Pneumocystis Colonization in a Non-human Primate Model of HIV Results in Pulmonary Obstructive Changes that Are Irreversible by Trimethoprim-Sulfamethoxazole Treatment

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Supplemental Digital Content

Supplemental Methods

Animals. Sixteen adult, cynomolgus macaques (*Macacca fascicularis*; weight, 5 to 8 kg) were used in this study. Prior to the initiation of this study, all animal experiments were approved by the IACUC of the University of Pittsburgh. Macaques underwent complete physical examination (pulmonary and cardiac auscultation, thoracic radiographs, computed tomography scanning, tuberculin skin testing, complete blood count, chemistry panel, and flow cytometric analysis of peripheral blood mononuclear and bronchoalveolar lavage cells) and were screened for simian retroviruses (SIV, simian retrovirus, and simian T-lymphocyte associated retrovirus) prior to study admission, for verification that they were free of any preexisting disease. Macaques were housed in an AAALAC-accredited, biosafety level 2+ primate facility at the University of Pittsburgh.

Peripheral blood and bronchoalveolar lavage (BAL) fluid collection. Peripheral blood samples were collected at baseline on all macaques. Serial peripheral blood samples from SHIV-infected monkeys were collected weekly for the first 4 weeks after SHIV infection and monthly thereafter. Briefly, plasma was

isolated by centrifugation from 10 mL EDTA-treated whole blood; red blood cells were lysed, and peripheral blood leukocytes (PBL) were washed with sterile PBS. Plasma aliquots were stored at −80 °C prior to assay. PBL were counted, stained, and fixed for analysis by flow cytometry, as described [1].

BAL fluid samples were collected at baseline and monthly on all animals. BAL fluid was processed for cell isolation as previously described [1-4]. Briefly, macaques were sedated with intramuscular ketamine (10 mg/kg) for blood collection and bronchoscopy. Local anesthetic (2% lidocane) was applied to the larynx before insertion of a pediatric fiberoptic bronchoscope into the trachea. The scope was directed into the right primary bronchus and wedged into a distal subsegmental bronchus, which approximated the diameter of the bronchoscope. Four 10-mL aliquots of 0.9% saline were instilled and then aspirated. Lavage fluid for a single animal was pooled, and recovered cells were counted by a hemocytometer. Aliquots of unfractionated BAL fluid were used for bacterial, fungal, and viral culture (Antech Diagnostics, Lake Success, NY) and PCR detection of *Pneumocystis* DNA. Remaining fluid was filtered through a 40-µm-pore-size cell strainer, and a hemocytometer was used to perform manual cell counts. Cells (10^5) were removed, stained with modified Giemsa stain (Dade Behring, Newark, DE), and differential counts were performed manually [1]. Remaining cells were pelleted and supernatant fluid was collected and stored at −80°C.

Nested PCR of BAL fluid. Baseline and monthly BAL fluid cell lysate samples were examined for the presence of *Pneumocystis* DNA by nested PCR of the mitochondrial large subunit rRNA gene (mtLSU), as described previously [5,6]. Five microliters of first-round PCR product was amplified using P1 and P2 primers [7]. To control for DNA quality and presence of PCR inhibitors, PCR for β-globin was also performed on BAL samples at each time point [1].

***Pneumocystis* kexin antibody endpoint titer determination.** Briefly, a partial fragment of the macaque-derived, *Pneumocystis kexin* gene in the pBAD expression vector [2] was used to produce recombinant kexin for enzyme-linked immunosorbent assay (ELISA). The target kexin protein is a conserved region of the sequence, approximately 90 amino acids in length ([2]; GenBank accession no. EU918304). Plasma samples were heat inactivated (56°C, 30 min) prior to use for detection of KEX1 antibodies. Purified KEX1 (μg/ml in Na_2CO_3, pH 9.5) was used to coat 96-well microtiter plates (Immulon 4HBX; Thermo Fisher Scientific, Inc., Waltham, MA). Heat-inactivated plasma samples were diluted 1:100 in blocking buffer (PBS with 5% nonfat milk); 50-μl of diluted plasma was 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, 1:2,000 for IgM; Nordic Immunology, Tilburg, Netherlands) was used for detection, and plates were developed by standard methods. Normal (uninfected, Pc-negative, giving an OD_{450nm} reading of 0.02 or less) macaque plasma was used as negative control; sample from a Pc+ monkey with previously determined high KEX1 titer 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.

Determination of plasma SHIV viral load. Viral loads in plasma and BAL fluid supernatant were determined as described elsewhere [8]. Briefly, RNA was extracted from plasma and BAL fluid supernatant and was quantified as RNA copies per milliliter, using an adapted protocol for quantitative real-time PCR detection of the SIV *gag* sequence.

Albuterol Challenge of Macaques. For bronchodilator challenge, standard PFT were performed, followed by administration of one pediatric dosette of nebulized albuterol (3 ml of 0.083% albuterol)

(Nephron Pharmaceuticals Corp., Orlando, FL). PFT were repeated fifteen minutes after albuterol administration and compared to baseline values. Increases in function measurements greater than 12% over baseline values are considered significant bronchodilation [9].

Supplemental Results

Albuterol treatment of SHIV-infected macaques. To examine the reversibility of pulmonary obstruction in SHIV-infected, Pc-colonized macaques, animals were treated with the bronchodilator albuterol at 43 weeks post-SHIV infection (18 weeks of TMP-SMX treatment in the treated group). SHIV-infected, Pc-negative animals were also treated with albuterol, and pulmonary function was compared prior to and following bronchodilator treatment (Fig. S1). No significant improvement in PEF or $FEV_{0.4}$ measurements occurred following albuterol treatment in the Pc+/TMP-SMX-treated group (Fig. S1A-B), the Pc+/TMP-SMX-untreated group (Fig. S1C-D), or the Pc-/TMP-SMX-treated group of animals (Fig. S1E-F) (paired t-test or Wilcoxon signed rank test). Only one monkey (Pc+/TMP-SMX-) had an improvement in PEF or $FEV_{0.4}$ of greater than 12% following albuterol administration.

Table S1. Nested PCR-positive (*mtLSU*) samples collected from trimethoprim-sulfamethoxazole-treated and untreated SHIV-infected, *Pneumocystis*-colonized macaques.

Treatment Group	Number of PCR+ Samples prior to TMP-SMX treatment (7 samples taken per animal)	Number of PCR+ Samples during TMP-SMX treatment (9 samples taken per animal)
TMP-SMX-treated, Pc+ macaques (n=7)	4 (8.2% of 49 samples)	1 (1.6% of 63 samples)
Untreated, Pc+ macaques (n=4)	2 (7.1% of 28 samples)	12 (33.3% of 36 samples)

Figure Legend for Supplemental Figure

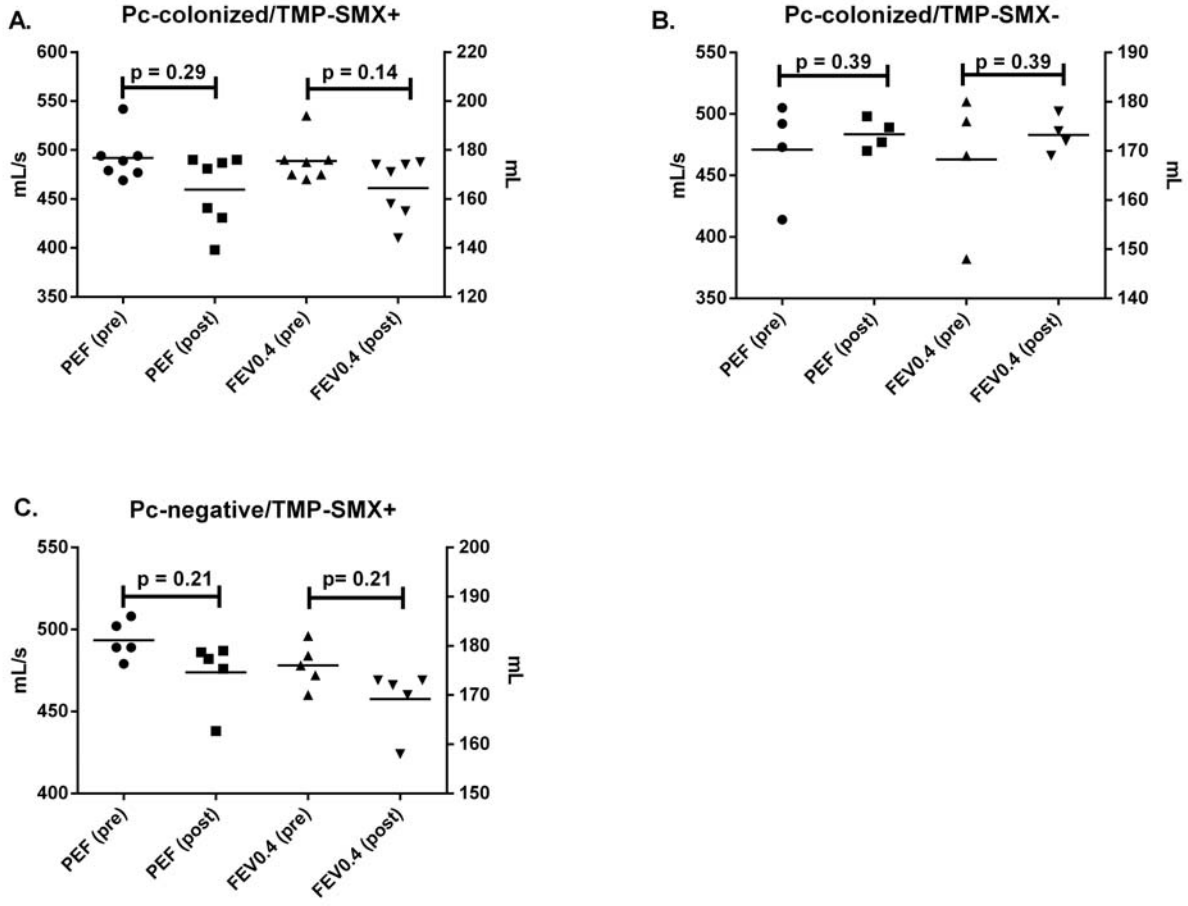

Figure S1. Pulmonary obstruction in SHIV-infected macaques is not reversible with bronchodilator treatment. At 43 weeks post SHIV-infection and *Pneumocystis* (Pc) exposure, peak expiratory flow (PEF) and forced expiratory volume in 0.4s ($FEV_{0.4}$) were measured prior to and fifteen minutes following bronchodilator administration in Pc-colonized, trimethoprim-sulfamethoxazole (TMP-SMX)-treated animals (**A-B**), Pc-colonized, TMP-SMX-untreated animals (**C-D**), and Pc-negative, TMP-SMX-treated animals (**E-F**). PEF and $FEV_{0.4}$ measurements taken pre- and post-bronchodilator treatment are given. No significant improvement in lung function was observed for any of the treatment groups ($p>0.05$, t-test or Wilcoxon signed rank test).

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