIV-infection of Rhesus monkeys (Kuhrt et al., 2010), coupled with the advantages of using a macaque model for examining serial time-points of longitudinal disease progression, provide strong rationale for the use of the SHIV-macaque model to investigate HIV-related B cell dysfunctions.

Although studies of HIV-infected subjects have reported diminished responses to vaccination with carbohydrate antigens and T-independent antigens, memory responses to other types of antigens, such as Pc-KEX1, require further investigation. In our experiments, similar effects of SHIV on B cell populations were seen in both the Pc-colonized and Pc-negative groups of animals, indicating overall B cell abnormalities did not contribute to Pc-colonization status. Instead, Pc-negative monkeys appear to possess a Pc-specific memory B cell pool, despite an early loss of total CD27+ (memory) B cells. These results suggest that exposure to Pc/Pc antigens, prior to immunosuppression, which results in high levels of circulating antibodies/plasma cells sufficient to prevent or delay Pc-colonization, contributes to the maintenance of a Pc-specific memory B cell pool following immunosuppression.

Exposure to Pc is likely frequent in humans (Peglow et al., 1990; Daly et al., 2002; Morris et al., 2008a) and non-human primates (Kling et al., 2009); thus, a study evaluating the humoral response to this pathogen is also qualitatively different than many studies of impaired humoral responses of HIV+ patients, which focus on recall responses to antigens or pathogens against which study participants have been vaccinated (Malaspina et al., 2005; Titanji et al.,

2006), or to antigens to which they are not likely to be naturally exposed (e.g., tetanus toxoid) (De Milito et al., 2004; Hart et al., 2007). Maintenance of the Pc-KEX1-specific antibody responses and B cell memory during SHIV-induced immunosuppression in monkeys that had high baseline Pc humoral immunity, suggests that responses to this antigen were not significantly affected by SHIV-infection. Thus, Pc-KEX1 is a potential model antigen for longitudinal analysis of the preservation of functional humoral responses in patients with defects in CD4+ T cell numbers, due to lentivirus infection or other immunosuppressive states (Duchini et al., 2003).

In the described experiments, we also investigated the potential clinical consequence of persistent Pc colonization and the role of a humoral response to Pc in prevention of pulmonary damage. The evidence we present here of an association of Pc-colonization with pulmonary obstruction supports the concept presented by others that persistent microbial colonization may be involved in perpetuating the inflammatory response, eventually leading to tissue destruction and clinical COPD (Sethi and Murphy, 2008). HIV+ patients, including those with high CD4+ T cells counts on ART (Morris et al., 2009), are at increased risk for Pc colonization (Morris et al., 2004a), as well as an accelerated form of emphysema (Diaz et al., 2000b), and additional evidence suggests a role for Pc colonization in development of COPD in HIV-negative patients (Calderon et al., 1996; Probst et al., 2000) The longitudinal study of pulmonary function in SHIV-infected macaques presented here supports these findings as well as confirm our previous studies proposing a role for Pc colonization and COPD development in the SHIV model (Norris et al., 2006; Shipley et al., in press).

Identifying a relationship between baseline anti-KEX1 antibody titers and susceptibility to Pc colonization and development of COPD extends these findings and supports recently

reported evidence of an association for Pc-kexin antibodies in protection from increased severity of obstruction in COPD patients (Morris et al., 2008a). The publication resulting from this study is presented in Appendix C of this document. This was a cross-sectional pilot study to determine the relationship of Pc KEX1 antibodies to severity of airway obstruction in a cohort of former and current smokers. Subjects included in the study were randomly selected from individuals enrolled in the Emphysema/COPD Research Center (ECRC) at the University of Pittsburgh. Of the 153 subjects included in the study, approximately 63 percent had detectable KEX1 antibody titers. This report that a majority of subjects had detectable KEX1 titers is also consistent with our findings from investigating KEX1 titers in immunocompetent macaques (Kling et al., 2009; reported in Chapter 2 of this dissertation). In this study of COPD patients, a low or undetectable anti-KEX1 antibody titer was an independent predictor of more-severe airway obstruction. A similar relationship was not observed with anti-influenza antibody titers, suggesting that the Pc KEX1 antibody association was not merely an indicator of a poor humoral immune response in these patients. Findings from this study further support the hypothesis that Pc is involved in the pathogenesis or progression of COPD and suggest that the KEX1 antibody assay may be useful in humans (Morris et al., 2008a).

Together with other preliminary work from our group demonstrating an association of low KEX1 antibody levels with subsequent PcP development in HIV+ subjects (Gingo et al., submitted), these findings support the implications of work presented in this dissertation, suggesting that Pc KEX1-antibodies are not only correlative with protection from Pc colonization in a macaque model of immunosuppression, but also relevant in human studies and may be a useful predictor of disease, in both the context of HIV and in COPD patients.

In summary, using a unique model of natural Pc colonization in SIV- or SHIV-immunosuppressed macaques, we have investigated the use of serology in both monitoring Pc colonization and in evaluating the correlation of antibodies to a particular Pc-antigen, kexin, with protection. We have demonstrated that, when examined serially, antibodies to Pc-kexin are more sensitive than nested PCR for detecting early Pc colonization. Additionally, we found that Pc-specific humoral immunity established prior to immunosuppression protects against subsequent Pc colonization and pulmonary obstruction, despite declining CD4+ T cell numbers and abnormalities in B cells populations. Although clinical use of Pc-kexin antibodies as a serum marker of active Pc colonization or PcP would be limited, as serial data are needed for appropriate interpretation, our data and supporting studies in human subjects indicate that instead, early evaluation of KEX1 antibodies may be a useful predictor of clinical outcome or severity of disease. Maintenance of a Pc-kexin-specific memory B cell pool following immunosuppression suggests that despite declines in total and subsets of memory B cells following SHIV-infection, memory B cell responses to this commonly-encountered antigen remain sufficiently intact. Furthermore, these experiments imply that Pc-kexin-specific antibodies may be capable of providing protection of immunocompromised individuals from developing pulmonary obstruction, supporting the hypothesis that Pc-colonization is associated with COPD development. These results demonstrate the importance of a Pc-specific humoral response in protection from Pc colonization and pulmonary damage and thereby provide a rationale for Pc-KEX1 vaccine development to protect at risk populations against this opportunistic pathogen.

Results from these studies suggest the existence of an adequate B cell memory response despite substantial loss of CD4+ T cells, suggesting that B cell help may be derived from

alternative sources under these conditions. These studies form a foundation for future experiments examining whether the B cell response to Pc-kexin can be augmented after SHIV-infection. This could be investigated by immunizing immunosuppressed monkeys with Pc-kexin protein, and examining whether there may be alternative sources of B cell help provided, such as via innate immune mechanisms. Generation of help from innate immunity sources could be explored through the use of adjuvants directed toward the innate arm of the immune response, such as alpha-galactosyl-ceramide (aGC) which has been shown to induce invariant NKT cell help for specific antibody responses to protein antigens (Galli et al., 2007). Another mechanism that might be explored is through CpG-TLR signaling. CpG DNA has been well studied with respect to stimulating innate immunity (Klinman, 2004; Klinman et al., 2004). However, whether these mechanisms of B cell help can function in the context of HIV immunosuppression has not been thoroughly examined, but could be uniquely explored using the SHIV-macaque model of immunosuppression.

To examine alternative mechanisms of B cell help and determine the efficacy of Pc-kexin immunization, macaques could be immunized with KEX1 in combination with different adjuvants (aGC, CpG, etc) to examine whether they can be vaccinated effectively with a T-dependent antigen while immunosuppressed. Studies using this model would examine the ability to induce a memory response to Pc-kexin in SHIV-infected macaques, and determine whether vaccination-induced enhancement of the Pc kexin-antibody response after SHIV-infection would have the capacity to prevent Pc colonization. We would use KEX1-specific ELISA and ELISpot to determine boosting of responses. Pc colonization would be determined as before, using nested PCR for detection of Pc DNA in the BAL fluid and ELISA for detection of anti-KEX1 antibodies. We would also monitor pulmonary function in these animals to investigate

development of obstructive lung disease as a correlate of Pc colonization. The longitudinal nature of the model gives us the capacity to examine baseline KEX1 antibody titers and follow the progression of individual animals following immunization. This model also allows us to potentially answer other questions regarding the nature of the immune response to Pc, such as examining whether long-lived plasma cells are generated in response to Pc exposure or vaccination. When circulating antibody levels are maintained by long-lived plasma cells, then the specific antibody levels do not generally correlate with the levels of specific memory B cells (Radbruch et al., 2006). Additionally, other correlates of protection from Pc colonization, such as cytokine production, could also be evaluated in these studies.

The data presented in this dissertation suggest that antibody responses to KEX1 can be augmented (via Pc exposure) in immunosuppressed monkeys. Thus, we expect that we will detect augmented antibody responses in macaque plasma following vaccination with Pc-kexin. Because we have observed that robust antibody responses to KEX1 correlate with protection from Pc colonization and subsequent airway obstruction, we hypothesize that augmented KEX1 responses will be protective in these animals. These studies would provide rationale for further investigation into strategies for Pc immunization of at-risk HIV-infected individuals. Additionally, results generated from these experiments may provide insight into potential mechanisms of alternative B cell help in the context of HIV-infection.

APPENDIX A

PUBLICATIONS

Kling, H.M., Shipley T.W., and K.A. Norris. SHIV-infected cynomolgus macaques exhibit abnormalities in peripheral blood B cell populations. *In preparation.*

Kling, H.M., Shipley T.W., Patil S., Kristoff J., Montelaro, R., Morris A., and K.A. Norris. Relationship of *Pneumocystis* Humoral Immunity to Prevention of Colonization and COPD in a Primate Model of HIV Infection. *In preparation.*

Shipley T.W., Kling H.M., Morris A., Kristoff J., Shao X., and K.A. Norris. Comparison of lung tissue gene expression profiles from SHIV-infected cynomolgus macaques with and without *Pneumocystis* colonization-induced COPD. *In preparation.*

George, M.P., Brower, A., **Kling, H.M.**, Shipley, T.W., Kristoff, J., Reinhart, T.A., Murphey-Corb, M., Gladwin, M.T., Champion, H.C., Morris, A., and Karen, A. Norris. Pulmonary Vascular Lesions are Common in SIV- and SHIV-Env-infected Macaques. *AIDS and Human Retroviruses. In review.*

Shipley T.W., **Kling, H.M.**, Morris A., Patil. S, Kristoff J., Guyach. S.E., Murphy J.M., Shao, X., Sciurba F.C., Rogers R.M., Richards T., Thomson P., Montelaro R.C., Coxson H.O., Hogg J.C., and K.A. Norris. Persistent *Pneumocystis* colonization leads to the development of chronic obstructive pulmonary disease (COPD) in a non-human primate model of AIDS. *Journal of Infectious Diseases. In press.*

Kling H.M., Shipley T.W., Patil S., Morris A., and K.A. Norris. *Pneumocystis* Colonization in Immunocompetent and Simian Immunodeficiency Virus-Infected Cynomolgus Macaques. *Journal of Infectious Diseases* 2009:199(1): 89–96.

Morris A., Netravali M., **Kling H.M**., Shipley T., Ross T., Sciurba F.C., K.A. Norris."Relationship of *Pneumocystis* Antibody Response and Severity of Chronic Obstructive Pulmonary Disease." *Clinical Infectious Disease*. 2008: 47(S2):e64–e68.

APPENDIX B

This section appeared as an online supplement for the publication of "*Pneumocystis* Colonization in Immunocompetent and Simian Immunodeficiency Virus-Infected Cynomolgus Macaques" in the *Journal of Infectious Diseases* 2009: 199(1).

B.1 SUPPLEMENTARY METHODS FROM CHAPTER 2

B.1.1 Western blot analysis.

A partial fragment of the macaque-derived, Pc kexin gene in the pBAD expression vector (gift from C.G. Haidaris, University of Rochester) was used to produce recombinant kexin protein (KEX1). Western blot *E. coli* Top10 (Invitrogen Corp., Carlsbad, CA) containing the pBAD-KEX1 plasmid was grown overnight at 37°C in Luria-Bertani (LB) broth, supplemented with carbenicillin (100 μg/mL), then diluted 1:20 in fresh LB broth with 100 μg/ml carbenicillin and grown at 37ºC to log phase (OD_{600} 0.7-0.8). KEX1 expression was induced by the addition of L-arabinose (0.01% final concentration) and continued culture for 4.5 h. at 37ºC. Cells were centrifuged for 10 minutes at 4,000 x g and cell pellets were frozen at -80ºC until use. Cells were lysed by thawing in extraction buffer (6 M guanidine-HCl, 50 mM Na_2HPO_4, 300 mM

NaCl pH 7.0) at room temperature for 20 min. After centrifugation (20 min at 7,240 x g), the supernatant fluid was applied to Talon metal affinity resin (Clontech Laboratories, Inc, Mountain View, CA) and KEX1 was eluted with 150 mM imidazole. Purified protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, blocked in 5% nonfat milk with 1% bovine serum albumin and 0.05% Tween 20 in phosphate-buffered saline (PBS), and incubated with plasma from a macaque with PCP, as well as with plasma from SIV-infected macaques from this study. Filters were washed (0.05% Tween, PBS), incubated with horseradish peroxidase-conjugated IgG secondary antibody, and developed according to standard protocols. Protein from the empty pBAD expression vector was prepared and purified in the same manner as KEX1 and used as a negative control in Western blots and ELISA.

B.1.2 ELISA controls.

Background was defined by wells coated with antigen, and to which the secondary antibody was added, but primary antibody was omitted. Previously we have tested plasma and BAL samples by ELISA for reactivity to protein prepared from the expression vector alone and observed negligible reactivity above background. All samples were tested at least twice on separate ELISA plates; reciprocal endpoint titers within one dilution (two-fold) were considered equivalent. ELISA reactivity was confirmed by Western blot in a small subset of samples (only one is shown in Fig 1).

APPENDIX C

RELATIONSHIP OF *PNEUMOCYSTIS* ANTIBODY RESPONSE TO SEVERITY OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Alison Morris,[1] Mahesh Netravali,[1] Heather M. Kling,[2] Timothy Shipley,[2] Ted Ross,[1] Frank C. Sciurba,[1] and Karen A. Norris[2]

Departments of [1]Medicine and [2]Immunology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

Note from the dissertation author: This manuscript is included here because results from this study are highly relevant to, and strongly support, the work presented in this dissertation.

This section was modified with permission from:

Alison Morris, Mahesh Netravali, Heather M. Kling, Timothy Shipley, Ted Ross, Frank C. Sciurba, and Karen A. Norris

Relationship of *Pneumocystis* Antibody Response and Severity of Chronic Obstructive Pulmonary Disease.

Clinical Infectious Disease. 2008: 47(S2):e64–e68.

C.1 ABSTRACT

Pneumocystis colonization has been associated with severity of chronic obstructive pulmonary disease (COPD). The relationship of *Pneumocystis* antibody status to COPD severity has not been investigated, but antibody levels might relate to both colonization susceptibility and COPD progression. We investigated anti-*Pneumocystis* antibody titers and airway obstruction in a cohort of patients with COPD. Undetectable anti-*Pneumocystis* antibody titer was an independent predictor of more-severe airway obstruction, although use of inhaled corticosteroids is a possible confounder of this effect.

C.2 INTRODUCTION

Smoking is the primary risk factor for chronic obstructive pulmonary disease (COPD), but factors that determine which smokers will develop significant disease are largely unknown. Infectious agents might play a role in accelerating progression of airway obstruction or in perpetuating its progression after discontinuation of tobacco exposure. *Pneumocystis jirovecii* is a fungal pathogen that causes pneumonia in immunocompromised individuals. The presence of *Pneumocystis* in the lungs, even at low levels, produces inflammatory changes similar to those seen in COPD (Di Stefano et al., 1998; Saetta et al., 1998). Colonization is highly prevalent in patients with COPD and correlates with disease severity (Calderon et al., 1996; Probst et al., 2000; Morris et al., 2004c).

Host defense against *Pneumocystis* is complex and involves both the humoral and cellular immune responses (Steele et al., 2005). $CD4^+$ T cells have historically been implicated in

susceptibility to colonization with *Pneumocystis*, but an antibody-mediated response is also likely to be important. Antibodies to the *Pneumocystis* endoprotease kexin (KEX1) may be particularly important, because immune responses to *Pneumocystis* kexin have been associated with control of *Pneumocystis* infection in animal models (Zheng et al., 2005; Wells et al., 2006a).

The serum KEX1 antibody response in patients with COPD has not been investigated and might be important for further clarifying the role of *Pneumocystis* in COPD by indicating a mechanism by which patients with COPD become colonized and by serving as a noninvasive marker of susceptibility to *Pneumocystis* colonization. We performed a cross-sectional pilot study to determine the relationship of *Pneumocystis* KEX1 antibodies to severity of airway obstruction in a cohort of former and current smokers.

C.3 PATIENTS, MATERIALS AND METHODS

Persons who were former or current smokers with a history of smoking at least 10 packs per year were randomly selected from individuals enrolled in the Emphysema/COPD Research Center at the University of Pittsburgh (Pittsburgh, PA). Participants were recruited for this registry from various areas of Pittsburgh and its suburbs. Exclusion criteria included current exacerbation, completely reversible airflow obstruction, a significant allergy history, or a history of clinical asthma. The University of Pittsburgh Institutional Review Board approved the study, and all participants provided informed consent.

Spirometry and measurement of single breath carbon monoxide diffusing capacity (DLCO) were performed at entry into the Emphysema/COPD Research Center, according to

American Thoracic Society criteria (Miller et al., 2005). The percentage of forced predicted expiratory volume in 1 s (FEV_1), forced vital capacity (FVC), and DLCO were calculated with use of standard reference equations (Crapo and Morris, 1981; Crapo et al., 1981). Plasma samples were obtained from patients at enrollment in the Emphysema/COPD Research Center registry and were stored at −80°C.

A partial fragment of the macaque-derived *Pneumocystis* kexin gene in the pBAD expression vector (gift from C. G. Haidaris, University of Rochester) was used to produce recombinant KEX1. *Escherichia coli* Top10 (Invitrogen), containing the pBAD-KEX1 plasmid, was grown overnight at 37°C in Luria-Bertani broth, supplemented with 100 μg/mL of carbenicillin, diluted 1:20 in fresh Luria-Bertani broth with 100 μg/mL of carbenicillin, and grown at 37°C to log phase (optical density of liquid medium at 600 nm, 0.7–0.8). KEX1 expression was induced by the addition of L-arabinose (0.01% final concentration) and continued culture for 4.5 h at 37°C. Cells were centrifuged for 10 min at 4000 *g*, and cell pellets were frozen at −80°C until use. Cells were lysed by thawing in extraction buffer (6 mol/L guanidine-hydrogen chloride, 50 mmol/L disodium hydrogen orthophosphate, and 300 mmol/L sodium chloride; pH, 7.0) at room temperature for 20 min. After centrifugation (20 min at 7240 *g*), the supernatant fluid was applied to Talon metal affinity resin (Clontech Laboratories), and KEX1 was eluted with 150 mmol/L imidazole. Purified protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose, blocked in 5% nonfat milk with 1% bovine serum albumin and 0.05% Tween 20 in PBS, and incubated with a plasma sample obtained from a macaque with *Pneumocystis* pneumonia. Microtiter plates (Immunolon 4HBX; Thermo Fisher Scientific) were coated with 5 μg/mL of purified KEX1 in sodium bicarbonate (pH, 9.5). Heat-inactivated plasma was diluted 1:100 in blocking buffer (PBS with

5% nonfat milk). Fifty microliters of plasma were plated into KEX1-coated wells, and serial dilutions up to 1:12,800 were made to determine end point titers. Goat antihuman immunoglobulin-conjugated horseradish peroxidase (1:10,000 for IgG; Sigma-Aldrich) was used for detection, and plates were developed by standard methods. Normal human plasma samples (*Pneumocystis* negative by antibody titer assay) were used as negative controls. The reciprocal end point titer was calculated as the highest dilution at which the optical density was the same or less than that of the control.

To determine whether patients with low KEX1 levels had a generalized defect in humoral immunity, plasma samples were also tested for antibodies to influenza with use of the hemagglutinin inhibition assay, adapted from the Centers for Disease Control and Prevention laboratory-based influenza surveillance manual (Bright et al., 2008). Antibody titers against A/Fujian/411/2002 (H3N2) and A/Wisconsin/65/2005 (H3N2) were determined.

Stata, version 8 (Stata), was used for analysis, and statistical significance was determined at $p < 0.05$. Variables were analyzed with use of either Student's *t* test and the Wilcoxon rank-sum test or the χ^2 test and Fisher's exact test. Demographic variables and antibody levels were determined for the entire cohort. Because we hypothesized that COPD severity would be associated with decreased antibody titers, we then examined the relationship of antibody levels as a continuous variable to pulmonary function parameters. Univariate analyses were also performed to determine clinical variables related to an undetectable KEX1 antibody titer (defined as a KEX1 titer <1:100). Multivariate linear regression was performed to determine independent predictors of FEV_1%, FEV_1-to-FVC ratio, and DLCO by including variables hypothesized to be causally related to the outcomes or that were statistically significant at $p = 0.1$ in univariate analyses. Models were run with log-transformed absolute anti-*Pneumocystis* reciprocal end point

titers and with antibody status as a dichotomous variable (detectable vs. undetectable antibody titer). We explored interactions between model variables, and normality of model residuals was assessed. Similar models were performed for influenza antibody titers.

C.4 RESULTS

One hundred fifty-three patients were included in the analysis (Table 1). The median anti-KEX1 antibody titer was 375 (range, 1–12,800), and 96 patients (62.7%) had detectable titers. Patients with undetectable antibody titers were similar to those with detectable titers with regard to age, sex, and number of patients who were currently smoking (Table 1). Patients with undetectable titers tended to have a lower pack-year smoking history (defined as the average number of packs smoked per day multiplied by the number of years that the person smoked), compared with patients with detectable titers (50.2 pack-years vs. 58.6 pack-years; p=0.06). Despite a somewhat lower pack-year smoking history, the patients with undetectable titers had a significantly lower FEV_1-to-FVC ratio (0.46 vs. 0.51; p=0.04) and tended to have a lower FEV_1% predicted, compared with patients with detectable titers (49.9% vs. 57.8%; p=0.08). The DLCO% predicted did not differ between groups. Compared with patients with detectable antibody titers, patients with undetectable titers were more symptomatic, according to the modified Medical Research Council dyspnea index scale (2.0 vs. 1.5; p=0.04), and were more likely to be using inhaled corticosteroids (52.6% vs. 32.3%; p=0.04). Patients with undetectable titers also had a lower mean (±SD) body mass index (defined as weight in kilograms divided by the square of the height in meters) than did patients who had a detectable antibody response (25.9 ± 4.2 vs. 28.4 ± 5.8);

however, most patients had a normal body mass index. Only 5 patients had an abnormally low body mass index (<19), and there was no relationship between a low body mass index and *Pneumocystis* antibody status. Data on injection or inhaled illicit drug use was not available; however, the population consisted of older, HIV-uninfected patients, and thus, the prevalence of drug use was likely to have been low. There were no differences in the percentages of patients who had been hospitalized or had experienced exacerbation in the previous year according to antibody status.

Table C1 Characteristics of patients with chronic obstructive pulmonary disease (COPD), by anti-*Pneumocystis* antibody status.

Characteristic	Patients			OR (95% CI)	*P*
	All (*n* = 153)	With undetectable antibody (*n* = 57)	With detectable antibody (*n* = 96)		
Age, mean years ± SD	63.3 ± 8.0	63.6 ± 8.4	63.1 ± 7.8	…	
Sex					
Male	86 (56.2)	30 (52.3)	56 (58.3)	…	
Female	67 (43.8)	27 (47.7)	30 (41.7)	…	
Current smoker	42 (27.5)	16 (28.1)	26 (27.1)	…	
Smoking history, median pack-years (range)[a]	55.4 (26.8)	50.2 (18.3)	58.6 (30.5)	1.01 (1.00–1.03)	.06
Mean FEV_1% predicted ± SD	54.9 ± 26.4	49.9 ± 25.6	57.8 ± 26.6	3.20 (0.88–11.5)	.08
Mean FEV_1:FVC ± SD	0.49 ± 0.17	0.46 ± 0.17	0.51 ± 0.17	8.23 (1.07–63.2)	.04
Mean DLCO% predicted ± SD	45.8 ± 19.0	45.2 ± 17.4	44.6 ± 20.2	…	
Median MMRC (range)	2 (0–4)	2 (0–4)	1.5 (0–4)	0.72 (0.53–0.97)	.04
Used inhaled corticosteroids	61 (39.9)	30 (52.6)	31 (32.3)	0.43 (0.22–0.84)	.01
Used oral corticosteroids	9 (5.9)	4 (7.0)	5 (5.2)	…	
Mean BMI ± SD	27.5 ± 5.4	25.9 ± 4.2	28.4 ± 5.8	…	.009
Exacerbation within the previous year	37 (24.2)	13 (22.8)	24 (25.)	…	
Hospitalization within the previous year	29 (19.0)	14 (24.6)	15 (15.7)	…	

NOTE. Data are no. (%) of patients, unless otherwise indicated. BMI, body mass index (calculated as weight in kilograms divided by the square of the height in meters); DLCO, diffusing capacity for carbon monoxide; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; GOLD, Global Initiative on Obstructive Lung Diseases; MMRC, modified Medical Research Council dyspnea index.

[a]Pack-years is defined as the average number of packs smoked per day multiplied by the number of years that the person smoked.

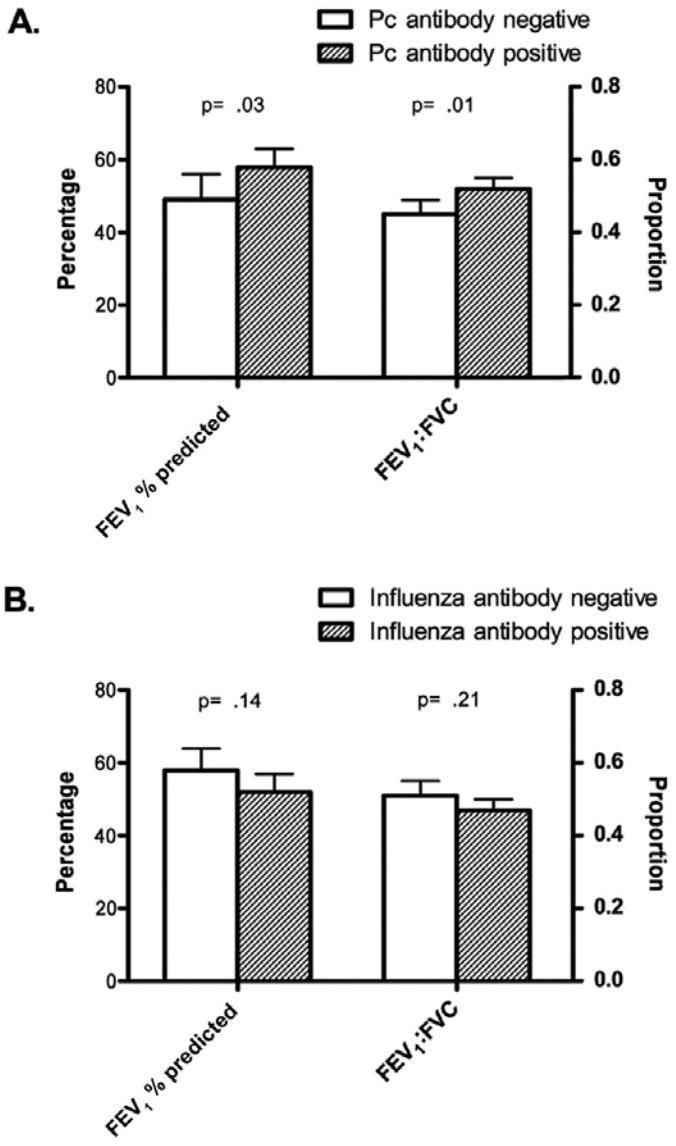

Figure C1. *A,* Adjusted mean spirometry values and 95% CIs for patients, by *Pneumocystis* (Pc) antibody status. *B,* Adjusted mean spirometry values and 95% CIs for patients, by anti-influenza antibody status. Data were adjusted for age, pack-year smoking history (defined as the average number of packs smoked per day multiplied by the number of years that the person smoked), current smoking status (forced expiratory volume in 1 s [FEV_1] and FEV_1-to–forced vital capacity [FVC] ratio), and sex (FEV_1-to-FVC ratio only).

C.5 DISCUSSION

To our knowledge, this study is the first to report the relationship between anti-*Pneumocystis* antibodies and the degree of COPD in smokers. We found that a low or undetectable anti-KEX1 antibody titer was an independent predictor of more-severe airway obstruction. We did not find a similar relationship with anti-influenza antibody titers, which suggests that the *Pneumocystis* antibody association is not merely a marker of a poor humoral immune response. This finding lends additional support to the hypothesis that *Pneumocystis* is involved in the pathogenesis or progression of COPD and suggests that the KEX1 antibody assay is a useful test in humans.

Previous studies demonstrated that an intact antibody response is important for protection from infection due to *Pneumocystis.* Studies have reported that HIV-infected patients have lower anti-*Pneumocystis* antibody levels than do HIV-uninfected blood donors (Daly et al., 2002; Bishop and Kovacs, 2003). Patients who have repeated episodes of *P. jirovecii* pneumonia fail to mount an antibody response to the *Pneumocystis* major surface glycoprotein (Daly et al., 2006). Although data regarding the anti-KEX1 antibody response in humans are limited, studies of rodent vaccine have indicated that immunization with KEX1 can result in a protective antibody response (Zheng et al., 2005; Wells et al., 2006a). The current study is, to our knowledge, the first to report that the majority of adults have a detectable anti-KEX1 titer and that this titer relates to COPD. Future studies will be necessary to determine whether a particular breakpoint can be used to distinguish patients colonized with *Pneumocystis* from those who are not colonized.

The association of low anti-*Pneumocystis* antibody titer with severity of airway obstruction is intriguing with regard to susceptibility to *Pneumocystis* colonization and

progression of COPD. Data from nonhuman primates infected with chimeric simian immunodeficiency virus/HIV indicate that low or undetectable baseline anti-KEX1 titers predict subsequent susceptibility to colonization with *Pneumocystis* (H. M. Kling, T. W. Shipley, S. Patil, A. Morris, K. A. Norris, unpublished observation). We previously demonstrated that the prevalence of colonization with *Pneumocystis* is increased in patients with COPD, compared with the rate among those with other types of end-stage lung diseases, and that colonization with *Pneumocystis* is associated with COPD severity, independent of smoking history (Morris et al., 2004c). Although we did not have direct data on colonization with *Pneumocystis* in these patients, the current findings suggest that low or undetectable anti-*Pneumocystis* antibody titers might increase susceptibility to colonization with *Pneumocystis,* which in turn might stimulate pulmonary inflammation and worsen obstruction.

This study had several limitations. First, antibody levels were measured at a single time, and we did not have corresponding data on colonization with *Pneumocystis.* We also were unable to determine the cause and effect of *Pneumocystis* infection in patients with COPD in our study. The organism might worsen disease or might be an indicator of disease severity. Also, *Pneumocystis* is likely to be one of several pathogens involved in the pathogenesis and progression of COPD, and other organisms, such as *Haemophilus influenzae* and adenovirus, may be important (Sethi, 2000; Retamales et al., 2001). Despite these limitations, our study involved necessary preliminary work to demonstrate that KEX1 antibodies are detectable in humans and are related to COPD. Future studies examining the time course of antibody response in patients with COPD, linking antibody levels to detection of *Pneumocystis* colonization in respiratory specimens, and determining the role of *Pneumocystis* in disease progression will be informative.

The use of inhaled corticosteroids was a confounding factor in the analysis and interpretation of the *Pneumocystis* antibody response. Patients with a low antibody response were more likely than others to be using inhaled corticosteroids, although use of oral corticosteroids had no relationship to antibody levels. Because patients with more-severe COPD are more likely to receive prescriptions for inhaled corticosteroids, it is impossible to completely separate this effect. We do not think that inhaled corticosteroid use affected the ability to mount a systemic humoral immune response, because use of inhaled corticosteroids was not associated with influenza antibody response; however, we cannot rule out the possibility that these medications altered the immune environment of the lung, thereby decreasing the *Pneumocystis* antibody response. Nonetheless, a decrease in the ability to generate an antibody response that is the result of inhaled corticosteroid use might still increase the risk of colonization with *Pneumocystis* and potentially result in worsening of COPD.

In summary, we found that adult smokers have a detectable anti-KEX1 *Pneumocystis* titer and that lower antibody levels are independently associated with more-severe airway obstruction. These findings lend additional support to a potential role of *Pneumocystis* in the progression of COPD and suggest that decreased antibody response might be an important mechanism by which smokers become colonized. If future studies correlate antibody response with susceptibility to colonization with *Pneumocystis*, serum antibody titer could be used as a noninvasive marker of risk of *Pneumocystis* colonization to identify patients susceptible to such colonization.

C.6 AUTHOR CONTRIBUTIONS AND ACKNOWLEDGEMENTS

Alison Morris (Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh) performed statistical analysis and prepared the manuscript. Mahesh Netravali (Division of Allergy and Infectious Diseases, University of Pittsburgh School of Medicine) performed the experiments as part of his fellowship project. Heather Kling (Molecular Virology and Microbiology Graduate Program, University of Pittsburgh School of Medicine) and Timothy Shipley (Immunology Graduate Program, University of Pittsburgh School of Medicine) provided a great deal of assistance in generating the kexin-antibody data. Ted Ross (Department of Medicine, University of Pittsburgh School of Medicine) generated the influenza antibody data. Frank C. Sciurba (Department of Medicine, University of Pittsburgh School of Medicine) provided assistance with data analysis. Karen A. Norris (Department of Immunology, University of Pittsburgh School of Medicine) provided scientific knowledge, insight and direction of the project. All authors contributed to scientific discussion regarding the project and critical reading and editing of the manuscript.

The authors thank Donald Carter and Chad Karoleski for technical assistance.

Financial support was provided by the National Institutes of Health (HL07837 to A.M. and P50 HL084948 to F.S.).

BIBLIOGRAPHY

(2003) Tables of Antibacterial Drug Dosages. In: L.K. Pickering (Ed) Red Book: 2003 Report of the Committee on Infectious Diseases, Vol. 2003. American Academy of Pediatrics, Elk Grove Village, IL, p. 699-712.

Abouya, Y.L., Beaumel, A., Lucas, S., Dago-Akribi, A., Coulibaly, G., N'Dhatz, M., Konan, J.B., Yapi, A. and De Cock, K.M. (1992) Pneumocystis carinii pneumonia. An uncommon cause of death in African patients with acquired immunodeficiency syndrome. Am Rev Respir Dis 145, 617-20.

Alvarez-Martinez, M.J., Miro, J.M., Valls, M.E., Moreno, A., Rivas, P.V., Sole, M., Benito, N., Domingo, P., Munoz, C., Rivera, E., Zar, H.J., Wissmann, G., Diehl, A.R., Prolla, J.C., de Anta, M.T., Gatell, J.M., Wilson, P.E. and Meshnick, S.R. (2006) Sensitivity and specificity of nested and real-time PCR for the detection of Pneumocystis jiroveci in clinical specimens. Diagn Microbiol Infect Dis 56, 153-60.

Amadori, A., Zamarchi, R., Ciminale, V., Del Mistro, A., Siervo, S., Alberti, A., Colombatti, M. and Chieco-Bianchi, L. (1989) HIV-1-specific B cell activation. A major constituent of spontaneous B cell activation during HIV-1 infection. J Immunol 143, 2146-52.

Amedee, A.M., Lacour, N., Gierman, J.L., Martin, L.N., Clements, J.E., Bohm, R., Jr., Harrison, R.M. and Murphey-Corb, M. (1995) Genotypic selection of simian immunodeficiency virus in macaque infants infected transplacentally. J Virol 69, 7982-90.

An, C.L., Gigliotti, F. and Harmsen, A.G. (2003) Exposure of immunocompetent adult mice to Pneumocystis carinii f. sp. muris by cohousing: growth of P. carinii f. sp. muris and host immune response. Infect Immun 71, 2065-70.

Bartlett, M.S., Angus, W.C., Shaw, M.M., Durant, P.J., Lee, C.H., Pascale, J.M. and Smith, J.W. (1998) Antibody to Pneumocystis carinii protects rats and mice from developing pneumonia. Clin Diagn Lab Immunol 5, 74-7.

Baskerville, A., Dowsett, A.B., Cook, R.W., Dennis, M.J., Cranage, M.P. and Greenaway, P.J. (1991) Pneumocystis carinii pneumonia in simian immunodeficiency virus infection: immunohistological and scanning and transmission electron microscopical studies. J Pathol 164, 175-84.

Baskin, G.B., Murphey-Corb, M., Watson, E.A. and Martin, L.N. (1988) Necropsy findings in rhesus monkeys experimentally infected with cultured simian immunodeficiency virus (SIV)/delta. Vet Pathol 25, 456-67.

Beck, J.M. and Harmsen, A.G. (1998) Lymphocytes in host defense against Pneumocystis carinii. Semin Respir Infect 13, 330-8.

Beck, J.M., Newbury, R.L., Palmer, B.E., Warnock, M.L., Byrd, P.K. and Kaltreider, H.B. (1996) Role of CD8+ lymphocytes in host defense against Pneumocystis carinii in mice. J Lab Clin Med 128, 477-87.

Benfield, T.L., Lundgren, B., Shelhamer, J.H. and Lundgren, J.D. (1999) Pneumocystis carinii major surface glycoprotein induces interleukin-8 and monocyte chemoattractant protein-1 release from a human alveolar epithelial cell line. Eur J Clin Invest 29, 717-22.

Bishop, L.R. and Kovacs, J.A. (2003) Quantitation of anti-Pneumocystis jiroveci antibodies in healthy persons and immunocompromised patients. J Infect Dis 187, 1844-8.

Board, K.F., Patil, S., Lebedeva, I., Capuano, S., 3rd, Trichel, A.M., Murphey-Corb, M., Rajakumar, P.A., Flynn, J.L., Haidaris, C.G. and Norris, K.A. (2003) Experimental Pneumocystis carinii pneumonia in simian immunodeficiency virus-infected rhesus macaques. J Infect Dis 187, 576-88.

Bright, R.A., Carter, D.M., Crevar, C.J., Toapanta, F.R., Steckbeck, J.D., Cole, K.S., Kumar, N.M., Pushko, P., Smith, G., Tumpey, T.M. and Ross, T.M. (2008) Cross-Clade Protective Immune Responses to Influenza Viruses with H5N1 HA and NA Elicited by an Influenza Virus-Like Particle. PLoS ONE 3, e1501.

Cagigi, A., Nilsson, A., De Milito, A. and Chiodi, F. (2008) B cell immunopathology during HIV-1 infection: lessons to learn for HIV-1 vaccine design. Vaccine 26, 3016-25.

Calderon, E.J., Regordan, C., Medrano, F.J., Ollero, M. and Varela, J.M. (1996) Pneumocystis carinii infection in patients with chronic bronchial disease. Lancet 347, 977.

Casanova-Cardiel, L. and Leibowitz, M.J. (1997) Presence of Pneumocystis carinii DNA in pond water. J Eukaryot Microbiol 44, 28S.

Chagas, C. (1909) Nova tripanozomiaze humana. Mem Inst Oswaldo Cruz 1, 159-218.

Chalifoux, L.V., King, N.W. and Letvin, N.L. (1984) Morphologic changes in lymph nodes of macaques with an immunodeficiency syndrome. Lab Invest 51, 22-6.

Chalifoux, L.V., Ringler, D.J., King, N.W., Sehgal, P.K., Desrosiers, R.C., Daniel, M.D. and Letvin, N.L. (1987) Lymphadenopathy in macaques experimentally infected with the simian immunodeficiency virus (SIV). Am J Pathol 128, 104-10.

Chong, Y., Ikematsu, H., Yamamoto, M., Murata, M., Yamaji, K., Nishimura, M., Nabeshima, S., Kashiwagi, S. and Hayashi, J. (2004) Increased frequency of CD27- (naive) B cells and their phenotypic alteration in HIV type 1-infected patients. AIDS Res Hum Retroviruses 20, 621-9.

Christensen, P.J., Preston, A.M., Ling, T., Du, M., Fields, W.B., Curtis, J.L. and Beck, J.M. (2008) Pneumocystis murina infection and cigarette smoke exposure interact to cause increased organism burden, development of airspace enlargement, and pulmonary inflammation in mice. Infect Immun 76, 3481-90.

Collins, R.L., Kanouse, D.E., Gifford, A.L., Senterfitt, J.W., Schuster, M.A., McCaffrey, D.F., Shapiro, M.F. and Wenger, N.S. (2001) Changes in health-promoting behavior following diagnosis with HIV: prevalence and correlates in a national probability sample. Health Psychol 20, 351-60.

Connors, M., Kovacs, J.A., Krevat, S., Gea-Banacloche, J.C., Sneller, M.C., Flanigan, M., Metcalf, J.A., Walker, R.E., Falloon, J., Baseler, M., Feuerstein, I., Masur, H. and Lane, H.C. (1997) HIV infection induces changes in CD4+ T-cell phenotype and depletions within the CD4+ T-cell repertoire that are not immediately restored by antiviral or immune-based therapies. Nat Med 3, 533-40.

Crapo, R.O. and Morris, A.H. (1981) Standardized single breath normal values for carbon monoxide diffusing capacity. Am Rev Respir Dis 123, 185-9.

Crapo, R.O., Morris, A.H. and Gardner, R.M. (1981) Reference spirometric values using techniques and equipment that meet ATS recommendations. Am Rev Respir Dis 123, 659-64.

Croix, D.A., Board, K., Capuano, S., 3rd, Murphey-Corb, M., Haidaris, C.G., Flynn, J.L., Reinhart, T. and Norris, K.A. (2002) Alterations in T lymphocyte profiles of bronchoalveolar lavage fluid from SIV- and Pneumocystis carinii-coinfected rhesus macaques. AIDS Res Hum Retroviruses 18, 391-401.

Crothers, K., Butt, A.A., Gibert, C.L., Rodriguez-Barradas, M.C., Crystal, S. and Justice, A.C. (2006) Increased COPD among HIV-positive compared to HIV-negative veterans. Chest 130, 1326-33.

Crotty, S., Aubert, R.D., Glidewell, J. and Ahmed, R. (2004) Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system. J Immunol Methods 286, 111-22.

Daly, K.R., Fichtenbaum, C.J., Tanaka, R., Linke, M.J., O'Bert, R., Thullen, T.D., Hui, M.S., Smulian, A.G. and Walzer, P.D. (2002) Serologic responses to epitopes of the major surface glycoprotein of Pneumocystis jiroveci differ in human immunodeficiency virus-infected and uninfected persons. J Infect Dis 186, 644-51.

Daly, K.R., Huang, L., Morris, A., Koch, J., Crothers, K., Levin, L., Eiser, S., Satwah, S., Zucchi, P. and Walzer, P.D. (2006) Antibody response to Pneumocystis jirovecii major surface glycoprotein. Emerg Infect Dis 12, 1231-7.

De Milito, A. (2004) B lymphocyte dysfunctions in HIV infection. Curr HIV Res 2, 11-21.

De Milito, A., Morch, C., Sonnerborg, A. and Chiodi, F. (2001) Loss of memory (CD27) B lymphocytes in HIV-1 infection. Aids 15, 957-64.

De Milito, A., Nilsson, A., Titanji, K., Thorstensson, R., Reizenstein, E., Narita, M., Grutzmeier, S., Sonnerborg, A. and Chiodi, F. (2004) Mechanisms of hypergammaglobulinemia and impaired antigen-specific humoral immunity in HIV-1 infection. Blood 103, 2180-6.

de Oliveira, A., Unnasch, T.R., Crothers, K., Eiser, S., Zucchi, P., Moir, J., Beard, C.B., Lawrence, G.G. and Huang, L. (2007) Performance of a molecular viability assay for the diagnosis of Pneumocystis pneumonia in HIV-infected patients. Diagn Microbiol Infect Dis 57, 169-76.

Dei-Cas, E., Brun-Pascaud, M., Bille-Hansen, V., Allaert, A. and Aliouat, E.M. (1998) Animal models of pneumocystosis. FEMS Immunol Med Microbiol 22, 163-8.

Delanoe, P. and Delanoe, M. (1912) Sur les rapports des kystes de Carini du poumon des rats avec le *Trypanosoma lewisi*. Compt Rend Acad Sci 155, 658.

Demanche, C., Wanert, F., Barthelemy, M., Mathieu, J., Durand-Joly, I., Dei-Cas, E., Chermette, R. and Guillot, J. (2005) Molecular and serological evidence of Pneumocystis circulation in a social organization of healthy macaques (Macaca fascicularis). Microbiology 151, 3117-25.

DeMilito, A., Morch, C., Sonnerborg, A., Chiodi, F. (2001) Loss of memory (CD27) B lymphocytes in HIV-1 infection. AIDS 15, 957-964.

Di Stefano, A., Capelli, A., Lusuardi, M., Balbo, P., Vecchio, C., Maestrelli, P., Mapp, C.E., Fabbri, L.M., Donner, C.F. and Saetta, M. (1998) Severity of airflow limitation is associated with severity of airway inflammation in smokers. Am J Respir Crit Care Med 158, 1277-85.

Diaz, O., Villafranca, C., Ghezzo, H., Borzone, G., Leiva, A., Milic-Emil, J. and Lisboa, C. (2000a) Role of inspiratory capacity on exercise tolerance in COPD patients with and without tidal expiratory flow limitation at rest. Eur Respir J 16, 269-75.

Diaz, P.T., Clanton, T.L. and Pacht, E.R. (1992) Emphysema-like pulmonary disease associated with human immunodeficiency virus infection. Ann Intern Med 116, 124-8.

Diaz, P.T., King, M.A., Pacht, E.R., Wewers, M.D., Gadek, J.E., Nagaraja, H.N., Drake, J. and Clanton, T.L. (2000b) Increased susceptibility to pulmonary emphysema among HIV-seropositive smokers. Ann Intern Med 132, 369-72.

Diaz, P.T., Wewers, M.D., Pacht, E., Drake, J., Nagaraja, H.N. and Clanton, T.L. (2003) Respiratory symptoms among HIV-seropositive individuals. Chest 123, 1977-82.

Duchini, A., Goss, J.A., Karpen, S. and Pockros, P.J. (2003) Vaccinations for adult solid-organ transplant recipients: current recommendations and protocols. Clin Microbiol Rev 16, 357-64.

Dunn, C.S., Beyer, C., Kieny, M.P., Gloeckler, L., Schmitt, D., Gut, J.P., Kirn, A. and Aubertin, A.M. (1996) High viral load and CD4 lymphopenia in rhesus and cynomolgus macaques infected by a chimeric primate lentivirus constructed using the env, rev, tat, and vpu genes from HIV-1 Lai. Virology 223, 351-61.

Edman, J.C., Kovacs, J.A., Masur, H., Santi, D.V., Elwood, H.J. and Sogin, M.L. (1988) Ribosomal RNA sequence shows Pneumocystis carinii to be a member of the fungi. Nature 334, 519-22.

Empey, K.M., Hollifield, M., Schuer, K., Gigliotti, F. and Garvy, B.A. (2004) Passive immunization of neonatal mice against Pneumocystis carinii f. sp. muris enhances control of infection without stimulating inflammation. Infect Immun 72, 6211-20.

Ezekowitz, R.A., Williams, D.J., Koziel, H., Armstrong, M.Y., Warner, A., Richards, F.F. and Rose, R.M. (1991) Uptake of Pneumocystis carinii mediated by the macrophage mannose receptor. Nature 351, 155-8.

Fournier, A.M., Baillat, V., Alix-Panabieres, C., Fondere, J.M., Merle, C., Segondy, M., Huguet, M.F., Reynes, J. and Vendrell, J.P. (2002) Dynamics of spontaneous HIV-1 specific and non-specific B-cell responses in patients receiving antiretroviral therapy. Aids 16, 1755-60.

Furuta, T., Fujita, M., Mukai, R., Sakakibara, I., Sata, T., Miki, K., Hayami, M., Kojima, S. and Yoshikawa, Y. (1993) Severe pulmonary pneumocystosis in simian acquired immunodeficiency syndrome induced by simian immunodeficiency virus: its characterization by the polymerase-chain-reaction method and failure of experimental transmission to immunodeficient animals. Parasitol Res 79, 624-8.

Gajdusek, D.C. (1957) Pneumocystis carinii; etiologic agent of interstitial plasma cell pneumonia of premature and young infants. Pediatrics 19, 543-65.

Galli, G., Pittoni, P., Tonti, E., Malzone, C., Uematsu, Y., Tortoli, M., Maione, D., Volpini, G., Finco, O., Nuti, S., Tavarini, S., Dellabona, P., Rappuoli, R., Casorati, G. and Abrignani, S. (2007) Invariant NKT cells sustain specific B cell responses and memory. Proc Natl Acad Sci U S A 104, 3984-9.

Garvy, B.A., Wiley, J.A., Gigliotti, F. and Harmsen, A.G. (1997) Protection against Pneumocystis carinii pneumonia by antibodies generated from either T helper 1 or T helper 2 responses. Infect Immun 65, 5052-6.

Gigliotti, F., Garvy, B.A., Haidaris, C.G. and Harmsen, A.G. (1998a) Recognition of Pneumocystis carinii antigens by local antibody-secreting cells following resolution of P. carinii pneumonia in mice. J Infect Dis 178, 235-42.

Gigliotti, F., Haidaris, C.G., Wright, T.W. and Harmsen, A.G. (2002) Passive intranasal monoclonal antibody prophylaxis against murine Pneumocystis carinii pneumonia. Infect Immun 70, 1069-74.

Gigliotti, F., Harmsen, A.G. and Wright, T.W. (2003) Characterization of transmission of Pneumocystis carinii f. sp. muris through immunocompetent BALB/c mice. Infect Immun 71, 3852-6.

Gigliotti, F. and Hughes, W.T. (1988) Passive immunoprophylaxis with specific monoclonal antibody confers partial protection against Pneumocystis carinii pneumonitis in animal models. J Clin Invest 81, 1666-8.

Gigliotti, F., Wiley, J.A. and Harmsen, A.G. (1998b) Immunization with Pneumocystis carinii gpA is immunogenic but not protective in a mouse model of P. carinii pneumonia. Infect Immun 66, 3179-82.

Gingo, M.R., Lucht. L., Daly, K., Djawe, K., Norris, K.A., Walzer, P.D., and A. Morris. Serologic Responses to *Pneumocystis* Proteins in Human Immunodeficiency Virus Patients With and Without *Pneumocystis jirovecii* Infection. *Submitted*.

Gottlieb, M.S., Schroff, R., Schanker, H.M., Weisman, J.D., Fan, P.T., Wolf, R.A. and Saxon, A. (1981) Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N Engl J Med 305, 1425-31.

Graham, S.M., Mtitimila, E.I., Kamanga, H.S., Walsh, A.L., Hart, C.A. and Molyneux, M.E. (2000) Clinical presentation and outcome of Pneumocystis carinii pneumonia in Malawian children. Lancet 355, 369-73.

Gritz, E.R., Vidrine, D.J., Lazev, A.B., Amick, B.C., 3rd and Arduino, R.C. (2004) Smoking behavior in a low-income multiethnic HIV/AIDS population. Nicotine Tob Res 6, 71-7.

Guarda, L.A., Butler, J.J., Mansell, P., Hersh, E.M., Reuben, J. and Newell, G.R. (1983) Lymphadenopathy in homosexual men. Morbid anatomy with clinical and immunologic correlations. Am J Clin Pathol 79, 559-68.

Hanano, R. and Kaufmann, S.H. (1998) Pneumocystis carinii and the immune response in disease. Trends Microbiol 6, 71-5.

Harmsen, A.G., Chen, W. and Gigliotti, F. (1995) Active immunity to Pneumocystis carinii reinfection in T-cell-depleted mice. Infect Immun 63, 2391-5.

Harmsen, A.G. and Stankiewicz, M. (1990) Requirement for CD4+ cells in resistance to Pneumocystis carinii pneumonia in mice. J Exp Med 172, 937-45.

Hart, M., Steel, A., Clark, S.A., Moyle, G., Nelson, M., Henderson, D.C., Wilson, R., Gotch, F., Gazzard, B. and Kelleher, P. (2007) Loss of discrete memory B cell subsets is associated with impaired immunization responses in HIV-1 infection and may be a risk factor for invasive pneumococcal disease. J Immunol 178, 8212-20.

Helweg-Larsen, J., Jensen, J.S., Dohn, B., Benfield, T.L. and Lundgren, B. (2002) Detection of Pneumocystis DNA in samples from patients suspected of bacterial pneumonia--a case-control study. BMC Infect Dis 2, 28.

Hidalgo, H.A., Helmke, R.J., German, V.F. and Mangos, J.A. (1992) Pneumocystis carinii induces an oxidative burst in alveolar macrophages. Infect Immun 60, 1-7.

Hogg, J.C. (2001) Role of latent viral infections in chronic obstructive pulmonary disease and asthma. Am J Respir Crit Care Med 164, S71-5.
Hogg, J.C. (2004) Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. Lancet 364, 709-21.
Huang, L., Crothers, K., Morris, A., Groner, G., Fox, M., Turner, J.R., Merrifield, C., Eiser, S., Zucchi, P. and Beard, C.B. (2003) Pneumocystis colonization in HIV-infected patients. J Eukaryot Microbiol 50 Suppl, 616-7.
Huang, L., Morris, A., Limper, A.H. and Beck, J.M. (2006) An Official ATS Workshop Summary: Recent advances and future directions in pneumocystis pneumonia (PCP). Proc Am Thorac Soc 3, 655-64.
Huggett, J.F., Taylor, M.S., Kocjan, G., Evans, H.E., Morris-Jones, S., Gant, V., Novak, T., Costello, A.M., Zumla, A. and Miller, R.F. (2008) Development and evaluation of a real-time PCR assay for detection of Pneumocystis jirovecii DNA in bronchoalveolar lavage fluid of HIV-infected patients. Thorax 63, 154-9.
Ikeogu, M.O., Wolf, B. and Mathe, S. (1997) Pulmonary manifestations in HIV seropositivity and malnutrition in Zimbabwe. Arch Dis Child 76, 124-8.
Jalil, A., Moja, P., Lambert, C., Perol, M., Cotte, L., Livrozet, J.M., Boibieux, A., Vergnon, J.M., Lucht, F., Tran, R., Contini, C. and Genin, C. (2000) Decreased production of local immunoglobulin A to Pneumocystis carinii in bronchoalveolar lavage fluid from human immunodeficiency virus-positive patients. Infect Immun 68, 1054-60.
Jensen, B.N., Lisse, I.M., Gerstoft, J., Borgeskov, S. and Skinhoj, P. (1991) Cellular profiles in bronchoalveolar lavage fluid of HIV-infected patients with pulmonary symptoms: relation to diagnosis and prognosis. Aids 5, 527-33.
Jiang, W., Lederman, M.M., Mohner, R.J., Rodriguez, B., Nedrich, T.M., Harding, C.V. and Sieg, S.F. (2008) Impaired naive and memory B-cell responsiveness to TLR9 stimulation in human immunodeficiency virus infection. J Virol 82, 7837-45.
Joag, S.V. (2000) Primate models of AIDS. Microbes Infect 2, 223-9.
Kaplan, J.E., Hanson, D., Dworkin, M.S., Frederick, T., Bertolli, J., Lindegren, M.L., Holmberg, S. and Jones, J.L. (2000) Epidemiology of human immunodeficiency virus-associated opportunistic infections in the United States in the era of highly active antiretroviral therapy. Clin Infect Dis 30 Suppl 1, S5-14.
Kaplan, J.E., Masur, H. and Holmes, K.K. (2002) Guidelines for preventing opportunistic infections among HIV-infected persons--2002. Recommendations of the U.S. Public Health Service and the Infectious Diseases Society of America. MMWR Recomm Rep 51, 1-52.
Kawagishi, N., Miyagi, S., Satoh, K., Akamatsu, Y., Sekiguchi, S. and Satomi, S. (2007) Usefulness of beta-D glucan in diagnosing Pneumocystis carinii pneumonia and monitoring its treatment in a living-donor liver-transplant recipient. J Hepatobiliary Pancreat Surg 14, 308-11.
Kazanjian, P., Locke, A.B., Hossler, P.A., Lane, B.R., Bartlett, M.S., Smith, J.W., Cannon, M. and Meshnick, S.R. (1998) Pneumocystis carinii mutations associated with sulfa and sulfone prophylaxis failures in AIDS patients. Aids 12, 873-8.
Keatings, V.M., Collins, P.D., Scott, D.M. and Barnes, P.J. (1996) Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. Am J Respir Crit Care Med 153, 530-4.

Kling, H.M., Shipley, T.W., Patil, S., Morris, A. and Norris, K.A. (2009) Pneumocystis colonization in immunocompetent and simian immunodeficiency virus-infected cynomolgus macaques. J Infect Dis 199, 89-96.

Klinman, D.M. (2004) Use of CpG oligodeoxynucleotides as immunoprotective agents. Expert Opin Biol Ther 4, 937-46.

Klinman, D.M., Currie, D., Gursel, I. and Verthelyi, D. (2004) Use of CpG oligodeoxynucleotides as immune adjuvants. Immunol Rev 199, 201-16.

Kolls, J.K., Habetz, S., Shean, M.K., Vazquez, C., Brown, J.A., Lei, D., Schwarzenberger, P., Ye, P., Nelson, S., Summer, W.R. and Shellito, J.E. (1999) IFN-gamma and CD8+ T cells restore host defenses against Pneumocystis carinii in mice depleted of CD4+ T cells. J Immunol 162, 2890-4.

Kovacs, J.A. and Masur, H. (2009) Evolving health effects of Pneumocystis: one hundred years of progress in diagnosis and treatment. JAMA 301, 2578-85.

Kovacs, J.A., Powell, F., Edman, J.C., Lundgren, B., Martinez, A., Drew, B. and Angus, C.W. (1993) Multiple genes encode the major surface glycoprotein of Pneumocystis carinii. J Biol Chem 268, 6034-40.

Koziel, H., Eichbaum, Q., Kruskal, B.A., Pinkston, P., Rogers, R.A., Armstrong, M.Y., Richards, F.F., Rose, R.M. and Ezekowitz, R.A. (1998) Reduced binding and phagocytosis of Pneumocystis carinii by alveolar macrophages from persons infected with HIV-1 correlates with mannose receptor downregulation. J Clin Invest 102, 1332-44.

Koziel, H., Li, X., Armstrong, M.Y., Richards, F.F. and Rose, R.M. (2000) Alveolar macrophages from human immunodeficiency virus-infected persons demonstrate impaired oxidative burst response to Pneumocystis carinii in vitro. Am J Respir Cell Mol Biol 23, 452-9.

Krajicek, B.J., Limper, A.H. and Thomas, C.F., Jr. (2008) Advances in the biology, pathogenesis and identification of Pneumocystis pneumonia. Curr Opin Pulm Med 14, 228-34.

Kuhrt, D., Faith, S.A., Leone, A., Rohankedkar, M., Sodora, D.L., Picker, L.J. and Cole, K.S. (2010) Evidence of early B-cell dysregulation in simian immunodeficiency virus infection: rapid depletion of naive and memory B-cell subsets with delayed reconstitution of the naive B-cell population. J Virol 84, 2466-76.

Kutty, G. and Kovacs, J.A. (2003) A single-copy gene encodes Kex1, a serine endoprotease of Pneumocystis jiroveci. Infect Immun 71, 571-4.

Lane, H.C., Masur, H., Edgar, L.C., Whalen, G., Rook, A.H. and Fauci, A.S. (1983) Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. N Engl J Med 309, 453-8.

Lane, H.C., Masur, H., Edgar, L.C., Whalen, G., Rook, A.H., Fauci, A.S. . (1983) Abnormalities of B-cell activation and immunoregulation in patients with acquired immunodeficiency syndrome. New England Journal of Medicine 309, 453-458.

Larsen, H.H., Huang, L., Kovacs, J.A., Crothers, K., Silcott, V.A., Morris, A., Turner, J.R., Beard, C.B., Masur, H. and Fischer, S.H. (2004) A prospective, blinded study of quantitative touch-down polymerase chain reaction using oral-wash samples for diagnosis of Pneumocystis pneumonia in HIV-infected patients. J Infect Dis 189, 1679-83.

Laursen, A.L., Jensen, B.N. and Andersen, P.L. (1994) Local antibodies against Pneumocystis carinii in bronchoalveolar lavage fluid. Eur Respir J 7, 679-85.

Laursen, A.L., Obel, N.S., Holmskov, U., Jensenius, J.C., Aliouat el, M. and Andersen, P.L. (2003) Activation of the respiratory burst by Pneumocystis carinii. Efficiency of different antibody isotypes, complement, lung surfactant protein D, and mannan-binding lectin. Apmis 111, 405-15.

Laursen, A.L., Rungby, J. and Andersen, P.L. (1995) Decreased activation of the respiratory burst in neutrophils from AIDS patients with previous Pneumocystis carinii pneumonia. J Infect Dis 172, 497-505.

Lebron, F., Vassallo, R., Puri, V. and Limper, A.H. (2003) Pneumocystis carinii cell wall beta-glucans initiate macrophage inflammatory responses through NF-kappaB activation. J Biol Chem 278, 25001-8.

Lee, L.H., Gigliotti, F., Wright, T.W., Simpson-Haidaris, P.J., Weinberg, G.A. and Haidaris, C.G. (2000) Molecular characterization of KEX1, a kexin-like protease in mouse Pneumocystis carinii. Gene 242, 141-50.

Leigh, T.R., Kangro, H.O., Gazzard, B.G., Jeffries, D.J. and Collins, J.V. (1993) DNA amplification by the polymerase chain reaction to detect sub-clinical Pneumocystis carinii colonization in HIV-positive and HIV-negative male homosexuals with and without respiratory symptoms. Respir Med 87, 525-9.

Li, J., Lord, C.I., Haseltine, W., Letvin, N.L. and Sodroski, J. (1992) Infection of cynomolgus monkeys with a chimeric HIV-1/SIVmac virus that expresses the HIV-1 envelope glycoproteins. J Acquir Immune Defic Syndr 5, 639-46.

Limper, A.H., Edens, M., Anders, R.A. and Leof, E.B. (1998) Pneumocystis carinii inhibits cyclin-dependent kinase activity in lung epithelial cells. J Clin Invest 101, 1148-55.

Limper, A.H., Thomas, C.F., Jr., Anders, R.A. and Leof, E.B. (1997) Interactions of parasite and host epithelial cell cycle regulation during Pneumocystis carinii pneumonia. J Lab Clin Med 130, 132-8.

Lokke, A., Lange, P., Scharling, H., Fabricius, P. and Vestbo, J. (2006) Developing COPD: a 25 year follow up study of the general population. Thorax 61, 935-9.

Louie, J.K., Hsu, L.C., Osmond, D.H., Katz, M.H. and Schwarcz, S.K. (2002) Trends in causes of death among persons with acquired immunodeficiency syndrome in the era of highly active antiretroviral therapy, San Francisco, 1994-1998. J Infect Dis 186, 1023-7.

Lucas, S.B., Peacock, C.S., Hounnou, A., Brattegaard, K., Koffi, K., Honde, M., Andoh, J., Bell, J. and De Cock, K.M. (1996) Disease in children infected with HIV in Abidjan, Cote d'Ivoire. BMJ 312, 335-8.

Lugli, E.B., Allen, A.G. and Wakefield, A.E. (1997) A Pneumocystis carinii multi-gene family with homology to subtilisin-like serine proteases. Microbiology 143 (Pt 7), 2223-36.

Lund, F.E. (2008) Cytokine-producing B lymphocytes-key regulators of immunity. Curr Opin Immunol 20, 332-8.

Lund, F.E., Hollifield, M., Schuer, K., Lines, J.L., Randall, T.D. and Garvy, B.A. (2006) B cells are required for generation of protective effector and memory CD4 cells in response to Pneumocystis lung infection. J Immunol 176, 6147-54.

Lund, F.E., Schuer, K., Hollifield, M., Randall, T.D. and Garvy, B.A. (2003) Clearance of Pneumocystis carinii in mice is dependent on B cells but not on P carinii-specific antibody. J Immunol 171, 1423-30.

Lundgren, B., Elvin, K., Rothman, L.P., Ljungstrom, I., Lidman, C. and Lundgren, J.D. (1997) Transmission of Pneumocystis carinii from patients to hospital staff. Thorax 52, 422-4.

Malaspina, A., Moir, S., Kottilil, S., Hallahan, C.W., Ehler, L.A., Liu, S., Planta, M.A., Chun, T.W. and Fauci, A.S. (2003) Deleterious effect of HIV-1 plasma viremia on B cell costimulatory function. J Immunol 170, 5965-72.

Malaspina, A., Moir, S., Orsega, S.M., Vasquez, J., Miller, N.J., Donoghue, E.T., Kottilil, S., Gezmu, M., Follmann, D., Vodeiko, G.M., Levandowski, R.A., Mican, J.M. and Fauci, A.S. (2005) Compromised B cell responses to influenza vaccination in HIV-infected individuals. J Infect Dis 191, 1442-50.

Marcotte, H., Levesque, D., Delanay, K., Bourgeault, A., de la Durantaye, R., Brochu, S. and Lavoie, M.C. (1996) Pneumocystis carinii infection in transgenic B cell-deficient mice. J Infect Dis 173, 1034-7.

Margolin, D.H., Saunders, E.F., Bronfin, B., de Rosa, N., Axthelm, M.K., Alvarez, X. and Letvin, N.L. (2002) High frequency of virus-specific B lymphocytes in germinal centers of simian-human immunodeficiency virus-infected rhesus monkeys. J Virol 76, 3965-73.

Margolin, D.H., Saunders, E.H., Bronfin, B., de Rosa, N., Axthelm, M.K., Goloubeva, O.G., Eapen, S., Gelman, R.S. and Letvin, N.L. (2006) Germinal center function in the spleen during simian HIV infection in rhesus monkeys. J Immunol 177, 1108-19.

Marty, F.M., Koo, S., Bryar, J. and Baden, L.R. (2007) (1->3)beta-D-glucan assay positivity in patients with Pneumocystis (carinii) jiroveci pneumonia. Ann Intern Med 147, 70-2.

Matsumoto, Y. and Yoshida, Y. (1984) Sporogony in Pneumocystis carinii: synaptonemal complexes and meiotic nuclear divisions observed in precysts. J Protozool 31, 420-8.

Medrano, F.J., Montes-Cano, M., Conde, M., de la Horra, C., Respaldiza, N., Gasch, A., Perez-Lozano, M.J., Varela, J.M. and Calderon, E.J. (2005) Pneumocystis jirovecii in general population. Emerg Infect Dis 11, 245-50.

Miller, M.R., Hankinson, J., Brusasco, V., Burgos, F., Casaburi, R., Coates, A., Crapo, R., Enright, P., van der Grinten, C.P., Gustafsson, P., Jensen, R., Johnson, D.C., MacIntyre, N., McKay, R., Navajas, D., Pedersen, O.F., Pellegrino, R., Viegi, G. and Wanger, J. (2005) Standardisation of spirometry. Eur Respir J 26, 319-38.

Mizuma, H., Litwin, S. and Zolla-Pazner, S. (1988) B-cell activation in HIV infection: relationship of spontaneous immunoglobulin secretion to various immunological parameters. Clin Exp Immunol 71, 410-6.

Mizuma, H., Zolla-Pazner, S., Litwin, S., el-Sadr, W., Sharpe, S., Zehr, B., Weiss, S., Saxinger, W.C. and Marmor, M. (1987) Serum IgD elevation is an early marker of B cell activation during infection with the human immunodeficiency viruses. Clin Exp Immunol 68, 5-14.

Moir, S. and Fauci, A.S. (2009) B cells in HIV infection and disease. Nat Rev Immunol 9, 235-45.

Moir, S., Ho, J., Malaspina, A., Wang, W., DiPoto, A.C., O'Shea, M.A., Roby, G., Kottilil, S., Arthos, J., Proschan, M.A., Chun, T.W. and Fauci, A.S. (2008) Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. J Exp Med 205, 1797-805.

Moir, S., Malaspina, A., Ogwaro, K.M., Donoghue, E.T., Hallahan, C.W., Ehler, L.A., Liu, S., Adelsberger, J., Lapointe, R., Hwu, P., Baseler, M., Orenstein, J.M., Chun, T.W., Mican, J.A. and Fauci, A.S. (2001) HIV-1 induces phenotypic and functional perturbations of B cells in chronically infected individuals. Proc Natl Acad Sci U S A 98, 10362-7.

Moir, S., Ogwaro, K.M., Malaspina, A., Vasquez, J., Donoghue, E.T., Hallahan, C.W., Liu, S., Ehler, L.A., Planta, M.A., Kottilil, S., Chun, T.W. and Fauci, A.S. (2003) Perturbations in

B cell responsiveness to CD4+ T cell help in HIV-infected individuals. Proc Natl Acad Sci U S A 100, 6057-62.
Morris, A., Alexander, T., Radhi, S., Lucht, L., Sciurba, F.C., Kolls, J.K., Srivastava, R., Steele, C. and Norris, K.A. (2009) Airway obstruction is increased in pneumocystis-colonized human immunodeficiency virus-infected outpatients. J Clin Microbiol 47, 3773-6.
Morris, A., Beard, C.B. and Huang, L. (2002) Update on the epidemiology and transmission of Pneumocystis carinii. Microbes Infect 4, 95-103.
Morris, A., Kingsley, L.A., Groner, G., Lebedeva, I.P., Beard, C.B. and Norris, K.A. (2004a) Prevalence and clinical predictors of Pneumocystis colonization among HIV-infected men. Aids 18, 793-8.
Morris, A., Lundgren, J.D., Masur, H., Walzer, P.D., Hanson, D.L., Frederick, T., Huang, L., Beard, C.B. and Kaplan, J.E. (2004b) Current epidemiology of Pneumocystis pneumonia. Emerg Infect Dis 10, 1713-20.
Morris, A., Netravali, M., Kling, H.M., Shipley, T., Ross, T., Sciurba, F.C. and Norris, K.A. (2008a) Relationship of pneumocystis antibody response to severity of chronic obstructive pulmonary disease. Clin Infect Dis 47, e64-8.
Morris, A., Sciurba, F.C., Lebedeva, I.P., Githaiga, A., Elliott, W.M., Hogg, J.C., Huang, L. and Norris, K.A. (2004c) Association of chronic obstructive pulmonary disease severity and Pneumocystis colonization. Am J Respir Crit Care Med 170, 408-13.
Morris, A., Sciurba, F.C. and Norris, K.A. (2008b) Pneumocystis: a novel pathogen in chronic obstructive pulmonary disease? Copd 5, 43-51.
Morris, A., Wachter, R.M., Luce, J., Turner, J. and Huang, L. (2003) Improved survival with highly active antiretroviral therapy in HIV-infected patients with severe Pneumocystis carinii pneumonia. Aids 17, 73-80.
Morris, A.M., Huang, L., Bacchetti, P., Turner, J., Hopewell, P.C., Wallace, J.M., Kvale, P.A., Rosen, M.J., Glassroth, J., Reichman, L.B. and Stansell, J.D. (2000) Permanent declines in pulmonary function following pneumonia in human immunodeficiency virus-infected persons. The Pulmonary Complications of HIV Infection Study Group. Am J Respir Crit Care Med 162, 612-6.
Morrow, M., Valentin, A., Little, R., Yarchoan, R. and Pavlakis, G.N. (2008) A splenic marginal zone-like peripheral blood CD27+B220- B cell population is preferentially depleted in HIV type 1-infected individuals. AIDS Res Hum Retroviruses 24, 621-33.
Nagase, H., Agematsu, K., Kitano, K., Takamoto, M., Okubo, Y., Komiyama, A. and Sugane, K. (2001) Mechanism of hypergammaglobulinemia by HIV infection: circulating memory B-cell reduction with plasmacytosis. Clin Immunol 100, 250-9.
Navin, T.R., Rimland, D., Lennox, J.L., Jernigan, J., Cetron, M., Hightower, A., Roberts, J.M. and Kaplan, J.E. (2000) Risk factors for community-acquired pneumonia among persons infected with human immunodeficiency virus. J Infect Dis 181, 158-64.
Nevez, G., Jounieaux, V., Linas, M.D., Guyot, K., Leophonte, P., Massip, P., Schmit, J.L., Seguela, J.P., Camus, D., Dei-Cas, E., Raccurt, C. and Mazars, E. (1997) High frequency of Pneumocystis carinii sp.f. hominis colonization in HIV-negative patients. J Eukaryot Microbiol 44, 36S.
Nevez, G., Magois, E., Duwat, H., Gouilleux, V., Jounieaux, V. and Totet, A. (2006) Apparent Absence of Pneumocystis jirovecii in Healthy Subjects. Clin Infect Dis 42, e99-e101.

Norris, K.A., Morris, A., Patil, S. and Fernandes, E. (2006) Pneumocystis colonization, airway inflammation, and pulmonary function decline in acquired immunodeficiency syndrome. Immunol Res 36, 175-87.

Norris, K.A., Wildschutte, H., Franko, J. and Board, K.F. (2003) Genetic variation at the mitochondrial large-subunit rRNA locus of Pneumocystis isolates from simian immunodeficiency virus-infected rhesus macaques. Clin Diagn Lab Immunol 10, 1037-42.

O'Riordan, D.M., Standing, J.E. and Limper, A.H. (1995) Pneumocystis carinii glycoprotein A binds macrophage mannose receptors. Infect Immun 63, 779-84.

Pascale, J.M., Shaw, M.M., Durant, P.J., Amador, A.A., Bartlett, M.S., Smith, J.W., Gregory, R.L. and McLaughlin, G.L. (1999) Intranasal immunization confers protection against murine Pneumocystis carinii lung infection. Infect Immun 67, 805-9.

Patil, S.P., Board, K.F., Lebedeva, I.P. and Norris, K.A. (2003) Immune responses to Pneumocystis colonization and infection in a simian model of AIDS. J Eukaryot Microbiol 50 Suppl, 661-2.

Pawar, S.N., Mattila, J.T., Sturgeon, T.J., Lin, P.L., Narayan, O., Montelaro, R.C. and Flynn, J.L. (2008) Comparison of the effects of pathogenic simian human immunodeficiency virus strains SHIV-89.6P and SHIV-KU2 in cynomolgus macaques. AIDS Res Hum Retroviruses 24, 643-54.

Peglow, S.L., Smulian, A.G., Linke, M.J., Pogue, C.L., Nurre, S., Crisler, J., Phair, J., Gold, J.W., Armstrong, D. and Walzer, P.D. (1990) Serologic responses to Pneumocystis carinii antigens in health and disease. J Infect Dis 161, 296-306.

Peters, S.E., Wakefield, A.E., Sinclair, K., Millard, P.R. and Hopkin, J.M. (1992) A search for Pneumocystis carinii in post-mortem lungs by DNA amplification. J Pathol 166, 195-8.

Pifer, L.L., Hughes, W.T., Stagno, S. and Woods, D. (1978) Pneumocystis carinii infection: evidence for high prevalence in normal and immunosuppressed children. Pediatrics 61, 35-41.

Pottratz, S.T. and Martin, W.J., 2nd. (1990) Mechanism of Pneumocystis carinii attachment to cultured rat alveolar macrophages. J Clin Invest 86, 1678-83.

Pottratz, S.T., Reese, S. and Sheldon, J.L. (1998) Pneumocystis carinii induces interleukin 6 production by an alveolar epithelial cell line. Eur J Clin Invest 28, 424-9.

Pottratz, S.T., Weir, A.L. and Wisniowski, P.E. (1994) Pneumocystis carinii attachment increases expression of fibronectin-binding integrins on cultured lung cells. Infect Immun 62, 5464-9.

Powles, M.A., McFadden, D.C., Pittarelli, L.A. and Schmatz, D.M. (1992) Mouse model for Pneumocystis carinii pneumonia that uses natural transmission to initiate infection. Infect Immun 60, 1397-400.

Probst, M., Ries, H., Schmidt-Wieland, T. and Serr, A. (2000) Detection of Pneumocystis carinii DNA in patients with chronic lung diseases. Eur J Clin Microbiol Infect Dis 19, 644-5.

Procop, G.W., Haddad, S., Quinn, J., Wilson, M.L., Henshaw, N.G., Reller, L.B., Artymyshyn, R.L., Katanik, M.T. and Weinstein, M.P. (2004) Detection of Pneumocystis jiroveci in respiratory specimens by four staining methods. J Clin Microbiol 42, 3333-5.

Proskocil, B.J., Sekhon, H.S., Clark, J.A., Lupo, S.L., Jia, Y., Hull, W.M., Whitsett, J.A., Starcher, B.C. and Spindel, E.R. (2005) Vitamin C prevents the effects of prenatal nicotine on pulmonary function in newborn monkeys. Am J Respir Crit Care Med 171, 1032-9.

Rabodonirina, M., Raffenot, D., Cotte, L., Boibieux, A., Mayencon, M., Bayle, G., Persat, F., Rabatel, F., Trepo, C., Peyramond, D. and Piens, M.A. (1997) Rapid detection of Pneumocystis carinii in bronchoalveolar lavage specimens from human immunodeficiency virus-infected patients: use of a simple DNA extraction procedure and nested PCR. J Clin Microbiol 35, 2748-51.

Radbruch, A., Muehlinghaus, G., Luger, E.O., Inamine, A., Smith, K.G., Dorner, T. and Hiepe, F. (2006) Competence and competition: the challenge of becoming a long-lived plasma cell. Nat Rev Immunol 6, 741-50.

Reimann, K.A., Li, J.T., Veazey, R., Halloran, M., Park, I.W., Karlsson, G.B., Sodroski, J. and Letvin, N.L. (1996) A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. J Virol 70, 6922-8.

Rennard, S.I., Basset, G., Lecossier, D., O'Donnell, K.M., Pinkston, P., Martin, P.G. and Crystal, R.G. (1986) Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. J Appl Physiol 60, 532-8.

Respaldiza, N., Montes-Cano, M.A., Dapena, F.J., de la Horra, C., Mateos, I., Medrano, F.J., Calderon, E. and Varela, J.M. (2005) Prevalence of colonisation and genotypic characterisation of Pneumocystis jirovecii among cystic fibrosis patients in Spain. Clin Microbiol Infect 11, 1012-5.

Retamales, I., Elliott, W.M., Meshi, B., Coxson, H.O., Pare, P.D., Sciurba, F.C., Rogers, R.M., Hayashi, S. and Hogg, J.C. (2001) Amplification of inflammation in emphysema and its association with latent adenoviral infection. Am J Respir Crit Care Med 164, 469-73.

Ruffini, D.D. and Madhi, S.A. (2002) The high burden of Pneumocystis carinii pneumonia in African HIV-1-infected children hospitalized for severe pneumonia. AIDS 16, 105-12.

Sadaghdar, H., Huang, Z.B. and Eden, E. (1992) Correlation of bronchoalveolar lavage findings to severity of Pneumocystis carinii pneumonia in AIDS. Evidence for the development of high-permeability pulmonary edema. Chest 102, 63-9.

Saetta, M., Di Stefano, A., Turato, G., Facchini, F.M., Corbino, L., Mapp, C.E., Maestrelli, P., Ciaccia, A. and Fabbri, L.M. (1998) CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 157, 822-6.

Savoia, D., Millesimo, M., Cassetta, I., Forno, B. and Caramello, P. (1997) Detection of Pneumocystis carinii by DNA amplification in human immunodeficiency virus-positive patients. Diagn Microbiol Infect Dis 29, 61-5.

Sethi, S. (2000) Bacterial infection and the pathogenesis of COPD. Chest 117, 286S-91S.

Sethi, S. and Murphy, T.F. (2008) Infection in the pathogenesis and course of chronic obstructive pulmonary disease. N Engl J Med 359, 2355-65.

Shellito, J., Suzara, V.V., Blumenfeld, W., Beck, J.M., Steger, H.J. and Ermak, T.H. (1990) A new model of Pneumocystis carinii infection in mice selectively depleted of helper T lymphocytes. J Clin Invest 85, 1686-93.

Shipley T.W., Kling, H.M., Morris A., Patil. S, Kristoff J., Guyach. S.E., Murphy J.M., Shao, X., Sciurba F.C., Rogers R.M., Richards T., Thomson P., Montelaro R.C., Coxson H.O., Hogg J.C., and K.A. Norris. Persistent *Pneumocystis* colonization leads to the development of chronic obstructive pulmonary disease (COPD) in a non-human primate model of AIDS. *Journal of Infectious Diseases. In press.*

Shirai, A., Cosentino, M., Leitman-Klinman, S.F. and Klinman, D.M. (1992) Human

immunodeficiency virus infection induces both polyclonal and virus-specific B cell activation. J Clin Invest 89, 561-6.
Sing, A., Roggenkamp, A., Autenrieth, I.B. and Heesemann, J. (1999) Pneumocystis carinii carriage in immunocompetent patients with primary pulmonary disorders as detected by single or nested PCR. J Clin Microbiol 37, 3409-10.
Smulian, A.G., Keely, S.P., Sunkin, S.M. and Stringer, J.R. (1997) Genetic and antigenic variation in Pneumocystis carinii organisms: tools for examining the epidemiology and pathogenesis of infection. J Lab Clin Med 130, 461-8.
Smulian, A.G., Sullivan, D.W., Linke, M.J., Halsey, N.A., Quinn, T.C., MacPhail, A.P., Hernandez-Avila, M.A., Hong, S.T. and Walzer, P.D. (1993) Geographic variation in the humoral response to Pneumocystis carinii. J Infect Dis 167, 1243-7.
Snider, G.L. (1985) Distinguishing among asthma, chronic bronchitis, and emphysema. Chest 87, 35S-39S.
Steele, C., Marrero, L., Swain, S., Harmsen, A.G., Zheng, M., Brown, G.D., Gordon, S., Shellito, J.E. and Kolls, J.K. (2003) Alveolar macrophage-mediated killing of Pneumocystis carinii f. sp. muris involves molecular recognition by the Dectin-1 beta-glucan receptor. J Exp Med 198, 1677-88.
Steele, C., Shellito, J.E. and Kolls, J.K. (2005) Immunity against the opportunistic fungal pathogen Pneumocystis. Med Mycol 43, 1-19.
Steele, C., Zheng, M., Young, E., Marrero, L., Shellito, J.E. and Kolls, J.K. (2002) Increased host resistance against Pneumocystis carinii pneumonia in gammadelta T-cell-deficient mice: protective role of gamma interferon and CD8(+) T cells. Infect Immun 70, 5208-15.
Swain, S.D., Lee, S.J., Nussenzweig, M.C. and Harmsen, A.G. (2003) Absence of the macrophage mannose receptor in mice does not increase susceptibility to Pneumocystis carinii infection in vivo. Infect Immun 71, 6213-21.
Tamburrini, E., Ortona, E., Visconti, E., Margutti, P., Mencarini, P., Zolfo, M., Marinaci, S. and Siracusano, A. (1997) Detection of Pneumocystis carinii in oropharyngeal washings by PCR-SHELA and nested PCR. J Eukaryot Microbiol 44, 48S.
Theus, S.A., Andrews, R.P., Steele, P. and Walzer, P.D. (1995) Adoptive transfer of lymphocytes sensitized to the major surface glycoprotein of Pneumocystis carinii confers protection in the rat. J Clin Invest 95, 2587-93.
Theus, S.A., Smulian, A.G., Steele, P., Linke, M.J. and Walzer, P.D. (1998) Immunization with the major surface glycoprotein of Pneumocystis carinii elicits a protective response. Vaccine 16, 1149-57.
Thomas, C.F., Jr. and Limper, A.H. (2004) Pneumocystis pneumonia. N Engl J Med 350, 2487-98.
Thomas, C.F., Jr. and Limper, A.H. (2007) Current insights into the biology and pathogenesis of Pneumocystis pneumonia. Nat Rev Microbiol 5, 298-308.
Titanji, K., Chiodi, F., Bellocco, R., Schepis, D., Osorio, L., Tassandin, C., Tambussi, G., Grutzmeier, S., Lopalco, L. and De Milito, A. (2005) Primary HIV-1 infection sets the stage for important B lymphocyte dysfunctions. Aids 19, 1947-55.
Titanji, K., De Milito, A., Cagigi, A., Thorstensson, R., Grutzmeier, S., Atlas, A., Hejdeman, B., Kroon, F.P., Lopalco, L., Nilsson, A. and Chiodi, F. (2006) Loss of memory B cells impairs maintenance of long-term serologic memory during HIV-1 infection. Blood 108, 1580-7.

Titanji, K., Nilsson, A., Morch, C., Samuelsson, A., Sonnerborg, A., Grutzmeier, S., Zazzi, M. and De Milito, A. (2003) Low frequency of plasma nerve-growth factor detection is associated with death of memory B lymphocytes in HIV-1 infection. Clin Exp Immunol 132, 297-303.

Vargas, S.L., Hughes, W.T., Santolaya, M.E., Ulloa, A.V., Ponce, C.A., Cabrera, C.E., Cumsille, F. and Gigliotti, F. (2001) Search for primary infection by Pneumocystis carinii in a cohort of normal, healthy infants. Clin Infect Dis 32, 855-61.

Vassallo, R., Kottom, T.J., Standing, J.E. and Limper, A.H. (2001) Vitronectin and fibronectin function as glucan binding proteins augmenting macrophage responses to Pneumocystis carinii. Am J Respir Cell Mol Biol 25, 203-11.

Vassallo, R., Thomas, C.F., Jr., Vuk-Pavlovic, Z. and Limper, A.H. (1999) Alveolar macrophage interactions with Pneumocystis carinii. J Lab Clin Med 133, 535-40.

Vestereng, V.H., Bishop, L.R., Hernandez, B., Kutty, G., Larsen, H.H. and Kovacs, J.A. (2004) Quantitative real-time polymerase chain-reaction assay allows characterization of pneumocystis infection in immunocompetent mice. J Infect Dis 189, 1540-4.

Vogel, P., Miller, C.J., Lowenstine, L.L. and Lackner, A. (1993) Evidence of horizontal transmission of Pneumocystis carinii pneumonia in simian immunodeficiency virus-infected rhesus macaques. J. Infect. Dis. 168, 836-843.

Vugmeyster, Y., Howell, K., Bakshi, A., Flores, C., Hwang, O. and McKeever, K. (2004) B-cell subsets in blood and lymphoid organs in Macaca fascicularis. Cytometry A 61, 69-75.

Wakefield, A.E. (1994) Detection of DNA sequences identical to Pneumocystis carinii in samples of ambient air. J Eukaryot Microbiol 41, 116S.

Wakefield, A.E., Banerji, S., Pixley, F.J. and Hopkin, J.M. (1990a) Molecular probes for the detection of Pneumocystis carinii. Trans R Soc Trop Med Hyg 84 Suppl 1, 17-8.

Wakefield, A.E., Pixley, F.J., Banerji, S., Sinclair, K., Miller, R.F., Moxon, E.R. and Hopkin, J.M. (1990b) Amplification of mitochondrial ribosomal RNA sequences from Pneumocystis carinii DNA of rat and human origin. Mol Biochem Parasitol 43, 69-76.

Wakefield, A.E., Pixley, F.J., Banerji, S., Sinclair, K., Miller, R.F., Moxon, E.R. and Hopkin, J.M. (1990c) Detection of Pneumocystis carinii with DNA amplification. Lancet 336, 451-3.

Walzer, P.D. (1999) Immunological Features of Pneumocystis carinii Infection in Humans. Clin. Diagn. Lab. Immunol. 6, 149-155.

Walzer, P.D. (2005) Immunological Features of Pneumocystis Infection in Humans. In: P.D. Walzer, Cushion MT (Ed) Pneumocystis Pneumonia, Vol. 194, Lung Biology in Health and Disease. Marcel Dekker, New York, p. 451-477.

Walzer, P.D., Kim, C.K., Linke, M.J., Pogue, C.L., Huerkamp, M.J., Chrisp, C.E., Lerro, A.V., Wixson, S.K., Hall, E. and Shultz, L.D. (1989) Outbreaks of Pneumocystis carinii pneumonia in colonies of immunodeficient mice. Infect Immun 57, 62-70.

Walzer, P.D., Perl, D.P., Krogstad, D.J., Rawson, P.G. and Schultz, M.G. (1974) Pneumocystis carinii pneumonia in the United States. Epidemiologic, diagnostic, and clinical features. Ann Intern Med 80, 83-93.

Wang, Y., Doucette, S., Qian, Q. and Kirby, J.E. (2007) Yield of primary and repeat induced sputum testing for Pneumocystis jiroveci in human immunodeficiency virus-positive and -negative patients. Arch Pathol Lab Med 131, 1582-4.

Wannamethee, S.G., Sirivichayakul, S., Phillips, A.N., Ubolyam, S., Ruxrungtham, K., Hanvanich, M. and Phanuphak, P. (1998) Clinical and immunological features of human

immunodeficiency virus infection in patients from Bangkok, Thailand. Int J Epidemiol 27, 289-95.
Weinberg, A. and Duarte, M.I. (1993) Respiratory complications in Brazilian patients infected with human immunodeficiency virus. Rev Inst Med Trop Sao Paulo 35, 129-39.
Weller, S., Braun, M.C., Tan, B.K., Rosenwald, A., Cordier, C., Conley, M.E., Plebani, A., Kumararatne, D.S., Bonnet, D., Tournilhac, O., Tchernia, G., Steiniger, B., Staudt, L.M., Casanova, J.L., Reynaud, C.A. and Weill, J.C. (2004) Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. Blood 104, 3647-54.
Wells, J., Haidaris, C.G., Wright, T.W. and Gigliotti, F. (2006a) Active immunization against Pneumocystis carinii with a recombinant P. carinii antigen. Infect Immun 74, 2446-8.
Wells, J., Haidaris, C.G., Wright, T.W. and Gigliotti, F. (2006b) Complement and Fc function are required for optimal antibody prophylaxis against Pneumocystis carinii pneumonia. Infect Immun 74, 390-3.
Wolff, L., Horch, S. and Gemsa, D. (1993) The development of Pneumocystis carinii pneumonia in germ-free rats requires immunosuppression and exposure to the Pneumocystis carinii organism. Comp Immunol Microbiol Infect Dis 16, 73-6.
Wright, T.W., Gigliotti, F., Finkelstein, J.N., McBride, J.T., An, C.L. and Harmsen, A.G. (1999) Immune-mediated inflammation directly impairs pulmonary function, contributing to the pathogenesis of Pneumocystis carinii pneumonia. J Clin Invest 104, 1307-17.
Wright, T.W., Notter, R.H., Wang, Z., Harmsen, A.G. and Gigliotti, F. (2001) Pulmonary inflammation disrupts surfactant function during Pneumocystis carinii pneumonia. Infect Immun 69, 758-64.
Wyder, M.A., Rasch, E.M. and Kaneshiro, E.S. (1998) Quantitation of absolute Pneumocystis carinii nuclear DNA content. Trophic and cystic forms isolated from infected rat lungs are haploid organisms. J Eukaryot Microbiol 45, 233-9.
Yanai, T., Simon, M.A., Doddy, F.D., Mansfield, K.G., Pauley, D. and Lackner, A.A. (1999) Nodular Pneumocystis carinii pneumonia in SIV-infected macaques. Vet Pathol 36, 471-4.
Zheng, M., Ramsay, A.J., Robichaux, M.B., Norris, K.A., Kliment, C., Crowe, C., Rapaka, R.R., Steele, C., McAllister, F., Shellito, J.E., Marrero, L., Schwarzenberger, P., Zhong, Q. and Kolls, J.K. (2005) CD4+ T cell-independent DNA vaccination against opportunistic infections. J Clin Invest 115, 3536-44.