ONLINE SUPPLEMENTAL DATA

Cigarette smoke and HIV synergistically affect lung pathology in cynomolgus macaques

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Methods

Animals

Female cynomolgus macaques (*M. fascicularis*) were purchased from Charles River Laboratories (Wilmington, MA) and the animals weighed between 2.5-3.0 kg. The reason we selected female macaques is that, with similar smoking history, women are far more likely to develop COPD than males (*1*). The animals were housed at the Primate Facility of Lovelace Respiratory Research Institute, Albuquerque, NM in accordance with the Guide for Laboratory Animal Practice under the Association for the Assessment and Accreditation for Laboratory Animal Care International. All experimental protocols carried out on these macaques were approved by the Institutional Animal Care and Use Committee. The animals were maintained at 12-hour light/dark cycle and received, unless exposed to cigarette smoke (CS), filtered fresh air (FA) with 10 to 15 air changes per hour. Room temperature and relative humidity were maintained according to the Guide for the Care and Use of Laboratory Animals. While all animals were weighed at baseline and at monthly intervals just before pulmonary function tests, animals subjected to CS and/or SHIV were also weighed at weekly intervals.

CS exposure, Simian HIV (SHIV) Infection, and cART Treatment

Macaques were exposed to cigarette smoke (CS) as described previously (*2, 3*). Briefly, the animals were placed into H2000 whole body exposure chambers and exposed to CS [250 mg/m^3 total suspended particulate matter (TPM)] for 6 hours per day, 5 days per week. Animals were started with an acclimatizing dose of 100 mg/m^3 TPM and increased to 250 mg/m^3 TPM within the first 5 exposure days. The dose of 100 and 250 mg/m^3/day corresponds to human equivalent of approximately 2 and 4 packs of cigarette/day, respectively (*3*). The reasons for using the indicated CS exposure concentration were: (a) there is strong association between the number of pack-years of smoking and development of CB and COPD (*4*), (b) under these conditions, cynomolgus macaques develop perceptible pathological changes in the lung within 3 months of CS exposure (*3*), and a number of published reports show that a variety of animal exposed chronically to similar or even higher concentrations of CS do not develop unexpected health consequences (reviewed in *5*). Of the 25 macaques, 11 received FA and 14 were exposed to CS for 11 weeks, at which time each group was subdivided into two subgroups. Seven animals from each group were injected intravenously with $SHIV_{89.6}$ ($5x10^4$ $TCID_{50}$), leading to 4 experimental groups of animals: FA (n = 4), CS (n = 7), SHIV (n = 7) and CS+SHIV (n = 7) as outlined schematically in Supplemental Figure 1. Thus, the animals were exposed to CS

for 27 weeks and SHIV + cART for 16 weeks. Animals in CS and CS+SHIV groups continued to receive CS until sacrifice (i.e., 16 weeks post SHIV infection).

SHIV infection in animals was determined by measuring plasma viral loads at one and two weeks post infection by using SHIV gag Taqman primers and probes as described previously (*6*) and expressed as number of copies/ml of plasma. The sensitivity of the assay was 50 vRNA copies/ml of plasma. Viral loads were also determined in BAL fluids after the sacrifice. At 2 weeks post SHIV infection, all SHIV-infected animals received a daily subcutaneous injection of cART (20 mg tenofovir + 30 mg emtricitabine/1 ml H_2O/kg body weight). Animals were weighed weekly and the treatment volumes were updated according to changes in the body weight.

Pulmonary Function Analysis

Pulmonary function was assessed by forced expiratory volume (FEV) at 0.1 sec ($FEV_{0.1}$) and 0.15 sec ($FEV_{0.15}$) as described previously (*3*). Briefly, animals were given ketamine (5 mg/kg) intramuscularly before bringing to the test laboratory where they were anesthetized with isoflurane by inhalation (5% for induction, 1.5-3% for maintenance). Blood oxygen saturation (SpO_2) and heart rate were monitored every 5 min by pulse oximetry and SpO_2 was maintained at >90% by adjusting ventilation. Body temperature was recorded every 10 to 15 minutes throughout anesthesia. Whole-body plethysmography was used on anesthetized animals and the transpulmonary pressure was measured through an esophageal catheter and the data was acquired digitally by a PC-based software system (Biopac Systems, Goleta, CA). The maneuver generates values for forced vital capacity (FVC), FEV, and flow rates at various intervals during exhalation.

Tracheal Wall Thickness Analysis

Trans-cutaneous ultrasound imaging of extrathoracic tracheal rings (TR) was performed by a single operator 14-MHz ultrasound source (M-Turbo® ultrasound system, FUJIFILM SonoSite, Inc.). Longitudinal views of the sagittal plane that spanned from the lower margin of the larynx up to the trachea inferiorly were recorded and offered the best anatomical details. Thickness of the first, second and third tracheal rings denoted as TR1, TR2 and TR3, respectively, were measured by two blinded readers and the mean tracheal thickness was normalized to body weights.

Bronchoalveolar Lavage (BAL) and Blood Collection

BAL was collected as described previously (*3*). Briefly, following pulmonary function assessment, a bronchoscope (BF-XP40, Olympus America Inc., Melville NY) was placed in approximately 4^{th}-6^{th} generation airway in one lung lobe. Three aliquots of 5ml saline were instilled and aspirated in succession and the process was repeated on the contralateral lung lobe. The aliquots were pooled, cleared by centrifugation, and stored at -80ºC until further use. In anesthetized animals, just before pulmonary function testing, blood was collected from femoral or peripheral veins and processed for harvesting plasma or serum samples.

BAL Inflammatory Cells

Total BAL cell numbers were enumerated and cytospin slides were prepared for each animal as described previously (*3*). Briefly, slides were stained with Hematoxylin/Giesma stain, and differential leukocytes were counted including the number of BAL macrophages, BAL polymorphonuclear neutrophils (PMNs), and BAL lymphocytes.

Histochemical Staining

Lung tissue sections (5 µ) were deparaffinized in xylene and hydrated in graded ethanol series and deionized water. Histochemical staining with either hematoxylin and eosin or Alcian Blue and periodic acid Schiff's reagent (AB-PAS) was carried out as described previously (*7*). Airway epithelial cell and mucous cells/mm basal lamina (BL) were determined by counting the number of nuclei and mucous cells, respectively, using NIH Image J software on the images captured by BZX700 All-in-One Microscopy system (Keyence Corp., Japan) and were divided by unit length of BL (mm).

Pulmonary Inflammation in Lung Sections

Lung tissue sections stained with hematoxylin and eosin were assessed for pulmonary inflammation by quantifying the number of lymphoid aggregates as described previously (*3*). Lymphoid aggregates in the parenchymal, peribronchial, and perivascular regions were evaluated using BZX700 Microscopy system (Keyence Corp., Japan).

Immunostaining and Fluorescent Imaging

For immunohistochemical staining, deparaffinized and hydrated lung tissue sections were washed in 0.05% v Brij-35 in PBS (pH 7.4) and immunostained for antigen expression as described previously (*8*). Briefly, the

antigens were unmasked by steaming the sections in 10 mM Citrate buffer (pH 6.0) followed by incubation in a blocking solution containing 3% BSA, 1% Gelatin and 1% normal donkey serum with 0.1% Triton X-100 and 0.1% Saponin. Serial sections were stained with antibodies to pan-Cytokeratin (Cell Signaling Technologies, Danvers, MA), HIV-1 gp120 (SinoBiologicals, Beijing, China), MUC5AC (Millipore Inc., Burlington, MA), MUC5B (Santa Cruz Biotech, CA), ZO-1 (Invitrogen Inc., Carlsbad, CA), occludin (Invitrogen Inc., Carlsbad, CA) or isotype control antibodies. The immunolabelled tissues were detected using respective secondary antibodies conjugated with fluorescent dyes (Jackson ImmunoResearch Lab Inc., West Grove, PA). Where indicated, the sections were stained with 4',6-diamidino-2-phenylindole (DAPI) containing Fluormount-G (SouthernBiotech, Birmingham, AL) to visualize nuclei. Immunofluorescent images were captured with BZX700 Microscopy system.

Quantitative Real-Time RT-PCR (qPCR)

Total RNA was isolated from the snap-frozen lung tissues using RNAeasy kit (Qiagen, Germantown, MD) as per manufacturer's instruction. RNA concentration was determined using the Synergy HTX Multi-Mode reader (BioTek, Winooski, VT) and cDNAs were synthesized using iScript advanced cDNA kit (BioRad, Hercules, CA). The primer/probe sets for *MUC5AC, MUC5B, SPDEF, FOXA3, FOXA2* and OCLN were obtained either from BioRad (Hercules, CA) or Qiagen (Germantown, MD) and cDNA amplified by q-PCR using the iTaq SYBR-green Master Mix (BioRad, Hercules, CA) was quantified in the Agilent Stratagene Mx3000P Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). Relative quantities were calculated by normalizing averaged CT values to *GAPDH* or *β-Actin* to obtain ΔCT, and the fold-change (ΔΔCT) over the control was determined as described previously (*9*).

Statistical Analysis

The data were normalized via natural log transformations and when the data was normally distributed, statistical significance among the groups was determined by one-way ANOVA with Bonferroni correction with multiple pairwise comparisons. When the data was not normally distributed, we used Kruskal-Wallis assessment on ranks followed by Dunn’s multiple comparison tests. $P \leq 0.05$ was considered statistically significant using Student’s *t*-test.

Study Approval

The animals were housed at the Primate Facility of Lovelace Respiratory Research Institute, Albuquerque, NM in accordance with the Guide for Laboratory Animal Practice under the Association for the Assessment and Accreditation for Laboratory Animal Care International. All experimental protocols carried out on these macaques were approved by the Institutional Animal Care and Use Committee.

References

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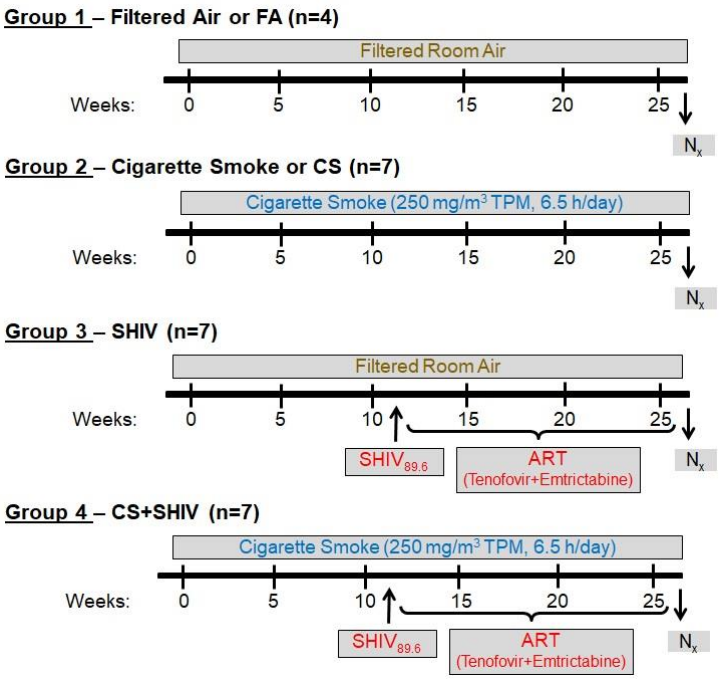
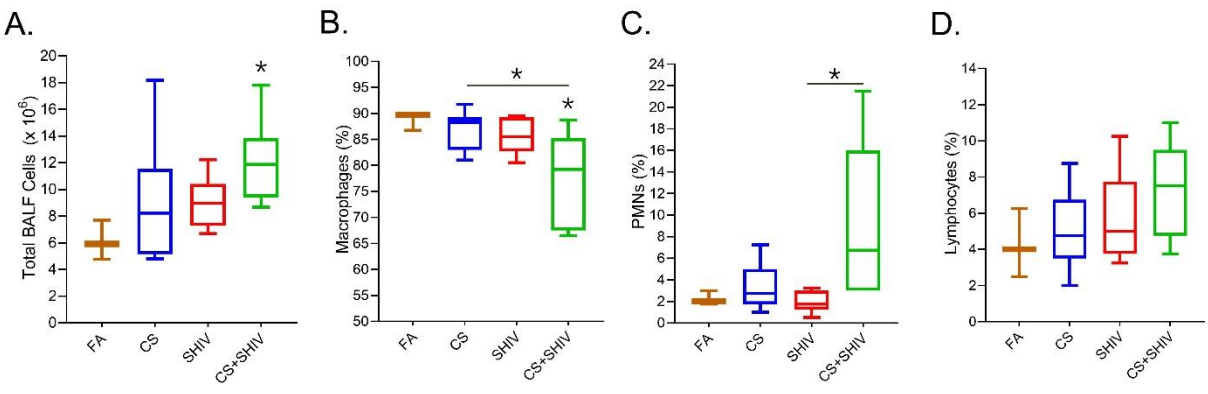
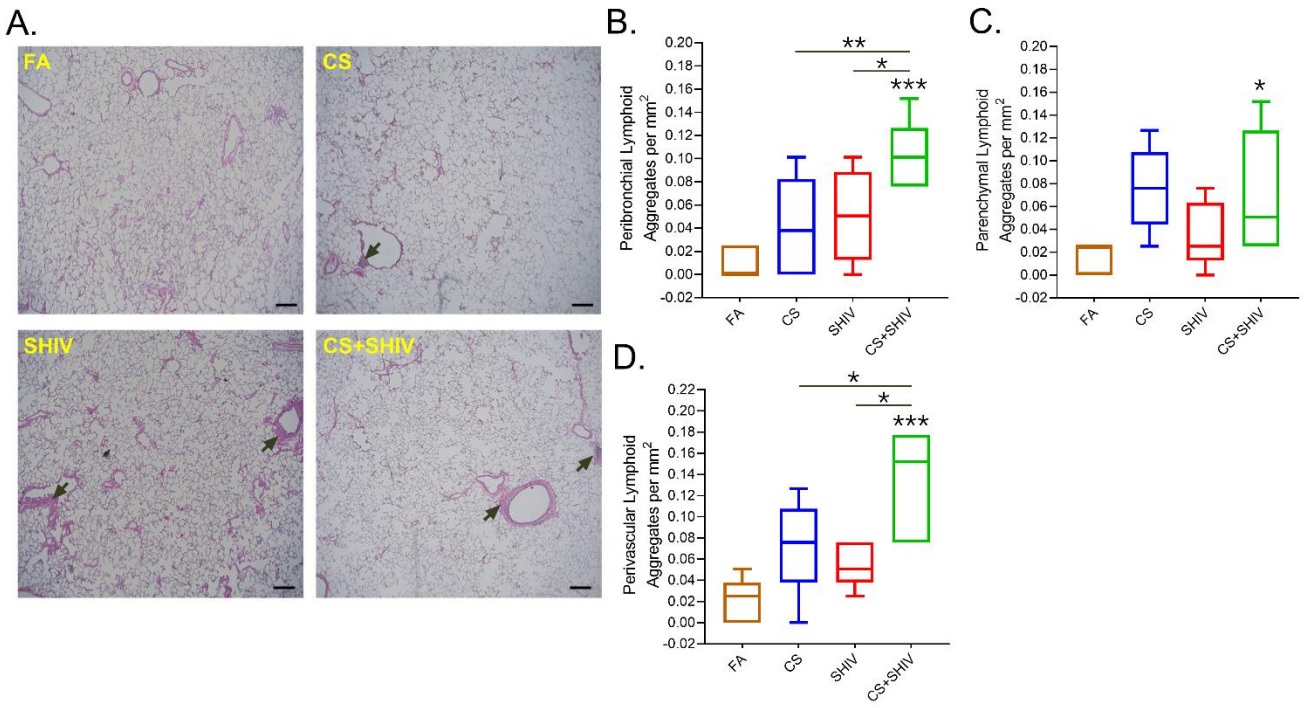


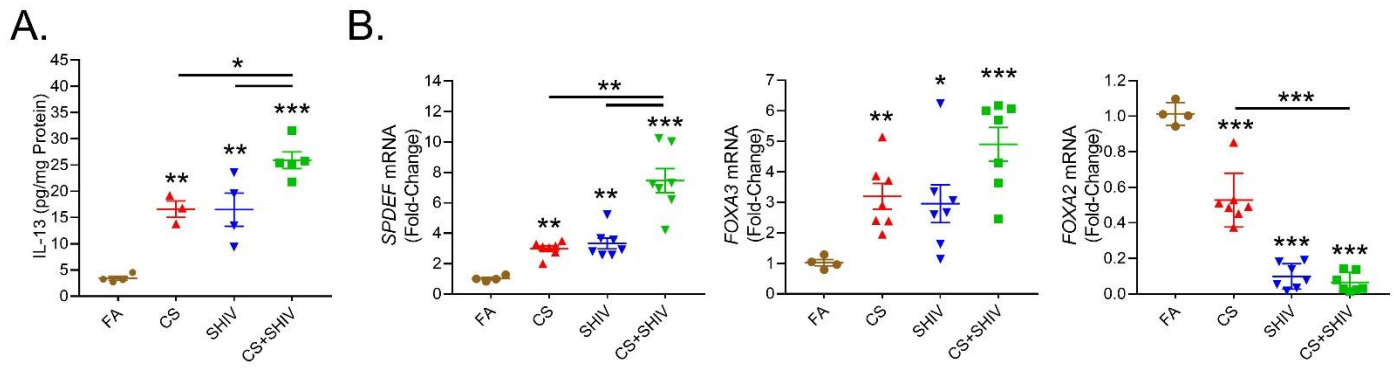
Supplemental Figure 1. Experimental design for nonhuman primate model of chronic cigarette smoke exposure and HIV infection. The study had 4 experimental groups of animals: FA (n = 4), CS (n = 7), SHIV (n = 7) and CS+SHIV (n = 7). For first 11 weeks, 11 cynomolgus macaques were exposed to FA and 14 were exposed to CS. At 11 weeks, the groups were subdivided into two subgroups. Seven animals each from FA and CS groups were injected intravenously with $SHIV_{89.6}$ ($5x10^4$ $TCID_{50}$) and at 2 weeks post-infection were treated daily with cART (20 mg tenofovir + 30 mg emtrictabine/kg) for 16 weeks. Animals in CS and CS+SHIV groups continued to receive CS until necropsy for a total of 27 weeks.



Supplemental Figure 2. CS exposure and SHIV infection increase total cellularity and neutrophil count of bronchoalveolar lavage (BAL). A. Total BAL leukocyte counts in BAL samples from each group and differential leukocyte counts were enumerated including BAL macrophage (**B**) polymorphonuclear neutrophils (**C**) and BAL lymphocytes (**D**). Data shown as mean ± SEM with n=4-7/group and analyzed by 1-way ANOVA; *p<0.05.



Supplemental Figure 3. CS exposure and SHIV infection increases the number of lymphoid aggregates in the lung. A. Representative micrographs of hematoxylin and eosin stained lung sections from each group, scale – 200 µ. Quantitation of lymphoid aggregates (shown by arrowheads) in peribronchial (**B**), parenchyma (**C**), and perivascular (**D**) regions per unit area of the lung tissue (mm^2). Data shown as mean ± SEM with n=4-7/group and analyzed by 1-way ANOVA; *p<0.05; **p<0.01; ***p<0.001.



Supplemental Figure 4. CS exposure and SHIV infection synergistically upregulate IL-13 expression and modulate mucous regulating transcription factors. A. Quantification of IL-13 by ELISA analysis in lung tissue homogenates from each group. **B.** Quantification of mRNA levels of mucous phenotype regulating transcription factors i.e. *SPDEF*, *FOXA3* and *FOXA2* in each group. Data shown as mean ± SEM with n=4-7/group and analyzed by 1-way ANOVA; *p≤0.05; **p<0.01; ***p<0.001.