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

Running Title: RSV Strain 2-20 Pathogenesis

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Abstract word count: 230

Manuscript word count: 5658
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 1 day post-infection (p.i.). This increased viral load correlated with higher viral antigen levels in the bronchiolar epithelium and greater histopathologic changes 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.
INTRODUCTION

Respiratory syncytial virus (RSV) is the most important cause of bronchiolitis and viral pneumonia in children. Each year in the USA, RSV causes lower respiratory tract illness (LRI) in 20-30% of infants and leads to the hospitalization of approximately 1% of infants at a cost of $300-400 million (20, 22, 28). The incidence and disease severity of RSV can vary from year to year (49). Dominant circulating RSV strains are generally replaced each year, likely by a process involving immune selection (5, 6, 55, 56). 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 (35, 43). We are interested in RSV-induced mucin expression in mice because mucus overabundance contributes to airway obstruction in severe RSV disease in children (2, 34, 46, 58). IL-13 is a cytokine linked to mucus production (73). The line 19 RSV strain induces lung IL-13 and airway mucin expression in BALB/cJ mice, whereas the A2 and Long RSV strains do not (35, 43). 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 a related isolate RSV A2001/3-12 (3-12). As RSV 2-20 and 3-12 are genotypically similar and 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 (19, 43). First-passage (P1) HEp-2 supernatants of RSV antigenic subgroup A clinical isolates were obtained from the Vanderbilt Vaccine Clinic (Nashville, TN) (76). Ten-fold serial dilutions of the P1 supernatants were used to infect HEp-2 cells in 6-well plates. For each isolate, supernatant from the most dilute inoculum exhibiting >50% CPE was harvested for sequential passage in HEp-2 cells. The isolates were passaged by limiting dilution 9 times in HEp-2 cells. HEp-2 cells in flasks were infected with the supernatants from the 9th limiting dilution passage (P10 overall). Master stocks (P11) and working stocks (P12) were generated as described (19). 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 inches from a 40-watt UV light on ice for 4 hours. 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 (Hyclone, ThermoFisher),
and a penicillin G/streptomycin sulfate/amphotericin B solution (Invitrogen). BEAS-2B human bronchial epithelial cells were a gift from Dr. Pierre Massion (Vanderbilt University, Nashville, TN) and cultured in Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum and penicillin G/streptomycin sulfate/amphotericin B solution. Female, 6 to 8 wk old BALB/cJ mice were obtained from Jackson Laboratories. IL13−/− mice were obtained from Dr. Gurjit K. Hershey at Cincinnati Children’s Hospital Medical Center (48). All mice were maintained under specific pathogen-free conditions. Six to eight week old female mice were lightly anaesthetized and infected intranasally (i.n.) with RSV or with mock-infected cell culture supernatant as described (19).

**Sequencing 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 passaged 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 F genes, cDNA was reverse transcribed using primer F-r, and F genes were PCR-amplified using primers FStuI and FSphI, as described (43). In order to sequence the G genes, cDNA was reverse transcribed using primer G-r

(CCATTGTTATTGCCCCAGA). PCR was performed using primer SHfEcoRI (AGGAATTCGGAAGCACACAGCTACACG) and primer GrStuI (GGTCAAGGCCTTTTGTGATAATATG), and generated approximately 1.6 kb amplicons containing the SH and the G genes. The EcoRI and StuI 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 AlignX software (neighbor-joining method) within VectorNTI.

**Multi-step virus growth curves.** Subconfluent HEp-2 or BEAS-2B cells in 6-well dishes were infected in triplicate with RSV strains A2, 2-20, or 3-12 at an MOI of 0.5 in 750 µl. After 1 hr adsorption at room temperature on a rocking platform, the cells were washed with medium, and fresh medium was added. Supernatants were harvested 24 hr, 48 hr, 72 hr, and 96 hr from each well, clarified by centrifugation, and RSV was titrated in duplicate by plaque assay on HEp-2 cells as described (19).

**Quantification of lung viral load.** Lungs were harvested from BALB/cJ mice infected with 10^5 PFU of RSV. We used a Beadbeater (Biospec Products, Bartlesville, OK) to homogenize the lungs. 2-ml deep 96-well plates (Axygen Scientific, Union City, CA) were loaded with 1 ml of zirconium beads (Biospec Products), sealed with rubber lid (Axiomat, Axygen Scientific), and autoclaved. Sterile MEM (500 µl) was added to each well, and the plate was briefly centrifuged then kept in deep ice. For each mouse, the left lung lobe was harvested, weighed, cut into 4-5 pieces, and placed into a well of the 2-ml deep 96-well plate on ice. An additional 400 µl MEM was added to each well, and the plate was sealed with the Axiomat lid. The plate was shaken in the Beadbeater for one min followed by one minute plunge in ice/water slurry. This was repeated 9 times for a total of 10-one minute homogenizations. The plate was centrifuged at 2000 RPM for 5 min at 4° C. Lung homogenates were immediately serially diluted and used to inoculate subconfluent HEp-2 cells in 24-well dishes. After 1 hr adsorption at room temperature on a rocking platform, the cells were overlayed with MEM/10% FBS/penicillin G/streptomycin sulfate/amphotericin B solution/0.75% methylcellulose. After five days, the
overlay media was removed and the cells fixed with methanol. Plaques were visualized by immunodetection as described (41).

**IL-13 ELISA and gob-5 Western blot.** Lungs were removed and snap-frozen in liquid nitrogen. IL-13 and gob-5 Western blots were performed as described (43). Briefly, lungs were homogenized in 1 ml of modified radioimmunoprecipitation assay buffer [120 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% TritonX-100, 0.1% SDS, 1% deoxycholate, 10 mM DTT, 1mM EDTA, 0.2mM PMSF, 1% protease inhibitor cocktail (P8340, Sigma)]. Homogenates were clarified by centrifugation at 2000 x g. IL-13 was quantified by enzyme-linked immunosorbent assay (ELISA) (Quantikine, R&D Systems, Minneapolis, MN). Gob-5 and actin were detected by Western blotting (43).

**Histopathology.** Heart and lung samples were fixed in 10% formalin overnight. Lungs were transferred to 70% ethanol then embedded in paraffin blocks. Tissue sections (5 µm) were stained with hematoxylin and eosin stains (H&E) to assess histologic changes. A pathologist blinded to the groups scored lymphocytes, neutrophils, macrophages, and eosinophils on a 0-4 scale for peribronchiolar, perivascular, interstitial, and alveolar spaces. Perivascular edema was scored on a 0-4 scale such that if a vessel had edema equal to its thickness, it was given a score of 1. If a vessel had edema twice as thick as the vessel, the score was 2, etc. All vessels were analyzed, and lungs were assigned the score of the vessel with the most edema. Slides were also scored for the presence or absence of bronchiolar exudates containing necrotic cell debris. Additional sections were stained with periodic acid-Schiff (PAS) stain to assess goblet cell hyperplasia as a measure of mucin expression. PAS-stained slides were digitally scanned using a MIRAX MIDI microscope with a 20X objective having a 0.85 numerical aperture (Carl Zeiss Microimaging Inc., Thornwood, NY) (45). Areas of airway epithelium were annotated using
Histoquant software (3D Histech, Budapest, Hungary). All airways involved in the tissue sections were analyzed. PAS-positive areas within the airway epithelium were identified by Histoquant software.

**Immunofluorescence in lung tissue.** Three μm thick sections of paraffin-embedded lung tissues were incubated at 37 °C overnight as described (57). The sections were deparaffinized in Clear Rite-3 (ThermoFisher) then rehydrated in a series of graded alcohols. Slides were placed in a plastic coplin jar with Antigen Unmasking Solution (Vector Labs, Burlingame, CA), and the jar was placed in a pressure chamber (Pascal, Dako USA, Carpinteria, CA). The slides were heated at 125 °C for 30 sec, passively cooled to 90 °C, heated at 90 °C 10 seconds, then removed from the pressure chamber and cooled 20 minutes on the bench. Slides were then washed in PBS, treated with PBS/0.2% Tween-20 for 30 min, washed three times with PBS, and blocked 1 hr with PBS/10% normal rabbit serum. Slides were washed three times with PBS then treated for 15 min with an avidin-blocking reagent and 15 min with a biotin-blocking reagent (Avidin/Biotin Blocking Kit, Vector Labs). The tissues were probed one hour at room temperature in a humidified chamber with a goat polyclonal Ab to RSV (AB1128, Millipore) diluted 1:400 in PBS/5% rabbit serum. After washing three times with PBS, the slides were treated 30 min with 5 μg/ml biotinylated anti-goat IgG in PBS/5% rabbit serum. The slides were washed three times in PBS then 20 μg/ml fluorescein-avidin (Vector Labs) in PBS was applied for 10 min. The tissues were washed three times in PBS and mounted with Prolong Gold antifade with DAPI according to the manufacturer’s instructions (Invitrogen). The slides were digitally scanned using a MIRAX MIDI fluorescence microscope (Carl Zeiss Microimaging).

**Flow cytometric analysis of lung mononuclear cells.** Mice were euthanized intraperitoneally (i.p.) with sodium pentobarbital (8.5 mg/kg body weight) and lungs were
harvested 8 d p.i. Cells were isolated using ficoll gradients and counted with a hemocytometer. Cells were stimulated for 6 hours with PMA/ionomycin and stained with the following antibodies: anti-CD8, anti-CD3, and anti-IFN-γ. 1x10^6 cells were analyzed using a LSR II flow cytometer (BD Biosciences). The total numbers of IFN-γ-expressing CD8^+ T cells in lungs were determined by multiplying the percentage of lymphocytes (defined by forward and side scatter properties in flow cytometry) that were CD3^-CD8^+ IFN-γ^+ by the total number of mononuclear cells isolated. Data were analyzed using FlowJo software (Tree Star).

**Methacholine Challenge.** We measured airway hyperresponsiveness (AHR) 9 days p.i. (35, 43). The mice to be tested were anesthetized with i.p. pentobarbital. The trachea was cannulated with a 20 gauge metal stub adapter. The animal was placed on a small animal ventilator, flexiVent (SCIREQ, Montreal, Canada), with 150 breaths/min and a tidal volume of 10 ml/kg body weight. Airway responsiveness was assessed by administering incremental concentrations of aerosolized methacholine (0, 30, 60, 100 mg/ml in saline) via an in-line ultranebulizer (Aeroneb, SCIREQ, Montreal, Canada). The SCIREQ software calculates the resistance by dividing the change in pressure by the change in flow (units = cm H_2O/ml/sec).

**Pulse Oximetry.** For breath distension measurements, mice were anesthetized using an isoflurane (2.5%)/oxygen (2L/min) mixture provided by an anesthesia machine (VetEquip, Pleasanton, CA). Mice were anesthetized at a time in a plexiglass box connected to the anesthesia machine. One mouse at a time was removed from the plexiglass box and placed on its back on a pad with its nose in an isoflurane/oxygen nosecone connected to the anesthesia machine. The anesthetized mouse’s feet were restrained to a pad with tape. A rodent pulse oximeter (MouseOx, Starr Life Sciences Corp., Oakmont, PA) was used. The MouseOx sensor was applied to the mouse’s thigh, and the mouse was covered with a cloth to reduce ambient
light. The machine measured arterial $O_2$ saturation, heart rate, pulse rate, pulse distension, and breath distension approximately every 0.1 sec (MouseOx Software Version 4.0). Each mouse was analyzed for 1 to 5 minutes. Only time points in which all of these physiological parameters were successfully measured were included in analyses, approximately 30 sec 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 then averaging these values per group (8 to 10 mice).

**Statistical analyses.** Unless otherwise indicated, groups were compared by one-way ANOVA and Tukey multiple comparison tests. $P < 0.05$ was considered significant. Data values below 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 the accession numbers JF279544 and JF279545. The sequence data for 3-12 F and G was submitted under the accession numbers JF279546 and JF279543.

**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 one week p.i. (19, 44, 53). We compared weight loss in BALB/cJ mice that were mock-infected or infected with RSV laboratory strains A2, line 19, Long, or RSV clinical isolates 2-20, 3-12, 12-35, 3-4, 3-2, or 12-21. The clinical isolates 2-20 and 3-12 caused significant weight loss 2 d p.i. (Fig. 1A and 1B). 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 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 10^7 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 (early weight loss), 2-20 (early and late bimodal weight loss), and 12-35 (late weight loss). Across multiple experiments, there was no consistent difference in the degree of early weight loss at d 2 p.i. caused by 2-20 and 3-12 (compare Fig. 1A to Fig. 1B). Infection with UV-inactivated 2-20 does 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 (35, 43, 67). We quantified IL-13 levels in BALB/cJ mice that were mock-infected or infected with RSV strains A2, line 19, Long, 2-20, 3-12, 12-35, 3-2, 12-21, or 3-4. RSV line 19, 2-20, 12-35, and 3-4 infections resulted in significantly elevated lung IL-13 expression (Fig. 3). RSV 2-20 and 12-35 infections resulted in higher levels of lung IL-13 than RSV line 19 infection (Fig. 3). Gob-5 protein levels are a marker of pulmonary mucus expression. Gob-5 has been identified as a key molecule in the induction of murine allergic airway inflammation and is selectively expressed in the setting of AHR (50). RSV strain line 19 infection increases lung gob-5 levels (24, 25, 35, 43). We determined gob-5 levels at 8 d p.i. in lung homogenates of BALB/cJ mice mock-infected or infected with 10^5 PFU of A2, line 19, Long, 2-20, 3-12, 12-35, 3-2, 12-21, or 3-4 RSV strains.
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 d 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 2000-2001 RSV season, 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 sequence, 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 one 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, G in P20). There was one 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, 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-GA7) that have remained distinct clusters of circulating RSV in several studies (1, 5, 6, 40, 52, 55, 56, 61). We performed multisequence alignments of the 270 nt C-terminal region of the G genes of 2-20, 3-12, and published C-terminal RSV G
sequences (7, 15, 33, 38, 40, 47, 55, 56, 60, 66, 71, 74). 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, 2000-2001) (60). Comparing the predicted full-length G proteins, RSV 2-20 and 3-12 G differ in sequence by 16 amino acids. The predicted 2-20 and 3-12 F protein sequences differ by six amino acids. RSV 2-20 and 3-12 are distinct antigenic subgroup A, GA2 clade strains that were circulating in Nashville, Tennessee 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 hr p.i. in HEp-2 cells than RSV A2 and 2-20 (Fig. 5A), as did the five other clinical isolates (see materials and methods). Only the yield of RSV 2-20 was similar to 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, 29, 64, 68). 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 semi-permissive for RSV replication. It was previously shown that the non-mucogenic A2 RSV strain has a higher viral load than the mucogenic line 19 RSV strain in BALB/cJ mice at 4 and 6 d p.i. (35). We compared viral loads of RSV strains A2, line 19, 2-20, and 3-12. We infected BALB/cJ mice with $5 \times 10^5$ PFU of these RSV strains and performed lung viral load, 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 d 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 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 1 d p.i. and exhibited peak viral loads and clearance similar to 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 d 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 one d p.i. (Fig. 6), we also performed immunofluorescence for RSV antigens in lung tissues 1 d 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 these H&E stains (1 d p.i.), the airways shown in 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 $5 \times 10^5$ PFU of RSV A2, 2-20, or 3-12 over a time course. Lungs were harvested days 1, 2, 4, 6, and 8 d 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 d 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. 7, yellow bars). The perivascular edema scores of two experiments combined were the following: 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 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 d p.i. than mock-infected or A2-infected mice (Fig. 8, arrows). Increased bronchiolar luminal debris 1 d 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 (43). 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 semi-quantitative scoring system to measure PAS positivity in RSV-infected mice (43). 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 airways in line 19- and 2-20-infected mice...
had ≥ 10% PAS positivity (Fig. 9B). Thus, the majority of airways in line 19- and 2-20-infected mice were unaffected. Many of the PAS+ airways in line 19- and 2-20-infected mice were smaller airways (Fig. 9A and 9C). Airways from infected IL13−/− mice were also analyzed. 2-20 infection of wildtype BALB/cJ mice induced significantly more mucus than 2-20 infection of IL-13 knockout mice (Fig. 10). Thus, RSV line 19 and 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 (51). A correlate of pulmonary obstruction and air trapping in humans is pulsus paradoxus, an exaggeration of normal variation in the pulse volume with respiration that can be caused by labored breathing (23, 31, 59). We observed labored breathing in RSV 2-20-infected mice (data not shown). We used a rodent pulse oximeter to quantify pulsus 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 and 3-12 RSV strains 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 (35, 53, 54, 67). 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 (30, 63). We used flow cytometry and intracellular staining to quantify IFN-γ-producing CD8+ T cells after infection with 10⁵ PFU of RSV strains A2, 2-20, 3-12, and line 19. We found that A2 infection resulted in significantly more lung IFN-γ-producing CD8+ T cells in the lung than RSV strains 2-20 and line 19 at 8 d p.i. (P < 0.05) (Fig. 12). We also infected IL13−/− mice with RSV 2-20 and used flow cytometry and intracellular staining to quantify IFN-γ-producing CD8+ T cells. There was no difference in number of IFN-γ-producing CD8+ T cells in the lung between wt and IL13−/− mice (Fig. 13). Taken together, the IL-13 and mucus-inducing RSV strains 2-20 and line 19 had lower numbers of IFN-γ-expressing CD8+ T cells than nonmucogenic RSV strains 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 (35, 43). 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 disease phenotypes and severity. Future studies with strain-chimeric RSVs may shed light on mechanisms of RSV 2-20-induced phenotypes (43).
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 (26). An attenuated A2 RSV strain (cp-RSV) was found to differ from its parental strain (HEK-7) by five amino acids (10, 11, 75). 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 (20, 21). Within antigenic subgroups, RSV strains can be further classified into clades based on sequence of a hypervariable region of the G gene (6, 55, 56). Generally, annual RSV epidemics consist of a dominant clade that is replaced the following season (6, 55, 56, 65). RSV clade differences have been associated with disease severity (16, 39). 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, higher antigen load in the airway epithelium, and caused more perivascular edema and damage to the airway epithelium at 1 d p.i. than laboratory RSV strains. One possibility for increased clinical isolate viral load at 1 d p.i. is that RSV clinical isolates have greater in vivo stability than RSV A2. However, RSV 2-20 and 3-12 do not exhibit greater 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 (72, 77).

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 compared to 3-12 infection. RSV 2-20 infection also resulted in lower numbers of IFN-γ-expressing CD8+ T cells in the lung compared to A2 and 3-12 at 8 d p.i. The Th1 and Th2 responses to RSV are important because Th2-type responses are associated with RSV immunopathology (46). Mixed Th1/Th2 responses are observed in RSV-infected children, with some groups reporting a skew towards Th2 inflammation in severe RSV disease and other groups reporting a lack of association between Th2 cytokine levels and RSV disease (12, 42, 69). If RSV strain differences contribute to differential immune responses, this may confound comparison of these reports because different RSV strains may have been circulating in the studies. The role of Th2-type responses in primary RSV infection is not completely defined. It is established in RSV vaccine-enhanced disease that Th2 cells mediate immunopathology (70). In primary A2 strain RSV infection in 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, 45). In primary A2 strain infection, IL-13 was shown to play a protective role in limiting viral load and disease severity (78). Additionally, mucin expression during 2-20
infection was shown to be IL-13-dependent (Fig. 10). In line19 infection, mucus and AHR were also shown to be IL-13-dependent (36). TH2 responses to RSV infection may be a double-edged sword in promoting viral clearance and Ab responses but 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 TH1 type cytokines (32). Conversely, IFN-γ antagonizes IL-13 and IL-4 production and inhibits mucus production (8, 13). In allergic inflammation, IFN-γ inhibits TH2 responses by activating the epithelium (8). In our model, however, 2-20-infected IL13−/− 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 TH2-type response. RSV A2 infection of BALB/cJ and STAT1-deficient mice results in basophil IL-4 expression (45).

Recently, RSV was shown to induce alternatively activated macrophages to produce IL-13 and IL-4 (62). Additional studies will be required to determine the contribution of innate immune cells to RSV 2-20 pathogenesis.

RSV 3-12-infected mice had approximately as many IFN-γ-expressing T cells in the lung as RSV A2-infected mice (Fig. 12). This was surprising because CD8+ T cells contribute to weight loss in A2-infected mice (17), but RSV 3-12 did not cause weight loss (Fig. 1) (18).

There were no differences between total CD8+ T cells in the lung and total RSV-specific (M282-90 tetramer) CD8+ T cells in the lung between A2- and 3-12-infected mice (data not shown).

Comparing 2-20 to 3-12 pathogenesis, mice infected with 3-12 had equivalent numbers (or slightly more in some experiments) of IFN-γ-expressing T cells to 2-20-infected mice (Fig. 12). As 2-20 was more pathogenic than 3-12 as measured by weight loss, airway mucus, and lung dysfunction, T cells may play a protective role in RSV clinical isolate strain pathogenesis.
Although T cells clearly cause immunopathology in RSV A2-infected mice, the role of T cells in human RSV disease is not known. Peak levels of activated T cells correlate with recovery from RSV disease, whereas neutrophil influx to the lung correlates with immunopathology (27, 37). Investigating the pathogenesis of RSV clinical isolates in mice may enable further insights into the role of neutrophils and T cells in RSV disease.
ACKNOWLEDGMENTS

We thank Pierre Massion for the BEAS-2B cells. This work was supported by NIH HL090664 (Peebles), NIH AI070672 (Peebles), 1I01BX000624 (Peebles), NIH GM07569 (Morrow), NIH AI077507 (Moore), and AI087798 (Moore).
FIGURE LEGENDS

FIG. 1. Differential weight loss patterns with RSV clinical isolates. (A) BALB/cJ mice were mock-infected (n=4) or infected with 5 × 10^5 PFU of A2, 2-20, or 3-12 (n=8/group). (B) BALB/cJ mice were mock-infected (n=6) or infected with 10^5 PFU of A2 (n=8), 2-20 (n=10), 12-35 (n=10), 3-12 (n=10), line 19 (n=8), or Long (n=6). Weight loss ± SEM is shown. *, at day 2, RSV 2-20 and 3-12 were significantly lower (P < 0.05, ANOVA) than other RSV strains. †, at day 6, RSV 2-20 and 12-35 were significantly lower (P < 0.05, ANOVA) than other RSV strains.

FIG. 2. Weight loss after UV-inactivation of RSV 2-20. BALB/cJ mice were mock-infected (n=8), infected with 1.2 x 10^6 PFU of 2-20 (n=8), or infected with UV-inactivated 2-20 (n=8). Weight loss ± SEM is shown.

FIG. 3. BALB/cJ mice were mock-infected (n=3) or infected with 10^5 PFU of the indicated RSV strain (n=4/group). IL-13 protein levels were quantified by ELISA in left lung lobe homogenates. The dashed line represents the limit of detection. *, P < 0.05 compared to mock. †, significantly higher (P < 0.05, ANOVA) than line 19.

FIG. 4. Gob-5 Western blotting. BALB/cJ mice were mock-infected with 10^5 PFU of RSV strains A2, line 19, Long, or clinical isolate strains (n=5/group). Lungs were harvested 8 d p.i. Each lane contains 100 µg total lung homogenate protein pooled from five mice. The membrane was probed with anti-gob-5 then stripped and re-probed with anti-actin as a loading control.
FIG. 5. In vitro growth of RSV strains A2, 2-20, and 3-12. Infectious yield in supernatants of (A) HEp-2 cells and (B) BEAS-2B cells infected at MOI 0.5 with RSV A2, 2-20, or 3-12. Error bars represent SEM of three separate infections. *, titers from cells infected with RSV 3-12 are significantly less than titers from cells infected with A2 or 2-20 (P < 0.05 ANOVA).

FIG. 6. Viral load of RSV strains A2, 2-20, and 3-12. BALB/cJ mice were infected with 5 × 10^5 PFU of A2, 2-20, or 3-12 (n=5/group). Lungs were harvested at the indicated days p.i. and infectious RSV was titrated by an immunodetection plaque assay. Data are means ± SEM. *, at day 1 p.i. 2-20 and 3-12 were significantly higher than A2 and line 19 (P < 0.05, ANOVA). **, at day 4 p.i. A2 was significantly higher than 2-20, 3-12, and line 19 (P < 0.05, ANOVA). †, at day 6, A2 was significantly higher than 2-20, 3-12, and line 19 (P < 0.05, ANOVA). The dotted line represents the limit of detection.

FIG. 7. RSV antigen in bronchiolar epithelium. BALB/cJ mice were infected with 5 × 10^5 PFU of A2, 2-20, or 3-12 (n=5/group). Lungs harvested day 1 p.i. were probed for RSV by immunofluorescence as described in the Materials and Methods. The left column shows RSV-positive cells (FITC green) 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 left column, showing airways corresponding to those in the immunofluorescence images. Black arrowheads indicate necrotic cell debris. Scale bar in upper left corners represents 50 µm.
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=4-5/group. Lungs harvested 1 d.p.i., stained with H&E, and analyzed for histologic changes as described in the 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 in upper left represents 50 µm.

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 8 d.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 the Materials and Methods. (A) Example of airway epithelium (annotated by 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 shown per group. Each round symbol represents one airway. Capped lines indicate significant differences between groups, $P < 0.05$ (ANOVA). (C) Same % PAS data as in (B) plotted with size of all airways. Airway depicted in (A) is indicated by an arrow.

FIG. 10. RSV 2-20 induced mucin expression is IL-13-dependent. BALB/cJ mice or IL-13 knockout mice were mock infected (n = per group) or infected with $10^5$ PFU of 2-20. Lungs were harvested 8 d.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 the Materials and Methods. % PAS
positivity is plotted per group. Capped lines indicated significant differences between groups, \( P < 0.001 \) (ANOVA).

FIG. 11. Lung dysfunction caused by RSV 2-20. (A) BALB/cJ mice were mock-infected (n=4) or infected with \( 10^5 \) PFU of A2, 2-20, or 3-12 RSV. Breath distension of peripheral arteries, a measure of pulsus paradoxus and pulmonary obstruction, was quantified at the indicated days non-invasively by pulse oximetry as described in the Materials and Methods. (B) BALB/cJ mice were mock-infected or infected with \( 10^5 \) PFU of 2-20 or line 19 (n=10/group). AHR was measured 9 d p.i.. Means and SEM are shown. * \( P < 0.05 \) comparing 2-20 or line 19 to mock at 100 mg/ml methacholine dose. * \( P < 0.05 \) comparing 2-20 to mock, A2, and 3-12 groups.

FIG. 12. RSV A2 and 3-12 induced higher levels of IFN-\( \gamma \)-producing CD8\(^+\) T cells than 2-20 and line 19. BALB/cJ mice (n = 5 per group) were mock-infected or infected with \( 10^5 \) PFU of A2, line 19, 2-20, or 3-12. Lungs were harvested 8 d p.i. and cells were isolated using a Ficoll gradient. 1x10\(^6\) cells were stained with fluorescently-conjugated anti-CD3, anti-CD8, and anti-IFN-\( \gamma \) antibodies and then analyzed by flow cytometry. Total cells were calculated as described in the Materials and Methods. Each symbol represents one mouse. Means and SEM are shown for each group. Brackets indicate significant differences between groups (\( P < 0.05 \), ANOVA)

FIG. 13. Lower IFN-\( \gamma \)-producing CD8\(^+\)T cell levels in 2-20 infected mice are not due to IL-13 production. IL-13 KO mice (n=4 for mock, n=5 per group for RSV infection) or wt BALB/cJ mice (n=2 per group) were mock-infected or infected with \( 10^5 \) PFU of 2-20. Lungs were harvested d 8 p.i. and cells were isolated using a ficoll gradient. Cells were stained with fluorescently-conjugated anti-CD3, anti-CD8, and anti IFN-\( \gamma \) antibodies and then analyzed by...
flow cytometry. Total cells were calculated as described in the Materials and Methods. Means and SEM are shown for each group.


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A  HEp-2 0.5MOI

BAS2-B 0.5MOI

Hours Post-Infection

PFU/ml

A2  2-20  3-12