Relationship of *Pneumocystis jiroveci* Humoral Immunity to Prevention of Colonization and Chronic Obstructive Pulmonary Disease in a Primate Model of HIV Infection

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Pulmonary colonization by the opportunistic pathogen *Pneumocystis jiroveci* is common in HIV+ subjects and has been associated with development of chronic obstructive pulmonary disease (COPD). Host and environmental factors associated with colonization susceptibility are undefined. Using a simian-human immunodeficiency virus (SHIV) model of HIV infection, the immunologic parameters associated with natural *Pneumocystis jiroveci* transmission were evaluated. SHIV-infected macaques were exposed to *P. jiroveci* by cohousing with immunosuppressed, *P. jiroveci*-colonized macaques in two independent experiments. Serial plasma and bronchoalveolar lavage (BAL) fluid samples were examined for changes in antibody titers to recombinant *Pneumocystis*-kexin protein (KEX1) and evidence of *Pneumocystis* colonization by nested PCR of BAL fluid. In experiment 1, 10 of 14 monkeys became *Pneumocystis* colonized (Pc+) by 8 weeks post-SHIV infection, while 4 animals remained *Pneumocystis* colonization negative (Pc−) throughout the study. In experiment 2, 11 of 17 animals became *Pneumocystis* colonized by 16 weeks post-SHIV infection, while 6 monkeys remained Pc−. Baseline plasma KEX1-IgG titers were significantly higher in monkeys that remained Pc−, compared to Pc+ monkeys, in experiments 1 (P = 0.013) and 2 (P = 0.022). Pc− monkeys had greater percentages of *Pneumocystis*-specific memory B cells after SHIV infection compared to Pc+ monkeys (P = 0.037). After SHIV infection, Pc+ monkeys developed progressive obstructive pulmonary disease, whereas Pc− monkeys maintained normal lung function throughout the study. These results demonstrate a correlation between the KEX1 humoral response and the prevention of *Pneumocystis* colonization and obstructive lung disease in the SHIV model. In addition, these results indicate that an effective *Pneumocystis*-specific memory B-cell response is maintained despite progressive loss of CD4+ T cells during SHIV infection.

Despite advances in treatment strategies and the introduction of antiretroviral therapy (ART), pulmonary diseases remain a leading cause of morbidity and mortality in HIV-infected patients (34). Both emphysema and chronic obstructive pulmonary disease (COPD) have been reported at an increased frequency in HIV-infected patients (7, 15) and, unlike many AIDS-associated opportunistic infections, HIV-associated COPD may be increasing due to the prolonged life expectancy of the HIV+ population with ART and the high smoking rate in this population (5, 24). Although cigarette smoking is a primary risk factor for the development of COPD, it is interesting that HIV+ nonsmokers may also be at increased risk of disease (14). In addition, the observation that most smokers do not develop COPD (33) indicates that other factors may play a role in disease development.

Evidence has accumulated suggesting a role for infectious agents as cofactors in the pathogenesis and exacerbation of COPD (58), 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 (44, 58). 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 (16).

Our laboratory and others have accumulated evidence in humans and in animal models that the fungal opportunistic pathogen, *Pneumocystis jiroveci* (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) (45), and recent studies suggest that low-level asymptomatic carriage of *Pneumocystis* may be associated with lung damage. An increased frequency of *Pneumocystis* colonization has been reported in HIV+ patients, including those on ART (41), and we have recently shown that HIV+ subjects who were *Pneumocystis* colonized (Pc+) have worse airway obstruction than HIV+ subjects who were Pc− (40). An association between *Pneumocystis* colonization and COPD has also been shown in HIV− subjects (3, 52).

Animal models also support the role of *Pneumocystis* colonization in the pathogenesis of COPD. In a model of immunocompetent mice, cigarette exposure and *Pneumocystis* colo-
nization resulted in greater pulmonary function deficits compared to cigarette exposure alone (4). Our laboratory has reported that in a simian immunodeficiency virus (SIV)-primate model of HIV infection, *Pneumocystis* colonization results in pulmonary inflammation, pulmonary function deficits, and anatomic emphysema (2, 6, 47, 59). Factors that influence susceptibility to *Pneumocystis* colonization are not clearly understood.

Immunologic control of *Pneumocystis* infection is strongly correlated with CD+ T-cell responses, although B cells and antibodies also play a role in prevention of PcP (19–23, 25, 38, 62). There is a high frequency of *Pneumocystis*-specific sero-prevalence in immunocompetent adults (1, 9), as well as in nonhuman primates (11, 30), suggesting the persistence of serological memory or *Pneumocystis*-specific, long-lived plasma cells in response to natural *Pneumocystis* exposure. Antibodies to the *Pneumocystis* endoprotease kexin (KEX1) may be particularly important, because immune responses to *Pneumocystis* KEX1 have been associated with control of *Pneumocystis* infection in immunosuppressed murine models (62, 63).

In the present 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 *Pneumocystis* exposure and examined the relationship between anti-*Pneumocystis* humoral immunity, the development of *Pneumocystis* colonization, and the development of COPD.

This study was presented in part as a poster at the American Thoracic Society International Conference, New Orleans, LA, 14 to 19 May 2010, and the American Thoracic Society International Conference, Toronto, Canada, 16 to 21 May 2008. A portion of the study was also presented as an oral presentation at the International Workshops on Opportunistic Protists in Boston, MA, 28 to 31 May 2008).

**MATERIALS AND METHODS**

**Animals.** Adult, Chinese origin cynomolgus macaques (*Macaca fascicularis*), weighing between 5 and 8 kg, were used in the present 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 a 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 cells (PBMC) and bronchoalveolar lavage) were screened for simian retroviruses (SIV, simian retrovirus, and simian T-cell leukemia virus) to verify that they were free of any preexisting 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 (National Research Council, 1996) and the Provisions of the Animal Welfare Act. Prior to the initiation of the present study, all animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

**Study design.** Two independent experiments were performed using 14 monkeys (experiment 1) and 17 monkeys (experiment 2). Monkeys were intravenously inoculated with 10^7.5–50% tissue culture infectious doses of SHIV89.6P (a gift from Opendra Narayan, University of Kansas), which induces CD4+ T-cell lymphopenia and AIDS-like disease with wasting and opportunistic infections (50, 54). To promote natural transmission of *Pneumocystis*, SHIV-infected macaques were continuously exposed by cohousing in the same room with 10 to 20 SIV- or SHIV-immunosuppressed macaques, which served as a *Pneumocystis* source (30). None of the macaques (source or recipients) contracted acute PcP during the study. Determination of *Pneumocystis* colonization status was performed by detection of *Pneumocystis* DNA in the BAL fluid samples by nested PCR and anti-*Pneumocystis* KEX1 serology (2, 30). *Pneumocystis* colonization was defined as a positive nested PCR of BAL fluid and at least a 3-fold change in plasma anti-*Pneumocystis* KEX1 titers (2, 30). In addition, BAL samples were stained for organisms by modified Giemsa and silver staining (2).

**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 after SHIV infection and monthly thereafter. BAL fluid samples were collected monthly. Samples were collected and processed as described previously (2, 30). Briefly, plasma was isolated from 10 ml of EDTA-treated whole blood by centrifugation. PBMC were purified over a Percoll gradient (Amersham Bioscience, Piscataway, NJ) and washed with sterile phosphate-buffered saline (PBS) (59). 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 (6, 30). Unfractionated BAL fluid aliquots were used for bacterial, fungal, and viral culture (Antech Diagnostics, Pittsburgh, PA) and PCR detection of *Pneumocystis* DNA. Remaining fluid was filtered through a 40-µm pore-size cell strainer, and manual cell counts were performed by using a hemocytometer. A total of 10^6 cells were removed and stained with modified Giemsa stain (Dade Behring, Newark, DE), and differential counts were performed manually (6). The remaining cells were pelleted and supernatant fluid was collected and stored at −80°C. Recovered cells were prepared for flow cytometry as described previously (2, 6).

**Endpoint antibody titer determination.** A partial fragment of the macaque-derived, *Pneumocystis kexin* gene in the pBAD expression vector (a gift from C. G. Haidaris, University of Rochester) (30) was used to produce recombinant human endoprotease kexin-1 protein (ELISA antigen, AcroTest). The antigen protein is a conserved region of the sequence, approximately 105 amino acids in length (30; GenBank accession no. EU918304). ELISA was performed as previously described (30). Plasma and BAL fluid supernatant were heat inactivated (60°C, 30 min) prior to use in ELISA for detection of *Pneumocystis*-specific antibodies. BAL fluid samples were normalized based on the plasma urea concentration (55). Microtiter plates (Immunolon 4HBX; Thermo Fisher Scientific, Waltham, MA) were coated with purified KEX1 at 5 µg/ml in PBS (pH 9.5). Heat-inactivated plasma samples were diluted 1:100 in blocking buffer (PBS with 5% nonfat milk). BAL fluid supernatant samples were diluted according to normalized concentrations in PBS. Then, 50-µl portions of diluted plasma or BAL fluid supernatant were plated into KEX1-coated wells, and serial dilutions were made to determine endpoint titers. Goat anti-mouse immunoglobulin-conjugated horseradish peroxidase (1:10,000 for IgG, 1:2,000 for IgM; Nordic Immunology, Tilburg, Netherlands) was used for detection, and plates were developed by standard methods. Normal (uninfected, Pce− as determined by antibody titer) macaque plasma or BAL fluid supernatant was used as negative control, and samples 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 (OD) values for the test sample were the same or less than the normal sample OD.

**Nest PCR of BAL fluid.** BAL fluid cell lysate samples were analyzed for the presence of *Pneumocystis* DNA by nested PCR of the mitochondrial large subunit rRNA gene (mtLSU), as described previously (2, 49). Nested PCR was performed on 5 µl of the first round product using primers P1 and P2 (56). PCR for β-globin was also performed on BAL samples to control for DNA quality and presence of PCR inhibitors (6).

**Flow cytometry.** Stained, fixed cells from whole blood and BAL fluid were analyzed by flow cytometry (6). 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-human CD4-allophycocyanin (clone SP34), all purchased from BD Pharmingen (San Diego, CA). Acquisition was performed on BD LSRII flow cytometer using BD FACSDiva software. A forward-scatter/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).

**Plasma SHIV viral load determination.** Virus loads in plasma and BAL fluid supernatant were determined as described elsewhere (50). 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.

**Determination of SHIV antibody titers.** Anti-Gag antibody titers were measured by ELISA in serial plasma samples. Samples were assayed as described elsewhere (50) 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 antibodies were detected by using anti-monkey IgG-horseradish peroxidase and TM blue substrate. Reactions were stopped with 1 N sulfuric acid.

ELISPOT assay for quantification of IgG- and KEX1-specific ASCs. For detection of plasma cells, freshly isolated PBMC were assayed directly ex vivo. Antibody-secreting cell (ASC) enzyme-linked immunosorbent (ELISPOT) assays were adapted from a protocol published elsewhere (8). Briefly, ELISPOT plates (nonsterile Multiscreen IP HTS plates; Millipore, Billerica, MA) were coated with either purified recombinant keratin protein (KEX1; 5 μg/ml), keyhole limpet hemocyanin (Pierce ImmuneX mLH, 2.5 μg/ml; Thermo-Scientific, Rockford, IL), or affinity-purified anti-monkey IgG (5 μg/ml; Rockland, Inc., Gilbertsville, PA) at 4°C overnight. The plates were washed with PBS plus 0.05% Tween 20 (PBS-T) (once) and PBS (three times) and then blocked in complete R-10 for at least 2 h at 37°C in 5% CO₂. The blocking medium was removed, 10⁵ 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 in 5% CO₂ overnight, and then the cells were washed away with PBS and PBS-T (four times each). For detection, biotin-conjugated secondary antibody (50 μl per well; Rockland, Inc.) was then added, followed by dilution at 1:1,000 in PBS-T plus 10% fetal bovine serum (FBS), and the plates were incubated overnight at 4°C. The plates were removed and washed with PBS-T (five times), and 50 μl of streptavidin-horseradish peroxidase (BD Biosciences, San Diego, CA) diluted 1:1,000 in PBS-T plus 10% FBS was added to each well, followed by incubation for 60 min at room temperature. The plates were washed and then developed with AEC substrate (BD Biosciences; 50 μl per well) in the dark for 8 to 15 min at room temperature. The reaction was stopped with 1 N sulfuric acid. Plate images were acquired by using an Immunoscan CTL plate reader (CTL Technologies, Ltd., Shaker Heights, OH) and Image Acquire 4.5 software (CTL Technologies, Ltd.). Spots were enumerated by using Immunoscan 5.0 Professional software (CTL Technologies, Ltd.). KEX1-specific plasma cells are reported as ASCs (i.e., the number of spots) per 10⁵ PBMC.

Memory B-cell ELISPOT. PBMC from the animals in experiment 2 were assayed after a 6- to 7-day stimulation, as reported elsewhere (8). Briefly, cells were freshly isolated from whole blood and washed with PBS. The cells were cultured in complete Gibco RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and Pen/Step (R-10) in 24-well plates at a concentration of 0.5 × 10⁶ cells per well, along with Staphylococcus aureus protein A Cowan strain (SAC; 1:10,000; Sigma-Aldrich, St. Louis, MO), Phytolacca americana pokeweed mitogen (PWM Emory Stock, a gift from S. Crotty), and 2 μg of CpG ODN 2006 (5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′; Oligos, Etc., Wilsonville, OR)/ml. Cells were expanded in culture for 6 to 7 days (37°C, 5% CO₂) to induce differentiation into plasma cells, washed with R-10, and then assayed by ELISPOT assay for IgG- and KEX1-specific secreting cells, as described above. KEX1-specific memory B cells were expressed as a percentage of total IgG-secreting cells. PBMC were evaluated for KEX1-specific memory cells at approximately 9 to 12 months after SHIV infection.

Pulmonary function testing. To assess airflow obstruction, pulmonary function tests (PFTs) were performed at baseline and every other month after SHIV infection using whole-body plethysmography (55) and forced deflation technique (59). Intravenous propofol (7.5 to 12.5 mg/kg [body weight]) was used to anesthesize monkeys, and 2% lidocaine was given prior to intubation (3.5-mm endotracheal tube) to desensitize the oropharynx. A chest radiograph was obtained to verify endotracheal tube placement, which was monitored by using a disposable CO₂ detector (Pedi-cap; Nellcor, 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 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.

Statistical analyses. Statistical analyses were performed using Prism software or InStat software, both from GraphPad (La Jolla, CA). An unpaired two-tailed Student t-test was used to compare Pc⁺ and Pc⁻ monkeys, unless otherwise noted. A 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 analysis of variance (ANOVA) was used for comparison. Where indicated, correlation analysis was performed to compare pre-SHIV infection Pneumocystis-antibody titers with post-SHIV infection Pneumocystis antibodies. A P value of <0.05 was considered significant.

RESULTS

Natural Pneumocystis colonization of SHIV-infected macaques. Prior to SHIV infection, macaques were determined to be negative for Pneumocystis colonization by nested PCR of BAL fluid. Baseline anti-Pneumocystis 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 Pneumocystis (Pc⁺) by 8 weeks post-SHIV infection, whereas 4 monkeys remained Pneumocystis negative (Pc⁻) throughout the study (53 weeks). In experiment 2, 11 of 17 SHIV-infected monkeys became naturally colonized with Pneumocystis 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 after SHIV infection, (Fig. 1A and 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 [two-way repeated measures ANOVA]) or in experiment 2 (P = 0.326 [two-way repeated measures ANOVA]). In addition, peak plasma virus titers were similar between Pc⁺ and Pc⁻ monkeys in both experiment 1 (P = 0.749, Fig. 1B) and experiment 2 (P = 0.595, Fig. 1E). No significant differences in anti-Gag antibody responses were observed in Pc⁺ and Pc⁻ animals (P = 0.419, Fig. 1C), indicating that susceptibility to Pneumocystis colonization was not a result of a generalized humoral defect or more severe immunosuppression.

Anti-KEX1 antibodies and KEX1-specific ASCs at baseline correlate with protection from Pneumocystis colonization. Baseline anti-KEX1 antibody titers were compared between macaques that became naturally Pneumocystis colonized and those that remained Pc⁻ post-SHIV infection. Baseline plasma KEX1 IgG antibody titers were significantly higher in monkeys that did not become Pneumocystis colonized compared to Pneumocystis colonized monkeys in both experiments 1 (P = 0.013, Fig. 2A) and 2 (P = 0.022, Fig. 2B). A baseline KEX1-IgG reciprocal endpoint titer (RET) of <10,000 was associated with Pneumocystis colonization following immunosuppression (P = 0.011, Fisher exact test, Fig. 2C). Peripheral blood CD4⁺ T-cell numbers were not different between monkeys with a baseline KEX1 RET greater than 10,000 and monkeys with a baseline KEX1 RET less than 10,000 at baseline (P = 0.10) or study endpoint (P = 0.53) in experiment 1 or baseline (P = 0.12) or endpoint (P = 0.24) in experiment 2 (Student t test for all comparisons, data not shown). PBMC were examined for KEX1-specific ASCs by plasma cell ELISPOT assay, and a similar trend was observed. Pc⁻ animals had significantly greater numbers of KEX1-specific ASCs at baseline than did animals that became Pneumocystis colonized after SHIV infection (P = 0.018, Fig. 2D).

Representative longitudinal profiles of Pneumocystis anti-KEX1 antibody production, peripheral blood CD4⁺ T-cell numbers, and nested PCR results are shown in Fig. 3. Macaques with a low baseline anti-KEX1 titer (<1:4,000), however, exhibited evidence of Pneumocystis colonization generally by 8 to 12 weeks post-SHIV inoculation (Fig. 3A). These an-
Animals had increases in anti-KEX1 titers of at least 3-fold over baseline, generally by 3 to 4 weeks post-SHIV infection. The rise in anti-KEX1 titers was followed by nested-PCR detection of *Pneumocystis* DNA in the BAL fluid, approximately 5 to 12 weeks later (Fig. 3A and data not shown). Macaques with high baseline anti-KEX1 titers (1/H11350:1:10,691) maintained a high titer throughout infection but did not exhibit either a 3-fold increase over baseline titer or a positive nested PCR for *Pneumocystis*, which would indicate active colonization (Fig. 3B). Earlier detection of anti-KEX1 antibodies in the BAL fluid supernatant correlates with protection against *Pneumocystis* colonization.

To determine whether KEX1-specific antibodies could be detected in the BAL fluid supernatant and to investigate whether KEX1-specific antibodies correlated with protection from *Pneumocystis* colonization, BAL fluid supernatant anti-KEX1 titers were determined (Fig. 4). There was a significant positive correlation between baseline plasma anti-KEX1 IgG and peak BAL fluid IgA titers after SHIV infection (Fig. 4A, P = 0.043, R² = 0.279). In addition, the kinetics of a class-switched anti-KEX1 response was examined in BAL fluid. Earlier detection of KEX1-specific IgA was observed in monkeys that resisted *Pneumocystis* colonization (Fig. 4B, P = 0.041, Fisher exact test). Monkeys in which *Pneumocystis* KEX1-specific IgA antibodies were detected in the BAL fluid by 4 weeks post-SHIV infection were significantly less likely to become *Pneumocystis* colonization than monkeys in which *Pneumocystis*-specific IgA was not detectable in the BAL fluid supernatant by this time point (Fig. 4B). The timing (detection post-SHIV infection) and levels of KEX1-specific IgG or IgM antibodies in BAL was not significantly different between Pc⁺ and Pc⁻ monkeys (data not shown).

**KEX1-specific memory response correlates with protection from *Pneumocystis* colonization.** In experiment 2, PBMC were evaluated to assess the *Pneumocystis*-specific memory response in SHIV-infected monkeys. PBMC were evaluated at approximately 9 to 12 months post-SHIV infection, and Pc⁺ monkeys had significantly higher percentages of KEX1-specific memory B cells than Pc⁻ monkeys (Fig. 5). Percentages of KEX1-specific memory B cells did not correlate with numbers of peripheral blood CD4⁺ T cells (P = 0.58, correlation analysis [data not shown]). These results suggest that a stronger *Pneumocystis*-specific memory response persists during SHIV infection and correlates with protection from natural *Pneumocystis* colonization in immunosuppressed macaques.

**Pc⁺ monkeys exhibit evidence of airway obstruction.** Previous studies have shown an association between persistent *Pneumocystis* colonization and COPD in HIV-infected and non-HIV-infected human subjects and in animal models (4, 40, 42, 43, 59). We therefore investigated whether *Pneumocystis* colonization correlated with preserved lung function in long-term SHIV-infected macaques. PFTs were performed at baseline and at 1- to 2-month intervals, up to 50 weeks post-SHIV infection. Declines in peak expiratory flow (PEF) and forced expiratory volume in 0.4 s (FEV₀.₄) were evaluated to assess progression of airway obstruction. Declines in these parameters are indicative of obstructive disease.

In experiment 1, six of eight Pc⁺ monkeys demonstrated significant decreases in PEF and FEV₀.₄ from baseline, whereas no significant declines from baseline were observed in Pc⁻ monkeys (Fig. 6A and B). PEF in Pc⁺ monkeys declined from 526.8 ml/s at baseline to 452.9 ml/s at study endpoint (P = 0.020, paired Student t test) compared to Pc⁻ monkeys that did
not exhibit a significant change from baseline ($P = 0.854$, paired Student $t$ test). $P_c$ monkeys declined significantly in $FEV_{0.4}$, from a mean of 188.6 ml at baseline to 165.8 ml at 10 months post-SHIV infection ($P = 0.023$, paired Student $t$ test), whereas $P_c$ monkeys did not exhibit significant declines in $FEV_{0.4}$ (Fig. 6B).

In experiment 2, $P_c$ monkeys also showed significant declines in pulmonary function parameters from baseline levels. From baseline to 10 months post-SHIV infection, $P_c$ monkeys declined from a mean $PEF$ of 555.3 ml/s to 484.4 ml/s ($P = 0.002$, paired Student $t$ test), whereas $P_c$ monkeys did not decline significantly from baseline ($P = 0.320$, paired Student $t$ test, Fig. 6C). $FEV_{0.4}$ values exhibited a similar trend, with a mean of 198.1 ml at baseline to 173.0 ml at 10 months post-SHIV infection for the $P_c$ monkeys ($P = 0.002$, paired Student $t$ test, Fig. 6D). Again, no significant changes were observed for the $P_c$ group of animals ($P = 0.122$, paired Student $t$ test, Fig. 6D).

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 posttreatment (data not shown).

**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% 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 in which KEX1-IgA was detected later than 4 weeks post-SHIV infection exhibited a greater decline in $PEF$ (average decline of 12% ± 4.3%), compared to monkeys in which KEX1-IgA was detected by week 4 (on average, no significant decline, Fig. 7B). 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.

**DISCUSSION**

SIV and SHIV infection of macaques are valuable models of HIV infection (18, 29, 32, 54) and of HIV-associated opportunistic infection (2, 6, 30, 50). We have previously used SIV and SHIV models to characterize natural transmission and persistence of *Pneumocystis* colonization (30, 59). In previous studies, persistent *Pneumocystis* colonization of SHIV-infected macaques was associated with inflammatory responses in the lungs and the development of COPD-like changes in lung...
function (47, 59). In the present study, we used a SHIV model to (i) determine the relationship between baseline anti-KEX1 antibody titers, susceptibility to *Pneumocystis* colonization, and the development of COPD and (ii) to test the hypothesis that an effective, *Pneumocystis*-KEX1-specific, B-cell response is maintained despite persistent SHIV-induced immunosuppression.

In the present study, we showed that higher baseline anti-KEX1 titers and higher numbers of KEX1-specific ASCs prior to immunosuppression correlated with prevention or delay of *Pneumocystis* colonization after SHIV immunosuppression. These data also suggest that, conversely, a low KEX1-antibody titer prior to immunosuppression predicts susceptibility to *Pneumocystis* colonization. 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 *Pneumocystis* exposure. Macaques with a high baseline anti-KEX1 IgG response maintained this response throughout SHIV infection and remained free from detectable *Pneumocystis* colonization. Differences in susceptibility to *Pneumocystis* colonization were not due to lack of environmental exposure to *Pneumocystis*, since all monkeys were housed together for the duration of the experiments, but only monkeys with lower baseline anti-KEX1 titers became *Pneumocystis* colonized. The failure of monkeys with low anti-KEX1 baseline titers to prevent *Pneumocystis* colonization was

![FIG. 3. Representative KEX1 antibody and CD4<sup>+</sup> T-cell profiles of macaques with low baseline KEX1 titers and high baseline KEX1 titers. Serial plasma anti-KEX1 titers and CD4 T-cell counts in two macaques with low (A) or high (B) baseline anti-KEX1 antibody titers were determined. BAL samples were tested at each time point for *Pneumocystis* DNA (by nested PCR). Time points marked by “+” had detectable *Pneumocystis* DNA.](http://iai.asm.org/)

![FIG. 4. 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). A total of 75% of Pc<sup>-</sup> macaques were producing KEX1-IgA in the BAL fluid by week 4 post-SHIV infection compared to only 10% of Pc<sup>+</sup> macaques (B, *P* = 0.041, Fisher exact test).](http://iai.asm.org/)
FIG. 5. Percentages of peripheral blood KEX1-specific memory B cells were significantly higher in Pc− monkeys than in Pc+ monkeys (experiment 2). PBMC from macaques in experimental group 2 were isolated at 9 to 12 months post-SHIV infection and stimulated in culture for 6 to 7 days and evaluated by using B-cell ELISPOT assays for total IgG and KEX1-specific IgG-secreting cells. KEX1-specific cells are expressed as a percentage of total IgG-secreting cells. The mean percentages of KEX1-specific memory cells for Pc− monkeys were significantly higher than for Pc+ monkeys (P = 0.037, Student t test).

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 Pneumocystis-specific humoral responses in murine models of PcP (19–22, 25, 62). In the experimental mouse model of infection, passive transfer of immune sera or monoclonal Pneumocystis-specific antibodies affords protection (19, 22). In addition, experimental immunization with Pneumocystis organisms/antigens of mice prior to T-cell depletion results in high levels of specific antibody production and clearance of Pneumocystis organisms following challenge (20, 48, 60). Murine studies have also provided evidence of the protective capacity of Pneumocystis-kexin (21, 62, 63).

The current study extends this knowledge by suggesting that Pneumocystis-KEX1 antibody responses may be involved in preventing or resolving Pneumocystis colonization in a highly relevant nonhuman primate model of Pneumocystis transmission and HIV infection (18, 32, 50, 54). These results are consistent with previous findings that SIV-infected macaques with high Pneumocystis-specific antibody titers prior to intrabronchial inoculation with Pneumocystis organisms were less likely to develop PcP compared to animals with lower antibody responses prior to Pneumocystis inoculation (2).

In addition to predicting resistance to Pneumocystis colonization, 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 Pneumocystis colonization and are consistent with clinical studies that showed mucosal antibodies to Pneumocystis are decreased in patients with PcP compared to HIV+ patients without PcP or with HIV-negative controls (27, 31).

In addition to the association of baseline KEX1 antibodies and ASCs with protection from Pneumocystis colonization, we also determined that Pc− animals maintained a significantly higher KEX1-specific B-cell memory response compared to animals that became Pneumocystis colonized. Murine models of Pneumocystis infection have demonstrated the importance of B cells in controlling Pneumocystis infection, since it has been shown that B-cell-deficient mice are highly susceptible to PcP (38), and other studies have demonstrated that antibody-independent B-cell effector functions may be important in the control of Pneumocystis infection (35, 36). Our results correlating baseline KEX1-specific antibody titers, ASCs, and persistence of antigen-specific memory cells to the prevention of Pneumocystis 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 Pneumocystis 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 (12, 28, 37, 39, 61). 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 (26) or splenic marginal zone-like peripheral blood populations (46), 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 numbers CD21+B cells, and a significant reduction in the percentages of IgM memory B cells (H. M. Kling et al., unpublished data). 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 (37, 61) or to antigens that they are not likely to be naturally exposed (i.e., tetanus toxoid) (13, 26). The present study explores humoral responses to a naturally acquired, ubiquitous organism to which most humans (9, 42, 51) and nonhuman primates (10, 30) have been continuously exposed before and after HIV or SHIV infection. The maintenance of the Pneumocystis-KEX1-specific antibody responses and B-cell memory during SHIV-induced immunosuppression in monkeys that had high Pneumocystis humoral immunity preinfection suggests that responses to this antigen were not significantly affected by SHIV infection. Thus, Pneumocystis-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 (17).

Our laboratory recently reported that the development of COPD in the SHIV-infected macaque cohort described here was associated with Pneumocystis colonization (59). COPD was evaluated by serial pulmonary function studies, morphometric analysis of alveolar enlargement, and quantitative analysis of emphysematous lung tissue by computed tomography. These studies demonstrated a correlation between Pneumocystis colonization and development of COPD, whereas macaques in-
fected with SHIV alone had normal lung function (59). The results of the present study extend these findings by demonstrating that humoral immunity to *Pneumocystis* correlates with protection from colonization and the development of obstruction.

We further investigated the potential clinical consequence of persistent *Pneumocystis* colonization and the correlation of a strong humoral response to *Pneumocystis* with the 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 (58). HIV+ patients are at increased risk for *Pneumocystis* colonization (41), as well as an accelerated form of emphysema (15), and additional evidence suggests a role for *Pneumocystis* colonization in the development of COPD in HIV-negative patients (3, 52).

**FIG. 6.** *Pneumocystis* colonization of immunosuppressed monkeys results in pulmonary obstruction, as measured by pulmonary function testing. Pulmonary function parameters were measured at baseline and 10 months post-SHIV infection, and changes were analyzed according to *Pneumocystis* colonization status. PEF (peak expiratory flow) and FEV$_{0.4}$ (forced expiratory volume in 0.4 s) for Pc$^+$ and Pc$^-$ animals in both experiment 1 (A and B) and experiment 2 (C and D) were compared by paired Student *t* test from baseline to 10 months post-SHIV infection. The corresponding *P* values for each comparison are given in each panel.
HIV+ subjects, the prevalence of *Pneumocystis* colonization is high and occurs even in patients with high CD4+ T cells counts on ART (40). Recently, Morris et al. demonstrated a link between *Pneumocystis* colonization and airway obstruction in HIV+ patients, and those who were colonized had a significantly lower spirometric values compared to noncolonized subjects (40). These results demonstrate a link between *Pneumocystis* 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 *Pneumocystis* colonization and COPD development in the SHIV model (47, 59). The present study extends these findings by identifying an association between baseline anti-KEX1 antibody titers and susceptibility to *Pneumocystis* 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 (42). In addition, preliminary studies from our group also have shown that low KEX1 antibody levels are associated with subsequent development of PcP in HIV+ subjects (M. R. Gingo, L. Lucht, K. Daly, K. Djawe, K. A. Norris, P. D. Walzer, and A. Morris, unpublished data).

There are limitations to our study. Although an association between *Pneumocystis* colonization and pulmonary function decline was observed, these studies do not definitively demonstrate a causal relationship. Furthermore, other organisms, in addition to *Pneumocystis*, may be involved in the pathogenesis or exacerbation of COPD (57, 58). Despite these limitations, these studies support previous reports linking *Pneumocystis* colonization and airway obstruction (4, 42, 47; Gingo et al., unpublished) and provide further evidence that antibodies to *Pneumocystis*-kexin may play a role in protection from *Pneumocystis* colonization. In addition, examination of the KEX1-specific memory B-cell response in SHIV-infected macaques was limited in the availability of cells from animals prior to infection. Further studies of the kinetics of the memory response will determine whether the magnitude of the *Pneumocystis*-specific memory response is affected by SHIV infection.

In summary, these results support the concept that *Pneumocystis*-specific humoral immunity established prior to immunosuppression is associated with improved resistance to subsequent *Pneumocystis* colonization and pulmonary obstruction, despite declining CD4+ T-cell numbers. These data further suggest that the humoral effector mechanisms responsible for this protection may 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 *Pneumocystis*-specific humoral response resistance to *Pneumocystis* colonization and prevention of pulmonary damage and support the feasibility of a *Pneumocystis*-KEX1 vaccine strategy for protection of high-risk populations.

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