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Differential Pathogenesis of Respiratory Syncytial Virus Clinical Isolates in BALB/c Mice

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Airway mucus is a hallmark of respiratory syncytial virus (RSV) lower respiratory tract illness. Laboratory RSV strains differentially induce airway mucus production in mice. Here, we tested the hypothesis that RSV strains differ in pathogenesis by screening six low-passage RSV clinical isolates for mucogenicity and virulence in BALB/cJ mice. The RSV clinical isolates induced variable disease severity, lung interleukin-13 (IL-13) levels, and gob-5 levels in BALB/cJ mice. We chose two of these clinical isolates for further study. Infection of BALB/cJ mice with RSV A2001/2-20 (2-20) resulted in greater disease severity, higher lung IL-13 levels, and higher lung gob-5 levels than infection with RSV strains A2, line 19, Long, and A2001/3-12 (3-12). Like the line 19 RSV strain, the 2-20 clinical isolate induced airway mucin expression in BALB/cJ mice. The 2-20 and 3-12 RSV clinical isolates had higher lung viral loads than laboratory RSV strains at day 1 postinfection (p.i.). This increased viral load correlated with higher viral antigen levels in the bronchiolar epithelium and greater histopathologic changes at 1 day p.i. The A2 RSV strain had the highest peak viral load at day 4 p.i. RSV 2-20 infection caused epithelial desquamation, bronchiolitis, airway hyperresponsiveness, and increased breathing effort in BALB/cJ mice. We found that RSV clinical isolates induce variable pathogenesis in mice, and we established a mouse model of clinical isolate strain-dependent RSV pathogenesis that recapitulates key features of RSV disease.

Respiratory syncytial virus (RSV) is the most important cause of bronchiolitis and viral pneumonia in children. Each year in the United States, RSV causes lower respiratory tract illness (LRI) in 20 to 30% of infants and leads to the hospitalization of approximately 1% of infants at a cost of $300 to $400 million (19, 21, 27). The incidence and disease severity of RSV can vary from year to year (47). Dominant circulating RSV strains are generally replaced each year, likely by a process involving immune selection (5, 6, 53, 54). RSV strain differences may contribute to year-to-year and/or patient-to-patient variations in clinical severity.

In BALB/cJ mice, laboratory RSV strains (A2, Long, and line 19) differ in their ability to cause pulmonary interleukin-13 (IL-13) and mucin expression (34, 41). We are interested in RSV-induced mucin expression in mice because mucous overabundance contributes to airway obstruction in severe RSV disease in children (2, 33, 44, 56). IL-13 is a cytokine linked to mucous production (71). In BALB/cJ mice, whereas the A2 and Long RSV strains do not (34, 41). However, the in vitro passage histories of RSV strains A2, Long, and line 19 are not defined and involve many serial passages. Thus, it is possible that mutations in these RSV laboratory strains determine pathogenesis phenotypes in the mouse model. RSV clinical isolates have not been studied extensively in vivo, and the role of RSV strain differences in pathogenesis is not clear.

Here, we tested whether RSV strains exhibit differential pathogenesis phenotypes in BALB/cJ mice using low-passage RSV clinical isolates. We derived six antigenic subgroup A RSV clinical isolates and generated low-passage stocks of these strains. Infection of BALB/cJ mice with these strains resulted in variable weight loss and airway mucin expression phenotypes. We found that RSV clinical isolate A2001/2-20 (2-20) induced higher lung IL-13 levels, airway mucin expression, and airway dysfunction than the related isolate RSV A2001/3-12 (3-12). As RSV 2-20 and 3-12 are genotypically similar and were isolated in the same year (2001), their divergent pathogenesis phenotypes suggest a role for RSV strain variation in differential disease severity observed during RSV epidemics.

MATERIALS AND METHODS

Viruses. The A2, line 19, and Long strains of RSV were propagated as described previously (19, 21). First-passage (P1) HEP-2 supernatants of RSV antigenic subgroup A clinical isolates were obtained from the Vanderbilt Vaccine Clinic (Nashville, TN) (74). Tenfold serial dilutions of the P1 supernatants were used to infect HEP-2 cells in six-well plates. For each isolate, supernatant from the most dilute inoculum exhibiting >50% cytopathic effect (CPE) was harvested.
for sequential passage in HEp-2 cells. The isolates were passaged by limiting dilution nine times in HEp-2 cells. HEp-2 cells in flasks were infected with the supernatants from the ninth limiting dilution passage (P10 overall). Membrane stocks (P11) and working stocks (P12) were generated as described previously (18). Six antigenic subgroup A clinical isolates strains were propagated: 2-20, 3-12, A1997/12-35 (12-35), A1998/3-2 (3-2), A1998/12-21 (12-21), and A2000/3-4 (3-4). Isolates are named with their subtype letter (A or B), followed by the year and month they were collected and their isolate number. Viral stocks were propagated and titrated by plaque assay in HEp-2 cells. A2001/2-20 was placed in a petri dish approximately 4 in. from a 40-watt UV light on ice for 4 h. UV-inactivated virus was titrated on HEp-2 cells to ensure inactivation.

**Cells and mice.** HEp-2 cells were cultured in minimal essential media (MEM) supplemented with l-glutamine, Earle’s salts, 10% fetal bovine serum (FBS) (HyClone; ThermoFisher), and a penicillin G-streptomycin sulfate-amphotericin B solution (Invitrogen). BEAS-2B human bronchial epithelial cells were a gift from Pierre Masson (Vanderbilt University, Nashville, TN) and were cultured in RPMI medium supplemented with 10% fetal bovine serum and penicillin G-streptomycin sulfate-amphotericin B solution. Female, 6- to 8-wk-old BALB/c mice were obtained from Jackson Laboratories. IL-13 null mice were obtained from Gurjit K. Hershey at Cincinnati Children’s Hospital Medical Center (46). All mice were maintained under specific-pathogen-free conditions. Female mice 5 to 6 wk of age were anesthetized intranasally with RSV or with mock-infected cell culture supernatant as described previously (18).

**Sequenceing RSV glycoprotein (G) and fusion (F) genes.** As described above, RSV clinical isolates A2001/2-20 (2-20) and A2001/3-12 (3-12) were passed by limiting dilution in HEp-2 cells. Total RNA was isolated from passage 2 (P2) and working stock (P12) supernatants using Trizol (Invitrogen). In order to sequence the G and F genes, chimeric reverse transcription PCR (RT-PCR) was performed amplifying using primers FStul and FSpHl, as described previously (41). In order to sequence the G gene, cDNA was reverse transcribed using primer G+ (CCATTGTTATTTGCCCCAGA). PCR was performed using primer SHecori (AGGAATTCCGAGACACAGTACAGC) and primer GStul (GGTCAAGGCCTTTTGTGATAATATG), and generated approximately 1.6-kb amplicons containing the SH (small hydrophobic) and the G genes. The EcoRi and Stul sites (underlined) facilitated cDNA cloning for future studies. RSV F and G PCR amplicons from P2 and P12 supernatants were gel purified and sequenced using primers complementary to these regions of the published RSV A2 and line 19 genomes. Sequences were analyzed using VectorNTI software (Invitrogen Corp.). Multisequence alignments were performed using AligX software (neighbor-joining method) within VectorNTI.

**Multistep virus growth curves.** Subconfluent HEp-2 or BEAS-2B cells in six-well dishes were infected in triplicate with RSV strains A2, 2-20, or 3-12 at a multiplicity of infection (MOI) of 0.5 in 750 μL. After 1 h of adsorption at room temperature on a rocking platform, the cells were washed with medium, and fresh medium was added. Supernatants were harvested at 24 h, 48 h, 72 h, and 96 h from each well and clarified by centrifugation, and RSV was titrated in duplicate by plaque assay on HEp-2 cells as described previously (15).

**Quantification of lung viral load.** Lungs were harvested from BALB/c mice infected with RSV. We infected 10-12 weeks old CD1 mice (Charles River, Bar Harbor, ME) with the RSV strain 2-20, and lungs were harvested at 8 days postinfection (dpi). Lungs were harvested from C57BL/6 mice infected with RSV. We infected 10-12 weeks old CD1 mice (Charles River, Bar Harbor, ME) with 106 cells were analyzed using an LSR II flow cytometer (BD Biosciences). For FACS analysis of lung mononuclear cell populations, we used an intraperitoneally (i.p.) with sodium pentobarbital (8.5 mg/kg body weight), and lungs were harvested at 8 days postinfection (p.i.). Cells were isolated using Ficoll gradients (P2000, Sigma) and counted with a hemocytometer. Cells were stimulated for 6 h with phorbol myristate acetate (PMA)-ionomycin and stained with the following reagents: anti-CD4, anti-CD8, anti-IFN-γ, anti-IL-12, anti-IL-17, and anti-TNF-α (BD Pharmingen). Tissues were harvested at 8 days postinfection, and cell suspensions were analyzed using FlowJo software (Tree Star). Mice were euthanized i.p. with sodium pentobarbital (8.5 mg/kg body weight), and lungs were harvested at 8 days postinfection (p.i.). Cells were isolated using Ficoll gradients (P2000, Sigma) and counted with a hemocytometer. Cells were stimulated for 6 h with phorbol myristate acetate (PMA)-ionomycin and stained with the following reagents: anti-CD8, anti-CD3, anti-IFN-γ, anti-IL-12, anti-IL-17, and anti-TNF-α (BD Pharmingen). Tissues were harvested at 8 days postinfection, and cell suspensions were analyzed using FlowJo software (Tree Star). Mice were euthanized i.p. with sodium pentobarbital (8.5 mg/kg body weight), and lungs were harvested at 8 days postinfection (p.i.). Cells were isolated using Ficoll gradients (P2000, Sigma) and counted with a hemocytometer. Cells were stimulated for 6 h with phorbol myristate acetate (PMA)-ionomycin and stained with the following reagents: anti-CD8, anti-CD3, anti-IFN-γ, anti-IL-12, anti-IL-17, and anti-TNF-α (BD Pharmingen). Tissues were harvested at 8 days postinfection, and cell suspensions were analyzed using FlowJo software (Tree Star). Mice were euthanized i.p. with sodium pentobarbital (8.5 mg/kg body weight), and lungs were harvested at 8 days postinfection (p.i.). Cells were isolated using Ficoll gradients (P2000, Sigma) and counted with a hemocytometer. Cells were stimulated for 6 h with phorbol myristate acetate (PMA)-ionomycin and stained with the following reagents: anti-CD8, anti-CD3, anti-IFN-γ, anti-IL-12, anti-IL-17, and anti-TNF-α (BD Pharmingen). Tissues were harvested at 8 days postinfection, and cell suspensions were analyzed using FlowJo software (Tree Star). Mice were euthanized i.p. with sodium pentobarbital (8.5 mg/kg body weight), and lungs were harvested at 8 days postinfection (p.i.). Cells were isolated using Ficoll gradients (P2000, Sigma) and counted with a hemocytometer. Cells were stimulated for 6 h with phorbol myristate acetate (PMA)-ionomycin and stained with the following reagents: anti-CD8, anti-CD3, anti-IFN-γ, anti-IL-12, anti-IL-17, and anti-TNF-α (BD Pharmingen). Tissues were harvested at 8 days postinfection, and cell suspensions were analyzed using FlowJo software (Tree Star).
Weight loss is a quantitative measure of RSV illness severity in BALB/cJ mice. The machine measured arterial O2 saturation, heart rate, pulse rate, pulse distension, and breath distension approximately every 0.1 s (MouseOx software, version 4.0). Each mouse was analyzed for 1 to 5 min. Only time points in which all of these physiological parameters were successfully measured were included in analyses, approximately 30 s to 2 min per mouse. Data were exported to Microsoft Excel for analysis. Breath distension for each group (mock or RSV strain) was calculated by averaging measurements for each mouse and then averaging these values per group (8 to 10 mice).

Statistical analyses. Unless otherwise indicated, groups were compared by one-way analysis of variance (ANOVA) and Tukey multiple comparison tests. A P value of <0.05 was considered significant. Data values below the limits of detection were assigned a value of half the limit of detection. Data are representative of at least three replicate experiments having consistent results.

Nucleotide sequence accession numbers. The 2-20 F and G sequences were submitted to GenBank under accession numbers JF279544 and JF279545, respectively. The sequence data for 3-12 F and G were submitted under accession numbers JF279546 and JF279547, respectively.

RESULTS

RSV clinical isolates caused differential disease severity. Weight loss is a quantitative measure of RSV illness severity in the BALB/cJ mouse model, and the A2 strain of RSV causes weight loss at 1 week p.i. (18, 42, 51). We compared weight loss in BALB/cJ mice that were mock infected or infected with RSV laboratory strains A2, Long, or with RSV clinical isolates 2-20, 3-12, 12-35, 3-4, 3-2, or 12-21. The clinical isolates 2-20 and 12-35 caused significant weight loss at 2 d p.i. (Fig. 1A and B). The 2-20 RSV strain uniquely exhibited a bimodal weight loss pattern in which the second peak of weight loss occurred slightly earlier than A2-induced weight loss (Fig. 1). Like the 2-20 strain, RSV clinical isolate strain 12-35 caused weight loss at 6 d p.i. (Fig. 1B). Compared to mock infection, RSV clinical isolate strains 3-4, 3-2, and 12-21 caused no weight loss in BALB/cJ mice (data not shown). RSV laboratory strains line 19 and Long caused no weight loss with a virus dose of 10^5 PFU (Fig. 1B). Thus, three of the six clinical isolates caused weight loss in BALB/cJ mice, and the weight loss pattern of those three strains was different: 3-12 produced early weight loss, 2-20 produced early and late bimodal weight loss, and 12-35 produced late weight loss. Across multiple experiments, there was no consistent difference in the degree of early weight loss at day 2 p.i. caused by 2-20 and 3-12 (compare Fig. 1A to B). Infection with UV-inactivated 2-20 did not cause weight loss (Fig. 2). Therefore, 2-20 weight loss required replication-competent virus. Over the time course, RSV 2-20 caused greater illness severity in BALB/cJ mice than the other RSV strains tested.

Lung IL-13 and gob-5 levels in mice infected with RSV laboratory and clinical isolate strains. In contrast to the A2 and Long RSV strains, the RSV strain line 19 induces lung IL-13 expression on day 8 p.i. and IL-13-dependent airway mucus expression (34, 41, 65). We quantified IL-13 levels in BALB/cJ mice that were mock infected or infected with RSV strain A2, Long, or with RSV clinical isolates 2-20, 3-12, 12-35, 3-4, 3-2, or 12-21. The clinical isolates 2-20 and 12-35 caused no weight loss in BALB/cJ mice (data not shown). RSV laboratory strains line 19 and Long caused no weight loss with a virus dose of 10^5 PFU (Fig. 1B). Thus, three of the six clinical isolates caused weight loss in BALB/cJ mice, and the weight loss pattern of those three strains was different: 3-12 produced early weight loss, 2-20 produced early and late bimodal weight loss, and 12-35 produced late weight loss. Across multiple experiments, there was no consistent difference in the degree of early weight loss at day 2 p.i. caused by 2-20 and 3-12 (compare Fig. 1A to B). Infection with UV-inactivated 2-20 did not cause weight loss (Fig. 2). Therefore, 2-20 weight loss required replication-competent virus. Over the time course, RSV 2-20 caused greater illness severity in BALB/cJ mice than the other RSV strains tested.

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in lung homogenates of BALB/cJ mice mock infected or infected with 10^5 PFU of the A2, line 19, Long, 2-20, 3-12, 3-2, 12-21, or 3-4 RSV strain. Of the clinical isolate strains, 12-35, 12-21, 3-4, and 2-20 induced relatively high levels of lung gob-5 (Fig. 4). gob-5 and IL-13 levels correlated well with each other in these experiments (Fig. 3 and 4). Taken together, RSV clinical isolate strains 2-20, 12-35, and 3-4 induced relatively high levels of gob-5 and IL-13 in BALB/cJ mice at 8 days p.i.

**RSV strain 2-20 and 3-12 G and F gene sequences.** Of the six RSV clinical isolates we screened for weight loss, IL-13 levels, and gob-5 levels, we chose 2-20 and 3-12 for further study because these two strains, both from the RSV season of 2000 to 2001, exhibited differential phenotypes. RSV 2-20 and 3-12, both antigenic subgroup A isolates, were obtained from the Vanderbilt Vaccine Clinic during the 2001 RSV season. In order to determine the effect of limiting-dilution culture on G and F sequences, we sequenced 2-20 and 3-12 G and F genes from P2 and final working stocks (P12). The G genes of RSV 2-20 and 3-12 were identical between passages 2 and 12. There was a 1-nucleotide (nt) difference between P2 and P12 F genes of RSV 2-20, resulting in a predicted amino acid change at position 76 (V in P2 and G in P20). There was a 1-nt difference between P2 and P12 F genes of RSV 3-12, resulting in a predicted amino acid change at position 101 (P in P2 and Q in P12). Mutations may have occurred in additional regions of these viruses, but they were not sequenced. Passage in HEp-2 did not alter partial (C-terminal) SH gene sequences of RSV 2-20 and 3-12 (data not shown). Thus, 10 passages (P2 to P12) had no effect on the G genes of 2-20 and 3-12 and altered one residue of the F genes for each virus.

We defined the genetic relatedness of 2-20 and 3-12 to each other and to other RSV isolates. Phylogenetic analysis of a hypervariable 270-nt region of the G gene divides subgroup A RSV isolates into clades (GA1 to GA7) that have remained relatively distinct clusters of circulating RSV according to several studies (1, 5, 6, 38, 50, 53, 54, 59). We performed multisequence alignments of the 270-nt C-terminal region of the G genes of the 2-20 3-12 strains and published C-terminal RSV G sequences (7, 15, 32, 36, 38, 45, 53, 54, 58, 64, 69, 72). Both RSV 2-20 and 3-12 are GA2 clade viruses. In this analysis, the virus most closely related to RSV 2-20 was RSV MON/1/94 (Uruguay, 1994) (15). The RSV most closely related to RSV 3-12 was RSV A2.20 (Germany, 2000 to 2001) (58). A comparison of the predicted full-length G proteins showed that RSV 2-20 and 3-12 G proteins differ in sequence by 16 amino acids. The predicted 2-20 and 3-12 F protein sequences differ by 6 amino acids. RSV 2-20 and 3-12 are distinct antigenic subgroup A, GA2 clade strains that were circulating in Nashville, TN, in 2001.

**In vitro replication of RSV 2-20 and 3-12 strains.** We compared the in vitro growth of RSV strains A2, 2-20, and 3-12. In HEp-2 cells commonly used for RSV propagation, RSV 3-12 grew to lower titers at 24 and 48 h p.i. than RSV A2 and 2-20 (Fig. 5A), as did the five other clinical isolates (see Materials and Methods). The yield of only RSV 2-20 was similar to that of RSV A2 (Fig. 5A). RSV primarily infects epithelial cells of the proximal airways. The human bronchial epithelial cell line BEAS-2B has been used to study host responses to RSV (4, 28, 62, 66). In BEAS-2B cells, RSV strains A2, 2-20, and 3-12 had equivalent growth kinetics (Fig. 5B).

**Viral load of RSV 2-20 and 3-12 in BALB/cJ mice.** BALB/cJ mice are semipermissive for RSV replication. It was previously shown that the nonmucogenic A2 RSV strain has a higher viral load than the mucogenic line 19 RSV strain in BALB/cJ mice at 4 and 6 days p.i. (34). We compared viral loads of RSV strains A2, line 19, 2-20, and 3-12. We infected BALB/cJ mice with 5 × 10^5 PFU of these RSV strains and performed lung viral load and time course experiments. The clinical isolate strains 2-20 and 3-12 had significantly higher viral loads than the A2 and line 19 strains at 1 day p.i. (Fig. 6). Like the line 19 strain, the 2-20 and 3-12 strains had lower viral loads than A2 at days 4 and 6 p.i. (Fig. 6). Although there was a trend that the 3-12 peak viral load was lower than that of 2-20 by approximately one-half log in replicate experiments, these differences did not reach statistical significance (Fig. 6). RSV 2-20 and 3-12 had high viral loads at 1 day p.i. and exhibited peak viral loads and clearances similar to those of the line 19 strain.
Localization of virus antigen. It has been shown that at the peak of viral load, RSV A2 strain antigen is detected exclusively in alveolar parenchyma of BALB/cJ mice (3). Using immunofluorescence, we analyzed the distribution of RSV antigen in lungs of BALB/cJ mice infected with RSV A2, 2-20, 3-12, and line 19. In agreement with published data, we found that RSV antigen was detected exclusively in alveolar regions at 4 days p.i., and this was the case with each RSV strain (data not shown). Because RSV 2-20 and 3-12 had higher viral loads than A2 and line 19 at 1 day p.i. (Fig. 6), we also performed an immunofluorescence assay for RSV antigens in lung tissues at 1 day p.i. At this time point, RSV antigen was detected in the bronchiolar epithelium (Fig. 6) and not in the alveolar regions (data not shown). Consistent with the viral load data, RSV strains 2-20 and 3-12 exhibited greater viral antigen than the A2 strain (Fig. 7). The RSV-positive cells are located in the bronchiolar epithelium as the airways are clearly evident in adjacent sections stained with H&E (Fig. 7). In H&E staining (1 day p.i.), the airways of 2-20- and 3-12-infected mice exhibited intraluminal necrotic cell debris, whereas the airways in mock- and A2-infected mice lacked this cell debris (Fig. 7).

Histologic features of RSV 2-20 infection. We analyzed lungs of BALB/cJ mice that were mock infected or infected with 5 × 10^{5} PFU of RSV A2, 2-20, or 3-12 over a time course. Lungs were harvested at 1, 2, 4, 6, and 8 days p.i. There were no differences in lung histologic features between mice infected with A2, 2-20, and 3-12 RSV strains on days 2, 4, 6, and 8 p.i. (data not shown). Mice infected with these RSV strains had indistinguishable inflammation, consisting of lymphocytes, neutrophils, and macrophages in perivascular and interstitial spaces on these days (data not shown). At 1 day p.i., however, mice infected with RSV 2-20 or 3-12 had greater perivascular edema than mice that were mock infected or infected with RSV A2 (Fig. 8, yellow bars). The perivascular edema scores of the two experiments combined were the following: for mock, 0.75 ± 0.25 (n = 4); A2, 2.1 ± 0.15 (n = 9); 2-20, 2.9 ± 0.11 (n = 9); and 3-12, 3.0 ± 0.0 (n = 9). Using ANOVA, the perivascular edema scores of 2-20- and 3-12-infected mice were significantly higher than those of A2- and mock-infected mice, and A2-infected mice had greater perivascular edema than mock-infected mice. BALB/cJ mice infected with RSV 2-20 or 3-12 also exhibited greater necrotic cellular debris in the airway epithelium at 1 day p.i. than mock-infected or A2-infected mice (Fig. 8, arrows). Increased bronchiolar luminal debris at 1 day p.i. in 2-20- and 3-12-infected mice (Fig. 8) correlates with increased viral load (Fig. 6) and antigen load in the bronchiolar epithelium (Fig. 7).

RSV strain 2-20 infection results in increased airway mucin expression. Overabundance of airway mucus is a key feature of severe RSV disease resulting in hospitalization. In contrast to the A2 and Long strains, the line 19 RSV strain induces airway mucin expression in BALB/cJ mice (41). We used periodic acid-Schiff (PAS) staining to measure mucin expression in the airway epithelium of BALB/cJ mice that were mock infected or infected with 10^{5} PFU of RSV strains A2, line 19, 2-20, or 3-12. We previously utilized a semiquantitative scoring system to measure PAS positivity in RSV-infected mice (41). Here, we used morphometric software (see Materials and Methods) to quantify PAS staining. RSV strains line 19 and 2-20 caused significantly increased airway mucin expression over mock-infected controls, whereas RSV strains A2 and 3-12 did not (Fig. 9B). There was no difference in the quantity of PAS staining between mice infected with line 19 and 2-20 (Fig. 9B). In these experiments, approximately 10% of the individual...
FIG. 7. RSV antigen in bronchiolar epithelium. BALB/cJ mice were infected with $5 \times 10^5$ PFU of A2, 2-20, or 3-12 (n = 5/group). Lungs harvested at day 1 p.i. were probed for RSV by immunofluorescence as described in Materials and Methods. The left column shows RSV-positive cells (fluorescein isothiocyanate [green] staining) in RSV A2-, 2-20-, and 3-12-infected mice and nuclei (DAPI blue counterstain). White arrowheads indicate FITC-positive cells. The right column (H&E) depicts lung sections adjacent to those in the immunofluorescence images. Black arrowheads indicate necrotic cell debris. Scale bar, 50 μm.
airways in line 19- and 2-20-infected mice had \( \geq 10\% \) PAS positivity (Fig. 9B). Thus, the majority of airways in line 19- and 2-20-infected mice were unaffected. Many of the PAS-positive airways in line 19- and 2-20-infected mice were smaller airways (Fig. 9A and C). Airways from infected IL-13 knockout mice were also analyzed. Infection of wild-type (wt) BALB/cJ mice with 2-20 induced significantly more mucus than 2-20 infection of IL-13 knockout mice (Fig. 10). Thus, RSV 2-20 infection resulted in significantly increased airway mucin expression in the small airways that was IL-13 dependent.

RSV 2-20 infection caused lung dysfunction in BALB/cJ mice. Airway dysfunction is the key feature of severe RSV bronchiolitis. A common radiologic finding in infants with severe RSV disease is pulmonary air trapping (49). A correlate of pulmonary obstruction and air trapping in humans is pulsatil paradoxus, an exaggeration of normal variation in the pulse volume with respiration that can be caused by labored breathing (22, 30, 57). We observed labored breathing in RSV 2-20-infected mice (data not shown). We used a rodent pulse oximeter to quantify pulsatil paradoxus as a measure of breathing effort in mock-infected and RSV-infected mice. The 2-20 strain of RSV caused increased breathing effort, whereas the A2 strain did not (Fig. 11A). As the 2-20 strain infection caused increased breathing effort, we also measured AHR in mice infected with 2-20 using methacholine challenge in mechanically ventilated mice. In these experiments, we used mock infection as the negative control and RSV line 19 infection as the positive control; the A2 strain has repeatedly been shown to not cause AHR in BALB/cJ mice (34, 51, 52, 65). We found that, similar to the line 19 RSV strain, the 2-20 RSV strain caused AHR in BALB/cJ mice (Fig. 11B). Taken together, the data show that RSV strain 2-20 caused significant airway dysfunction in BALB/cJ mice.

Lung CD8+ T cell responses to RSV strains A2, 2-20, and 3-12. CD8+ T cells play a role in RSV clearance as well as mediating illness by enhancing TH1 and inhibiting TH2 responses (29, 61). We used flow cytometry and intracellular cytokine staining to quantify IFN-\( \gamma \)-producing CD8+ T cells after infection with \( 10^5 \) PFU of RSV strains A2, 2-20, 3-12, and line 19. We found that A2 infection resulted in significantly more IFN-\( \gamma \)-producing CD8+ T cells in the lung than RSV strains 2-20 and line 19 at 8 days p.i. \( (P < 0.05) \) (Fig. 12). We also infected IL-13−/− mice with RSV 2-20 and used flow cytometry and intracellular cytokine staining to quantify IFN-\( \gamma \)-producing CD8+ T cells. There was no difference in number of IFN-\( \gamma \)-producing CD8+ T cells in the lung between wt and IL-13−/− mice (Fig. 13). Taken together, the IL-13- and mucus-inducing RSV strains 2-20 and line 19 had lower numbers of IFN-\( \gamma \)-expressing CD8+ T cells than

FIG. 8. Early histopathologic lesions of RSV 2-20 and 3-12 infection. BALB/cJ mice were mock infected (A) or infected with \( 5 \times 10^5 \) PFU of A2 (B), 2-20 (C), or 3-12 (D) (\( n = 4 \) to 5 per group). Lungs were harvested at 1 day p.i., stained with H&E, and analyzed for histologic changes as described in Materials and Methods. Representative airways are shown. Yellow bars indicate distance between vascular wall and adjacent tissue as a measure of perivascular edema. Arrows indicate intrabronchiolar exudates of necrotic cell debris. Scale bar, 50 \( \mu \)m.
nonmucogenic RSV strain A2, consistent with a hypothesis that Th1 and Th2 responses play a role in modulating RSV pathogenesis (Fig. 12).

DISCUSSION

The laboratory RSV strains A2 and Long are widely used in animal models of RSV pathogenesis. It was previously shown that, in contrast to A2 and Long, RSV strain line 19 induces airway mucus expression in BALB/cJ mice (34, 41). However, the passage histories of these RSV strains are not recorded. We have shown that the RSV clinical isolate strain 2-20 is more pathogenic in BALB/cJ mice than commonly used laboratory RSV strains and a closely related clinical isolate, 3-12. In contrast to RSV A2 and 3-12 infection in BALB/cJ mice, strain 2-20 caused lung dysfunction, as measured by increased breathing effort, airway resistance, and mucin expression. We cannot rule out the possibility that differences in pathogenesis phenotypes between RSV 2-20 and 3-12 strains are due to mutations in these viruses that arose during their low number of passages in vitro. Nevertheless, our findings support the hypothesis that RSV strain differences are a determinant of

FIG. 9. RSV A2001/2-20 induced high levels of pulmonary mucin expression. BALB/cJ mice were mock infected (n = 3 per group) or infected with 10^5 PFU of A2 (n = 4), line 19 (n = 4), 2-20 (n = 5), or 3-12 (n = 5). Lungs were harvested at 8 days p.i. and processed for PAS staining. The lung tissues were digitized, and each area of airway epithelium was annotated by hand as described in Materials and Methods. (A) Example of airway epithelium (indicated by a thin red line) in 2-20-infected mouse. The software detects PAS positivity based on color (thin red lines outlining PAS stain). (B) The percentage of area that was PAS positive for each airway was determined; >200 individual airways are shown per group. Each round symbol represents one airway. Capped lines indicate significant differences between groups (P < 0.05, ANOVA). (C) Same percent PAS data as in panel B plotted against the size of all airways. The airway depicted in panel A is indicated by an arrow.
disease phenotypes and severity. Future studies with strain-chimeric RSVs may shed light on mechanisms of RSV 2-20-induced phenotypes (41).

It is well established that minor genetic differences in viral genomes can have a large impact on pathogenesis. For example, the molecular basis for the highly pathogenic H5N1 influenza virus was found to be a single amino acid substitution (25). An attenuated A2 RSV strain (a cold-passaged mutant, cp-RSV) was found to differ from its parental strain (HEK-7) by 5 amino acids (10, 11, 73). Circulating RSV strains exhibit considerable genomic variation, especially in the viral glycoproteins (9). RSV has one serotype, within which there are two antigenic subgroups, A and B (9). RSV subgroup A is associated with greater illness than RSV subgroup B (19, 20). Within antigenic subgroups, RSV strains can be further classified into clades based on the sequence of a hypervariable region of the G gene (6, 53, 54). Generally, annual RSV epidemics consist of a dominant clade that is replaced the following season (6, 53, 54, 63). RSV clade differences have been associated with disease severity (16, 37). We found that two antigenic subgroup A strains within the same clade (GA2) exhibited differential virulence in a mouse model, suggesting that minor sequence variations affect pathogenicity.

Both RSV clinical isolates 2-20 and 3-12 had higher viral loads and higher antigen load in the airway epithelium and caused more perivascular edema and damage to the airway epithelium at 1 day p.i. than laboratory RSV strains. One possibility for increased clinical isolate viral load at 1 day p.i. is that RSV clinical isolates have greater \textit{in vivo} stability than RSV A2. However, RSV 2-20 and 3-12 do not exhibit greater \textit{in vitro} stability than A2 (data not shown). We speculate that RSV clinical isolates enter mouse airway epithelial cells more efficiently than laboratory RSV strains that have been adapted to cell culture lines. Future studies will be required to determine if this is the case. For example, differences in the F protein may lead to enhanced entry and fusion, resulting in greater epithelial damage. Bronchiolar cytopathology is an important feature of RSV bronchiolitis and is thought to contribute to airway obstruction (2). Airway epithelial damage in calves infected with bovine RSV (BRSV) contributes significantly to pathogenesis (70, 75). Many groups have focused on the later, adaptive immune response to RSV. It is likely that both early virus-induced epithelial damage and the ensuing cascade of innate and adaptive immune responses contribute to lung dysfunction. RSV clinical isolates described in this study provide a mouse model for investigating consequences of early airway histopathology.

Although both RSV strains 2-20 and 3-12 caused early epithelial cytopathology in mice and had similar viral loads over the time course, different cytokine responses that can be characterized as Th1 or Th2 were observed. RSV 2-20 strain infection resulted in dramatically higher lung IL-13 and mucin expression than 3-12 infection. RSV 2-20 infection also resulted in lower numbers of IFN-\(\gamma\)-expressing CD8\(^+\) T cells in the lung than infection with A2 and 3-12 at 8 days p.i. The Th1 and Th2 responses to RSV are important because Th12-type responses are associated with RSV immunopathology (44). Mixed Th1/Th2 responses are observed in RSV-infected children, with some groups reporting a skew toward Th12 inflammation in severe RSV disease and other groups reporting a lack of association between Th12 cytokine levels and RSV dis-
shown to downregulate TH1-type cytokines (31). Conversely, and mucus production are currently unknown. IL-13 has been involved in a TH2-type response. RSV A2 infection of mice, IL-4 suppresses IFN-γ expression, promotes RSV-specific Ab production, and contributes to pulmonary mononuclear cell inflammation, but IL-4 deficiency has no effect on viral load, viral clearance, or disease severity (14, 43). In primary A2 strain infection, IL-13 was shown to play a protective role in limiting viral load and disease severity (76). Additionally, mucin expression during 2-20 infection was shown to be IL-13 dependent (Fig. 10). In line 19 infection, mucus and AHR were also shown to be IL-13 dependent (35). T42 responses to RSV infection may be a double-edged sword in promoting viral clearance and Ab responses while contributing to pulmonary obstruction.

The mechanisms by which RSV strain 2-20 induces IL-13 and mucus production are currently unknown. IL-13 has been shown to downregulate T41-type cytokines (31). Conversely, IFN-γ antagonizes IL-13 and IL-4 production and inhibits mucus production (8, 13). In allergic inflammation, IFN-γ inhibits T42 responses by activating the epithelium (8). In our model, however, 2-20-infected IL-13−/− mice did not show a decrease in IFN-γ. Therefore, the decrease in IFN-γ-producing CD8+ T cells in 2-20-infected mice is not due to IL-13 production. In addition to T cells, other immune cells have been shown to be involved in a T42-type response. RSV A2 infection of BALB/c and STAT1-deficient mice results in basophil IL-4 expression (43). Recently, RSV was shown to induce alternatively activated macrophages to produce IL-13 and IL-4 (60). Additional studies will be required to determine the contribution of innate immune cells to RSV 2-20 pathogenesis.

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