SHORT COMMUNICATION

Distinct Chronic Post-Viral Lung Diseases upon Infection with Influenza or Parainfluenza Viruses Differentially Impact Superinfection Outcome

Geyon L. Garcia,^{*} Alex Valenzuela,^{*} Tomaz Manzoni,^{*} Andrew E. Vaughan,^{†‡} and Carolina B. López^{*}

From the Departments of Pathobiology^{*} and Biomedical Sciences,[†] School of Veterinary Medicine, and the Institute for Regenerative Medicine,[‡] University of Pennsylvania, Philadelphia, Pennsylvania

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Address correspondence to
Carolina B. López, Ph.D.,
Department of Pathobiology,
University of Pennsylvania
School of Veterinary Medicine,
380 South University
Avenue, 318 Hill, Philadel-
phia, PA 19104-4539. E-mail:
lopezca@upenn.edu.

Chronic obstructive pulmonary disease (COPD) and asthma remain prevalent human lung diseases. Variability in epithelial and inflammatory components that results in pathologic heterogeneity complicates the development of treatments for these diseases. Early childhood infection with parainfluenza virus or respiratory syncytial virus is strongly associated with the development of asthma and COPD later in life, and exacerbations of these diseases correlate with the presence of viral RNA in the lung. Well-characterized animal models of postviral chronic lung diseases are necessary to study the underlying mechanisms of viral-related COPD and asthma and to develop appropriate therapies. In this study, we cross-analyzed chronic lung disease caused by infection with Sendai virus (SeV) or influenza A virus in mice. Differences were observed in lesion composition and inflammatory profiles between SeV- and influenza A virus–induced long-term lung disease. In addition, a primary SeV infection led to worsened pathologic findings on secondary heterologous viral challenge, whereas the reversed infection scheme protected against disease in response to a secondary viral challenge >1 month after the primary infection. These data demonstrate the differential effect of primary viral infections in the susceptibility to disease exacerbation in response to a different secondary viral infection and highlight the usefulness of these viral models as tools to understand the underlying mechanisms that mediate distinct chronic postviral lung diseases. (*Am J Pathol* 2020, 190: 543–553; <https://doi.org/10.1016/j.ajpath.2019.11.003>)

Chronic obstructive pulmonary disease (COPD) and asthma are complex chronic diseases that affect the airways and distal lung compartments. Although the pathologic features of each disease are somewhat different, airway thickening, epithelial cell hyperplasia, excessive mucous production, and loss of functional alveoli are common to both. The World Health Organization reported that together COPD and asthma affected >486 million individuals worldwide across all age groups and caused 3.6 million deaths in 2015. Known risk factors for developing COPD or asthma include chemical irritants, tobacco smoke, pollution, and respiratory infections.¹ Infections with respiratory syncytial virus, human parainfluenza virus 1 and 4, and others at an early age are strongly associated with the development and exacerbation of COPD and

asthma,^{2,3} whereas presence of RNA from rhinovirus, influenza A or B virus, respiratory syncytial virus, or parainfluenza virus in adult's sputum samples are linked to severe episodes of COPD and asthma exacerbation.^{4,5} Susceptibility to these diseases appears stochastic and dependent on the primary and challenge pathogens.^{6–8} No cure exists for COPD or asthma, and patients are at constant risk of experiencing severe episodes triggered by commonly circulating viruses. The limited availability of therapies for these patients is in part the result of a lack of well-characterized animal models of postviral chronic lung

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diseases, which are essential to study the mechanisms of COPD and asthma development and exacerbation and to develop new targeted therapies.

The murine parainfluenza virus Sendai virus (SeV), which mimics human parainfluenza virus 1 pathogenesis,⁹ and the mouse-adapted influenza A virus (IAV) are good models to study postviral lung disease and infection susceptibility because both are closely related to commonly circulating human pathogens. In mice, IAV-induced disease persists for at least 98 days after initial infection and is characterized by expansion of bronchiolar basal-like stem cells into the parenchyma, alias bronchiolization of the parenchyma. Bronchiolization upon IAV infection involves a Sox2⁺p63⁺keratin 5⁺ (Krt5) stem cell program that prolongs lung repair via notch-mediated inhibition of basal stem cell differentiation.^{10–18} In contrast, SeV chronic disease only manifests after viral clearance and is characterized by dense patches of bronchiolized epithelia that increase in size over time.^{19,20} The mechanism by which this occurs remains unclear; however, it is thought to be a result of continuous IL-33 production from epithelial cells in the lung lesions.^{20,21}

The inflammatory nature of IAV-induced chronic disease has not been well characterized, although the damage associated with IAV-induced disease is thought to be a result of robust interferon- γ (IFN- γ) expression during the acute phase of infection.^{22–24} Pathogenesis in SeV-induced disease is attributed to an increased presence of alternatively activated macrophages (AAMs) in the lung after viral clearance and is dependent on accumulation of natural killer T cells and T cells polarized toward type 2 immune responses that promote a positive feedback loop.^{20,21}

Side-by-side characterization of the IAV and SeV post-viral chronic lung diseases were performed and the histopathologic changes, as well as the epithelial and inflammatory components of the lesions, were investigated. In addition, the effect of both infections on the pathogenesis of secondary heterologous viral challenge was evaluated. Striking differences were found in both the composition of the primary lesions and their effects on heterologous challenge. Post-IAV chronic lung disease was not associated with enhanced pathogenesis on challenge with SeV in mice. However, after heterologous challenge, SeV-specific chronic lung disease was significantly exacerbated. These data demonstrate that chronic IAV and SeV in mice serve as relevant, independent models for the study of long-term consequences of viral infections in the lung.

Materials and Methods

Mice, Virus Titration, and Infection

Six- to eight-week-old female C57BL/6 mice were bred in house or paired with mice supplied by Taconic Biosciences (Rensselaer, NY) for all experiments. SeV (strain 52) and mouse-adapted influenza A/Puerto Rico/8/1934 H1N1 (PR8) and A/X31 H3N2 (X31) stocks were expanded in

10-day-old embryonated chicken eggs (Charles River Laboratories, Wilmington, MA). Viral titer was determined using an end point dilution tissue culture infectious dose (TCID₅₀) standard infectivity assay in LLC-MK2 cell (SeV) and MDCK cells (IAV) (both from ATCC, Manassas, VA).²⁵ Virus stocks were diluted in 1× phosphate-buffered saline (PBS) (Gibco, Waltham, MA) and administered intranasally to anesthetized mice in 40- μ L doses ranging from 1×10^4 to 5×10^4 TCID₅₀ per mouse for SeV52, 40 to 60 TCID₅₀ per mouse for PR8, and 2×10^3 TCID₅₀ per mouse for X31. To quantify viral titers in infected mice, mice were euthanized, lung lobes were placed in 0.1% gelatin and homogenized, and viral titer was determined as described earlier in this paragraph.

Histologic Analysis

Euthanized mice were perfused with 10 mL of PBS, and lungs were extracted, fixed with 10% buffered formalin (Fisher Scientific, Hampton, NH), and then transferred to paraffin. Lung sections were analyzed by hematoxylin and eosin, periodic acid–Schiff, and trichrome staining. Chronic disease intensity was blindly scored (G.L.G.) on a scale of 0 to 3 for alveolitis, peribronchiolitis, airway smooth muscle hypertrophy, goblet cell hyperplasia, airway squamous epithelial metaplasia, and fibrosis. Percentage of airways and area of lung affected for each type of pathologic finding was determined. The raw percentage was multiplied by the intensity score and graphed as the weighted score.

Immunofluorescence and Imaging

Lungs were inflated with OCT compound (Tissue-Tek), submerged, and flash frozen for cryosectioning. Sections (11 μ m thick) were fixed in 4% paraformaldehyde (Electron Microscope Services, Hatfield, PA) diluted in 1× PBS and permeabilized in 0.02% Triton X-100 (Sigma), blocked with 1% naive sera, incubated with 1× True Black (Biotium, Hayward, CA) in 70% ethanol, and stained with anti-Sftpc at 1:2500 (Ab3786; EMB Millipore, Burlington, MA), anti-Krt5 at 1:500 (905901 or 905504; Biolegend, San Diego, CA), anti-Scgb3a2 at 1:200 (AF3465; R&D Systems Inc., Minneapolis, MN), anti-Ager at 1:400 (175410; R&D Systems Inc.), anti-CD45 at 1:100 (30-F11, Biolegend), or anti-CD11c-APC at 1:100 (N418; eBioscience Inc., San Diego, CA) antibodies at room temperature for 1 hour. Sections were washed in PBS and stained with secondary antibodies: goat anti-rabbit IgG AF488 (A11008; Invitrogen, Carlsbad, CA), donkey anti-rabbit IgG AF594 (A11037; Invitrogen), donkey anti-goat IgG AF488 (A11055; Invitrogen), donkey anti-goat IgG AF594 (A11058; Invitrogen), goat anti-chicken IgG AF488 (410802; Biolegend), or goat anti-rat IgG AF488 (405418; Biolegend). Nuclei were labeled with Hoechst (Invitrogen), and slides were mounted with Fluoromount-G (Invitrogen). Images were acquired using a Leica DM6000 Widefield

Fluorescent microscope using $\times 4$, $\times 5$, $\times 10$, and $\times 20$ objectives.

Lesion Surface Area Quantification and BALT Counts

Two to three representative mouse lung lobes were imaged with the $\times 5$ objective and stitched using Leica Application Suite X microscope imaging software version 3.3.016799 (Leica Microsystems, Wetzlar, Germany). A lesion was defined as a region of the lung that had chronic disease-like pathologic features, including inflammation, bronchus-associated lymphoid tissue (BALT), epithelial hyperplasia or dysplasia, and fibrosis. Volocity software version 6.3 (Quorum Technologies, Puslinch, ON, Canada) was used to quantify pixel counts for lesions and total lobes. Regions of BALT were blindly, manually counted (G.L.G.) for each condition.

RNA Extraction and RT-qPCR

Lungs were placed into TRIzol (Ambion Inc., Austin, TX), homogenized, and RNA was extracted. A total of 1 μg of RNA was reverse transcribed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA), and 10 ng/ μL of cDNA were used for quantitative RT-PCR (RT-qPCR). RT-qPCR was performed using SYBR Green reagents (Thermo Fisher Scientific, Waltham, MA) in a ViiA7 Real Time PCR System, and results were normalized to *Gapdh* forward 5'-CTCCCACTCTTCCACCTTCG-3', reverse 5'-CCACCACCCTGTTGCTGTAG-3', and corrected for *Tubal1* forward 5'-TGCCTTTGTGCACTGGTATG-3', reverse 5'-CTGGAGCAGTTTGACGACAC-3'. Genes analyzed include: *Chil3* forward 5'-TCTGGGTACAAGATCCCTGAA-3', reverse 5'-TCATATGGAGATTTATAGAGGGGACT-3'; *Muc5ac* forward 5'-GTGTCCCAAGATCAACTGTCC-3', reverse 5'-TGGTGCACCGTACATTTCTG-3'; *Il13* forward 5'-CCTCTGACCCTTAAGGAGCTTAT-3', reverse 5'-CGTTGCACAGGGGAGTCT-3'; *Il33* forward 5'-ACCCACGAAAA-GATATTCATAAAA-3', reverse 5'-CAAGCAAGGATCTCTTCCTAGAAT-3'; *Krt5* forward 5'-ATCCAGCGGTTCAGGACT-3', reverse 5'-GTCCAGGACCTTGTCTGCT-3'; *Trp63* forward 5'-ATGTTGTACCTGGAAAACAATGCC-3', reverse 5'-CAGGCATGGCAGGATAAC-3'; *Scgb1a1* forward 5'-CAGAGTCTGTTATGTGGCATC-3', reverse 5'-TAGGATTTTCTCCGTGAGCTTC-3'; *Ager* forward 5'-CTTGCTCTATGGGAGAGCTGTA-3', reverse 5'-GGAGGATTTGAGCCACGC-3'; *Sftpc* forward 5'-AGCAAAGAGGTCCTGATGGA-3', reverse 5'-ATGAGAAGGCGTTTGAGGTG-3'; *Arg1* forward 5'-GGAAAGCCAATGAAGAGCTG-3', reverse 5'-GATGCTTCCAACCTGCCAGAC-3'; *Cxcl1* forward 5'-GCACCCAAACCGAAGTCATA-3', reverse 5'-CTTGGGACACCTTTTAGCA-3'; *Il6* forward 5'-ACAGAAGAGTGGCTAAGGA-3', reverse 5'-CGCACTAGGTTTGCCGAGTA-3'; *IFN γ* forward 5'-GACTGTGATTGCGGG-

GTTGT-3', reverse 5'-GGCCCGGAGTGTAGACATCT-3'; *IAV NP* forward 5'-CAGCCTAATCAGACCAAATG-3', reverse 5'-TACCTGCTTCTCAGTTCAAG-3'; and *SeV NP* forward 5'-TGCCCTGGAAGATGAGTTAG-3', reverse 5'-GCCTGTTGGTTTGTGGTAAG-3'.

Statistical Analysis

Statistical significance was calculated using GraphPad Prism software version 7.0 (GraphPad Software, San Diego, CA). Group size was predetermined to be three to five mice per condition. Two-way analysis of variance with Bonferroni multiple comparison test was used to calculate significance for weight loss. One-way analysis of variance with Bonferroni multiple comparison test was used to calculate significance for lung titer, RT-qPCR, histopathologic scoring, and quantification of pathologic findings.

Results

Distinct Chronic Lung Pathologic in IAV and SeV Infections

To better characterize the long-term diseases elicited by parainfluenza and influenza viruses, side-by-side comparisons of morbidity and histopathologic features of mice infected with sublethal doses of the murine parainfluenza virus SeV strain 52 or the mouse-adapted IAV strains PR8 (highly pathogenic) or X31 (mildly pathogenic) were performed. Conditions that ensured that all mice were infected ($10\times$ the infectious dose 50) and that disease manifested as similar weight loss among groups (Figure 1A) were used. No significant differences were found in virus titers among the different infections at day 3 after infection despite X31 infection showing higher viral RNA than PR8 at this time, which corresponds to peak viral titers (Figure 1B).^{26,27} Groups of mice were sacrificed on day 33 after infection for histologic analysis of the lung. This end point was selected based on preliminary studies demonstrating early signs of disease by 3 weeks after infection and clear bronchiolization by day 33.^{19–21} Histologic analysis revealed that immune cells coalesced into BALT-like structures in IAV-infected lungs, whereas immune cells localized diffusely in SeV-infected lungs (Figure 1C). In addition, areas of bronchiolization of the lung parenchyma were evident in infections with SeV by day 33. Bronchiolization was present in IAV PR8 or X31 infected lungs with more intense disease shown in the PR8 group (Figure 1C). IAV pathologic findings were localized to peribronchiolar regions in the lung and adjacent to BALT structures. In SeV-infected lungs, foamy macrophages were seen on the periphery of the lesions. These cells have been previously characterized as AAMs responsible for mediating wound repair and chronic inflammation in the case of type 2 inflammatory disease.^{19,20,28,29} Lesions were observed in all lung lobes and localized to the adjacent parenchyma

surrounding distal airways (data not shown). In contrast, IAV lesions largely resembled basal cell pods^{16,17,30} and contained tertiary lymphoid tissue with large accumulations of lymphocytes and other mononuclear cells.

To further characterize the appearance of chronic postviral disease, groups of infected mice were analyzed for histopathologic findings at 5, 10, 21, and 49 days post infection (DPI).^{19–21} Although infiltrates were observed across all infections in early time points, by 21 DPI SeV-infected lungs remained affected primarily by goblet cell hyperplasia, whereas IAV-infected lungs were characterized by growing BALT structures and the appearance of basal cell pods. At 49 DPI, lesions in SeV-infected lungs were larger and denser, while maintaining the hallmarks of type 2 inflammation, including eosinophilia and goblet cell hyperplasia (Figure 1, D and F). Hematoxylin and eosin staining revealed these lesions to contain mostly epithelial cells, whereas periodic acid–Schiff stain revealed large accumulations of extracellular mucus associated with significant alveolitis, smooth muscle hypertrophy, and squamous epithelium metaplasia (Figure 1, E and F). Differences were also observed in the phenotypes of IAV-associated chronic lesions at this timepoint. Lesions in IAV-infected lungs became larger and of a mixed phenotype. In addition to BALT-like structures, some evidence of type 2 immunity–mediated pathologic findings, such as positive periodic acid–Schiff staining results, was detected within the basal stem cell pods. However, the density and overall cellular composition of post SeV- and IAV-induced lesions remained distinct. In IAV-infected lungs, trichrome staining revealed moderate deposition of collagen in areas with lesions, consistent with previously described fibrosis in chronic postinfection disease.²⁷ SeV infected lungs showed little to no collagen deposition (Figure 1, E and F). In conclusion, phenotypically distinct long-term histopathologic findings are observed in mice infected with SeV or IAV, despite similar viral titers and morbidity during acute infection.

Epithelial Composition of Chronic SeV and IAV Lesions

We hypothesized that the morphologic and histochemical differences observed in chronic lung diseases induced by

SeV or IAV were due to distinct epithelial or inflammatory events occurring after infection. The epithelial cell composition of the chronic lung lesions observed after infection with SeV52 and IAV PR8 was further characterized using fluorescent antibodies to visualize basal stem cells (Krt5), club cells (Scgb3a2), alveolar type 1 cells (Ager), and alveolar type 2 (Sftpc) epithelial cells (Figure 2A). The IAV PR8 strain and high-dose SeV52 were used to ensure robust disease. In agreement with previous reports,^{16,17,30} IAV lesions were predominately Krt5⁺ and excluded alveolar epithelial cells. However, paired analysis of SeV-disease lesions found that SeV lesions contained a dense mix of bronchiolar and alveolar epithelial cells. The expression of specific epithelial cell subpopulation markers was then quantified in the lung 49 DPI with SeV52 or IAV PR8 (Figure 2B). Although the relative mRNA levels of Scgb1a1, a protein secreted by club cells lining the airway, appeared to be higher in healthy animals than in SeV and IAV mice, levels of mRNA for epithelial stem cell markers (*Krt5* and *Trp63*) were significantly greater in SeV-infected mice compared with mock infected controls and IAV PR8-infected mice. In contrast, levels of mRNA for alveolar type 1 and type 2 cell markers (*Ager* and *Sftpc*, respectively), were greater in IAV-infected mice (Figure 2B). We hypothesize that differences in the epithelial cell composition of disease are a result of basal cell differentiation into parenchymal epithelium as previously reported.^{15,16} In agreement with this hypothesis, during SeV infection, higher basal cell mRNA correlates with the histologic findings of highly invasive and denser bronchiolized lesions than those observed on IAV infection. IAV lesions were diffuse throughout the lung with mild disruption to normal parenchymal structure. The differences observed in morphologic features and composition of lesions in SeV or IAV disease models begin to highlight the contrasting and variable nature of chronic postviral lung disease.

Distinct Chronic Inflammatory Environments in the Lung after SeV or IAV Infection

Notable differences were observed in the composition and distribution of the lung cellular infiltrate during chronic

Figure 1 Acute infection with Sendai virus (SeV) and influenza A virus (IAV) results in phenotypically distinct chronic epithelial diseases. Mice were inoculated with phosphate-buffered saline, 1×10^4 tissue culture infectious dose (TCID₅₀) per mouse SeV52, 40 to 60 TCID₅₀ per mouse PR8, or 2×10^3 TCID₅₀ per mouse of X31, doses that are approximately 10 times the infectious dose 50 (ID₅₀). **A:** Weight loss was tracked throughout the infection course. **B:** Lungs were harvested at 3 days post infection (DPI), and virus titer was determined for each lung sample by a standard infectivity assay as well as measuring for viral nucleoprotein RNA levels using quantitative RT-PCR. Data were normalized to *Gapdh* and *Tuba1a*. **C:** Lungs were harvested at 33 DPI and stained with hematoxylin and eosin (H&E) to determine global morphologic changes. **D:** Lungs were harvested at 5, 10, 21, and 49 DPI and stained with H&E. **E:** Lungs were harvested at 49 DPI and stained with periodic acid–Schiff (PAS) or trichrome as indicated. **C, D and E: Insets** show lesion components indicated by **arrowheads:** **black arrowheads** indicate alternatively activated macrophages; **white arrowheads**, eosinophilic crystal; **yellow arrowheads**, basal stem cell pod; **red arrowheads**, bronchus-associated lymphoid tissue. **F:** Representative sections from mice in each group shown in **D** and **E** at 49 DPI were blindly scored (G.L.G.) for histopathologic analysis. The percentages of airways and area of the lung affected were determined for each lung section, and a value of intensity ranging from 0 to 3 for each metric was determined by the amount of cellular infiltration, degree of smooth muscle thickening from slight to severe, percent area affected, and epithelial cell layering. Weighted scores are shown. Data are expressed as means $\pm$ SEM over time (**A, B, and F**). One-way analysis of variance with Bonferroni multiple comparison test was used to calculate statistical significance. $n = 3$ to 7 mice per group (**A**); $n = 3$ to 4 mice per group (**C**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Scale bars = 100 μ m. Stitched whole lobes were imaged with the $\times 5$ objective, whereas all other images in **C, D, and E** were obtained with the $\times 20$ objective. **Insets** are digital magnifications of specific areas.

Figure 2 Sendai virus (SeV)- and influenza A virus (IAV)-associated chronic lung lesions differ in epithelial cell composition. Groups of mice were infected with SeV52 [5×10^4 tissue culture infectious dose (TCID₅₀) per mouse] or IAV PR8 (60 TCID₅₀ per mouse). **A:** Lungs were harvested 49 days post infection. Lobes were serial sectioned and stained for basal stem cells [Sox2⁺p63⁺keratin 5⁺ (Krt5)], club cells (Scgb3a2), type 1 alveolar cells (Ager), and type 2 alveolar cells (Sftpc). **B:** Mouse lung homogenates were analyzed for expression of major epithelial subtype markers by quantitative RT-PCR. Data were normalized to *Gapdh* and *Tuba1a*, plotted as individual points, and graphed as means $\pm$ SEM. $n = 4$ to 5 mice per group. Data are representative of two to three experimental repeats. One-way analysis of variance with Bonferroni multiple comparison test was used to calculate statistical significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Scale bars = 100 μ m. Mock images were obtained using the $\times 10$ objective, and representative images for SeV52 and PR8 lung lesions were imaged using the $\times 4$ and $\times 20$ objective, respectively.

disease (Figure 1). To better characterize the inflammatory environments of SeV- and IAV-induced chronic diseases, mice were infected with SeV52 or IAV PR8, and lungs were harvested 49 DPI for initial analyses of major inflammatory cell populations contained in and around the lesions. Lung sections were stained for CD45 and CD11c to visualize the distribution and morphologic features of immune cells present during disease. SeV lesions contained diffuse clusters of large myeloid cells (CD45⁺/CD11c⁺), corresponding to previous descriptions of AAMs in these lesions (Figure 3A).^{19–21} In IAV lesions, smaller CD11c⁺ cells that resembled alveolar macrophages and a predominant CD45⁺ population, most likely T and B cells, clustered into BALT. Such structures were not seen in SeV-infected mice. A summary of the distinct epithelial and hematopoietic cell composition in post-SeV and post-IAV lesions is shown in Figure 3B.

Cytokine mRNA expression levels in the lung revealed a marked contrast in the inflammatory environments of each condition (Figure 3C). Although *Ifng* and *Cxcl1* expression was slightly increased in lungs from the IAV group,

confirming general inflammation, the inflammatory environment in the SeV group compared with the IAV group was strongly biased toward type 2 immunity shown by the induction of *Il33*, *Il13*, *Il6*, and the AAM markers *Chil3* and *Arg1* (Figure 3, C and D). Because the IAV model lacks long-term expression of *Il33* and canonical type 2 markers, such as markers of AAMs and eosinophilia, it can be concluded that IAV disease inflammation is not identical to SeV disease.

Distinct Disease Susceptibilities to Heterologous Virus Infection in SeV and IAV Chronic Disease Phenotypes

The distinct chronic lung structure and inflammation observed after SeV and IAV infection led us to hypothesize that disease outcomes would vary in mice infected with either of these viruses on a heterologous challenge. To test this hypothesis, mice that were mock infected with PBS or infected with SeV52 or IAV X-31 were challenged with the opposite virus at doses identical to the primary infection (Figure 4A). The less virulent IAV X31 strain was used for

Figure 3 Sendai virus (SeV) and influenza A virus (IAV) chronic postviral lung diseases associate with distinct inflammatory environments. **A:** Lungs harvested 49 days post infection (DPI) with SeV52 and IAV PR8 were further analyzed for CD45⁺ and CD11c⁺ cellular composition and morphologic features by immunofluorescence. **B:** Summary of differences between SeV and IAV chronic disease bronchiolization and immune cell composition. Quantitative RT-PCR was then used to analyze levels of *Ifng*, *Cxcl1*, and *Il6* (**C**), type 2 inflammatory markers (*Il33*, *Il13*, and *Muc5ac*), and markers of alternatively activated macrophages (*Chil3* and *Arg1*) (**D**) from lung homogenates 49 DPI. Data were normalized to *Gapdh* and *Tuba1a*, plotted as individual points, and graphed as means $\pm$ SEM. $n = 4$ to 5 mice per group. Data are representative of two to three experimental repeats. One-way analysis of variance with Bonferroni multiple comparison test was used to calculate statistical significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Scale bars = 100 μ m. Representative lesions were obtained using the $\times 5$ and $\times 20$ objectives.

these studies to ensure a large population of surviving mice at the time point of secondary challenge. Distinct signs of morbidity were apparent in the different groups. Primary infection with IAV resulted in protection from morbidity at the time of secondary challenge with SeV (Figure 4B). This was not seen in the reverse condition. Instead, primary infection with SeV correlated with partially worsened morbidity early after challenge (Figure 4C). Mouse lungs were harvested 7 days after challenge when weight loss reached the protocol approved limit. Histologic analysis of the lungs at this time point revealed exacerbation of the primary virus-induced disease (Figure 4D). The extent of whole lung pathologic features quantified by determining the percent area of lesions, including bronchiolization,

thickened airways, fibrosis, inflammation, and BALT, indicates a larger area of the lung compromised in mice infected with SeV first and then challenged with IAV (Figure 4F). The induction of worsened disease after challenge in mice first exposed to SeV correlated with enhanced levels of *Krt5* mRNA in the lungs (Figure 4G). BALT formation was again observed only in IAV-induced disease (Figure 4H) and may serve a protective role in the immune response to heterologous challenge. These data indicate that although mice first exposed to IAV are protected from heterologous infection, mice with chronic postviral type 2 inflammation and bronchiolization are at greater risk of morbidity and developing significant disease on challenge with an unrelated virus.

Discussion

IAV and SeV have been extensively used as tools to study lung repair and chronic inflammation.^{4,10–17,19–21,27,29–33} However, these infections have not been studied in parallel to compare the consequences that these infections have on chronic inflammation, lung damage, and susceptibility to secondary infection. We found that there are striking differences in the composition and extent of disease among chronic postviral lesions that develop after infection with SeV or IAV. These structural and phenotypic differences contribute to different responses to secondary challenge with an unrelated pathogen. Post-SeV chronic type 2 biased disease led to more extensive lung pathologic and enhanced morbidity upon challenge with IAV compared with the flipped infection scheme. These data indicate that SeV postviral disease better models human postviral conditions (COPD or asthma) that are associated with exacerbations on virus challenge.

Despite the cumulative nature of SeV lesions and their diverse composition, the underlying mechanisms of epithelial disease in this infection are not completely understood. This study found that chronic post-SeV lesions are composed of four major lung epithelial cell types: basal stem cells, club cells, and alveolar epithelial 1 and 2 cells. The basal stem cell component of these lesions would suggest similar epithelial mechanisms of disease between SeV and IAV. However, lesions in the IAV model have been characterized as alveolar epithelial deserts lacking both alveolar epithelial 1 (AEC1) and 2 cells.^{14,15} Trp63⁺/Krt5⁺ stem cell lesions in the IAV model are capable of further differentiating into bronchiolar and alveolar epithelial cells, thereby reestablishing alveolar structure.^{10,12,13,16,17} Further analysis needs to be conducted to better understand the ontology of SeV lesions as well as why these lesions appear to expand overtime and on challenge, although not in the IAV model.

Our data agree with reports indicating that post-SeV lesions exist in a highly type 2 polarized immune environment and are scattered with AAMs.^{19–21} These cells are thought to be capable of perpetually remodeling the lung. Efforts have been made to inhibit the pathogenic effects of T_H2 inflammation in the lung. Although depleting AAMs and

inhibiting type 2 signal via IL-33- and IL-13-specific antibodies can reduce type 2 inflammation,³⁴ the effects of these anti-inflammatory drugs have not been studied in the context of already established alveolar bronchiolization. Other cytokines, including IL-25 and thymic stromal lymphopoietin, are capable of inducing type 2 inflammation^{35–38}; however, these cytokines do not promote SeV postviral lung disease.^{19,20}

Unlike the SeV model, IAV chronic disease is thought to be induced during the acute phase of IAV infection during which a robust IFN- γ response causes dose-dependent damage and inflammation that sets the stage for dysplastic repair.^{22,39–41} Interestingly, other groups have reported long-term IFN- γ /IL-10-producing T cells⁴² and epithelial cells expressing interferon stimulated genes that survive infection in both influenza A and B infections,^{43–45} suggesting that IAV disease bares a semipermanent type 1 inflammatory signature.

Intriguingly, a recent study using the WSN/33 IAV strain capable of infecting multiple tissues reported type 2 inflammatory characteristics in the lung despite lack of evidence of type 2 inflammation hallmarks, including IL-33 expression and AAMs.²⁷ A similar phenotype was observed developing at day 49 on infection with influenza X31. Moreover, chemosensory tuft cells that drive type 2 pathology are present in the distal lung after PR8 infection coincident with chronic disease,⁴⁶ but the presence and role of these cells in post-SeV infection have not been determined. How type 2-like inflammation is driven during IAV infection remains unknown. On the basis of our histologic, transcriptional, and protein expression data, the CD45⁺ component in the SeV model is predominantly myeloid, whereas in the IAV model the CD45⁺ cells have lymphocytic characteristics. Although the major contributing inflammatory cell in the IAV disease model has yet to be determined, differences in inflammatory cell composition have implications in treatment decisions.

Understanding the chronic inflammatory and epithelial differences in these 2 distinct forms of COPD-like disease is essential for therapeutic design and for better predicting patient outcomes. Patients with chronic respiratory diseases

Figure 4 Sendai virus (SeV) chronic postviral lung diseases increase susceptibility to lethal influenza A virus (IAV) infection. **A:** Mice were infected intranasally with phosphate-buffered saline (PBS), 1×10^4 tissue culture infectious dose (TCID₅₀) per mouse of SeV52, or 2×10^3 TCID₅₀ per mouse of IAV X31 and monitored for weight loss throughout the course of infection. Mice were challenged 25 days post infection (DPI) with SeV52 or IAV X31 at doses identical to the primary infection. Tissue was collected 7 days after challenge for histologic analysis and quantitative RT-PCR. **B and C:** Weight loss was monitored during the acute phase of secondary challenge for IAV primary and SeV primary infection groups to track morbidity. Statistics given for **B** compare X31 plus SeV52 and PBS plus SeV52. Statistics given for **C** compare SeV52 plus X31 and PBS plus X31. **D:** Lungs were harvested and stained with hematoxylin and eosin. **E:** The $\times 5$ objective was used to whole lung stitch representative lung lobes in the SeV plus IAV and IAV plus SeV groups to show differences in total lung lobe pathologic features. **F:** The total area of lesions, including bronchus-associated lymphoid tissue (BALT), fibrosis, bronchiolization, and thickened airways in the lung, were quantified and graphed as a percentage of total lung pixel counts for two to three of five lobes per mouse. **G:** *Krt5* mRNA was determined by quantitative RT-PCR as a measure of bronchiolization in the lungs. Data were normalized to *Gapdh* and *Tuba1a*. **H:** Patches of BALT were manually counted for each group. Data are representative of ≥ 2 experimental repeats with an $n = 3$ to 5 per repeat. Data were plotted as means $\pm$ SEM over time (**B** and **C**). Data were plotted as individual points and graphed as means $\pm$ SEM (**F**, **G**, and **H**). Two-way analysis of variance with Bonferroni multiple comparison test was used to calculate statistical significance (**B** and **C**). One-way analysis of variance with Bonferroni multiple comparison test was used to calculate statistical significance (**F**, **G**, and **H**). $n = 4$ to 5 mice per group; $n = 4$ to 5 mice per group (**F**, **G**, and **H**). *** $P < 0.001$, **** $P < 0.0001$ versus X31 plus SeV52; † $P < 0.05$, †† $P < 0.01$ SeV52 plus X31 versus PBS plus X31; ‡ $P < 0.05$, ‡‡ $P < 0.01$, ‡‡‡ $P < 0.001$, and ‡‡‡‡ $P < 0.0001$. Scale bars = 100 μ m. Images were obtained with a $\times 5$ objective (**D**, main image); $\times 20$ objective (**D**, insets). P, PBS; S, SeV52; X, X31.

are at an increased risk of developing worsened disease or detrimental immune responses under the stress of secondary challenge.⁴⁷ *In vitro*, prior exposure of macrophages to type 1 or type 2 polarizing cytokines, IFN- γ , and IL-4 leads to altered cytokine production,⁴⁸ suggesting that the immune response in type 1 or type 2 biased lungs are differentially equipped to handle secondary virus challenge. The effect that chronic disease-driven biased immune responses have during heterologous challenge have not been studied in the context of disease pathogenesis. Secondary challenge studies have primarily focused on pathogen replication,^{6,8} immune cell proliferation and activation,^{6,49} and recruitment.^{8,50} These studies have not addressed the effect of heterologous challenge on long-term disease pathogenesis and exacerbation except in the cases of lymphocytic choriomeningitis virus⁸ and respiratory syncytial virus,⁶ where mice experience worsened disease or protection, respectively, after IAV primary challenge.

In conclusion, our data indicate that respiratory viruses are capable of eliciting inherently different forms of chronic disease, which in turn results in varying pathologic susceptibilities to secondary infection. Therefore, further elucidation of the inciting events and factors that maintain respiratory diseases, on epithelial and immune levels, across different models is essential to treat and prevent chronic disease and exacerbations.

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