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Human Respiratory Syncytial Virus Memphis 37 Causes Acute Respiratory Disease in Perinatal Lamb Lung

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Abstract

Respiratory syncytial virus (RSV) is the leading cause of hospitalization due to respiratory illness among infants and young children of industrialized countries. There is a lack of understanding of the severe disease mechanisms as well as limited treatment options, none of which are fully satisfactory. This is partly due to lack of a relevant animal model of perinatal RSV infection that mimics moderate to severe disease in infants. We and others have shown mild disease in perinatal lambs with either a bovine or a human A2 strain of RSV. The Memphis 37 clinical strain of human RSV has been used to produce mild to moderate upper respiratory disease in healthy adult volunteers. We hypothesized that the Memphis 37 strain of RSV would infect perinatal lambs and produce clinical disease similar to that in human infants. Perinatal (3- to 5-day-old) lambs were inoculated intranasally with 2 mL/nostril of 1×10^5 focus-forming units (FFU)/mL ($n = 2$) or 2.1×10^8 FFU/mL ($n = 3$) of RSV Memphis 37. Clinical signs, gross and histological lesions, and immune and inflammatory responses were assessed. Memphis 37 caused moderate to severe gross and histologic lesions along with increased mRNA expression of macrophage inflammatory protein. Clinically, four of the five infected lambs had a mild to severe increase in expiratory effort. Intranasally administered RSV strain Memphis 37 infects neonatal lambs with gross, histologic, and immune responses similar to those observed in human infants.

Key words: respiratory syncytial virus; lung; neonate; innate immunity

Introduction

Respiratory syncytial virus (RSV) is an enveloped RNA virus in the Paramyxoviridae family that causes variable levels of disease severity in all age groups from infants to the aged. Most commonly, RSV causes a mild upper or lower respiratory disease with cold-like symptoms, but in a small percentage of patients, particularly the very young, severe disease can occur. Nearly every child in the United States has been infected with RSV at least once by age 2 years.[1] Because of its ubiquity, even the low percentage of individuals that develop severe disease yields a significant number of hospital cases. In the United States, 85,000–144,000 infants with RSV infection are hospitalized annually.[2] This constitutes 20%–25% of the pneumonia cases and up to 70% of all bronchiolitis cases in the hospital.[3,4]

Current treatment of RSV infection is limited to supportive care and one of two therapies approved by the U.S. Food and Drug Administration that include either an inhaled nucleoside analog (Ribavirin) with limited treatment efficacy or a prophylactic monoclonal antibody (palivizumab), which is limited to use in high-risk infants.[5] Two major stumbling blocks in the development of preventative and treatment regimens have been the disastrous initial vaccine clinical trial[6] and the lack of an available, clinically relevant model of RSV infection. Rodents are a valuable model of RSV infection but undergo postnatal alveolar development as opposed to the prenatal alveologenesis that occurs in humans and sheep.[7–9] Additionally, sheep and humans share a number of upper and lower airway traits, including airway branching pattern, nasal lymphoid tissue distribution, alveolar size, submucosal gland type and distribution, cartilage distribution, sensory nerves, airway capillary physiology, mast cell distribution, mucus-secreting cells, number of Clara cells in bronchioles, histamine effects, and cough/wheeze response.[10] Natural RSV disease occurs in cattle and sheep with bovine respiratory syncytial virus (bRSV) and has a similar presentation: seasonal outbreaks of highly contagious, mild respiratory disease with infrequent severe disease that occurs in

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Table 1. Primer and Probe Sequences Used for Real-Time Reverse Transcription Quantitative Polymerase Chain Reaction, 5′ to 3′

M37 hRSV	Fwd	GCTCTTAGCAAAGTCAAGTTGAA CGA	bRSV	Fwd	CAGTCAAGAATATTATGCTTGGT CATG
	Rev	TGCTCCGTTGGATGGTGTATT		Rev	CCTAACTTTTGTGCATATTCATAG ACTTC
	Probe	6 FAM-ACACTCAAACAAGATCA ACTTCTGTCATCCAGC-TAMRA		Probe	6FAM-CAACCTGTTCCATTTCTGCT TGTACGCTG-TAMRA
CCSP	Fwd	CAGCCCTGACGAAGACATGA	IFN-γ	Fwd	TGGAGGACTTCAAAAGGCTGAT
	Rev	GGGTGTCTACCAGCGTCTTCA		Rev	GATGGCTTTGCGCTGGAT
	Probe	6FAM-AGAGGCAACAAGTC AG-MGBNFQ		Probe	6FAM-CAAATTCCGGTGGATGATCT GC-TAMRA
SP-A	Fwd	TGACCCTTATGCTCCTCTGGAT	TNF-α	Fwd	CAACCTGGGACACCCAGAAT
	Rev	GGGCTTCCAAGACAAACTTCCT		Rev	TCTCAAGGAACGTTGCGAAGT
	Probe	6FAM-TGGCTTCTGGCCTCGAGTG CG-TAMRA		Probe	6FAM-CAAGGGCCAGGGTTCTTACC GGAA-TAMRA
IL-6	Fwd	GCTGCTCCTGGTGATGACTTC	TGF-β	Fwd	TGTGTTCGTCAGCTCTACATTGAC
	Rev	GGTGGTGTCATTTTTGAAATCTTCT		Rev	TAGCCCTTGGGTTCGTGAAT
	Probe	6FAM-CTTTCCCTACC CCGGGTCCCCTG-MBGNFQ		Probe	6FAM-TCCAGCCCAGGTCCTTCCGG A-TAMRA
IL-8	Fwd	TTCCAAGCTGGTCTGTTGCT	MCP-1α	Fwd	GCTGTGATTTTCAAGACCATCCT
	Rev	TTGACAGAACTGCAGCTTCACA		Rev	GGCGTCCTGGACCCATTT
	Probe	6FAM-CCGCTTTCCTGCT CTCTGCAGCTC-TAMRA		Probe	6FAM-AAAGAGTTTTGTGCAGACCC CAACC-TAMRA
IL-10	Fwd	GTCGGAATGATCCAGTTTTACCT	MIP-1α	Fwd	CAGCAGCCAGTGCTCCAA
	Rev	GTCAGGCCCATGGTTCTCA		Rev	ACCTGCCGGCCTTTTTTG
	Probe	6FAM-AGGAGGTGATG CCACAGG-MGBNFQ		Probe	6FAM-CCTGGTGTCATCTTCCAGA-MGBNFQ
IFN-β	Fwd	TGGTTCTCCTGCTGTGTTTCTC	RANTES	Fwd	TGCTTCTGCCTCCCCATATG
	Rev	CGTTGTTGGAATCGAAGCAA		Rev	GGGCGGGAGATATAGGCAAA
	Probe	6FAM-ACCACAGCTCTTTCC AGGAGCTACA-TAMRA		Probe	6FAM-CACCACGCCCTGCT-MGBNFQ

The following targets were assessed: M37, M37 strain of respiratory syncytial virus; bRSV, bovine respiratory syncytial virus; CC10, Clara cell secretory protein; SP-A, surfactant protein A; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin 10; MIP-1α, macrophage inflammatory protein; MCP-1α, monocyte chemotactic protein; TNF-α, tumor necrosis factor alpha; IFN-β, interferon-β; IFN-γ, interferon-γ; TGF-β, tumor growth factor-β; RANTES, regulated on activation normal T-cell expressed and secreted.

conjunction with other pathogens.[11,12] A bovine model of RSV infection has been used by a number of groups and is useful in mimicking human disease, but it is somewhat limited by the cost of feed and housing and the typical birth of a single offspring in cattle. In addition, relatively large quantities of testing material (therapeutic compounds) are required due to the size of testing animals that may not be easily available at the preclinical stage of drug development. Finally, there is current use of a number of efficacious vaccines that decrease the severity of disease due to RSV in cattle, as opposed to the current situation in humans.[11] Sheep are a particularly attractive model because of their smaller size, reduced cost, and increased offspring/parity compared with cattle, and more a human-relevant pulmonary development and structure compared with rodents. Also, newborn and preterm lambs have increased RSV disease severity compared with older lambs,[13–15] and the histopathology of RSV lesions in lambs (and cattle) is similar to that seen in human infants.[13,16] Also, lambs can be deprived of colostrum, thus avoiding the effects of maternal antibodies.

In this study, perinatal lambs were intranasally inoculated with human RSV (hRSV) Memphis strain 37 (M37) by using an atomizer. This intranasal route was used to more closely mimic natural disease. A similar method has been used to cause upper airway disease in humans with M37.[17] Previously, our group has administered virus (bRSV or hRSV A2 strain) intratracheally or intrabronchially.[15,18,19] It was our hypothesis that a more physiological intranasal inoculation of M37 hRSV would produce clinical disease and pathology paralleling that in human neonates. Our results demonstrate that M37 can cause consistent, moderate disease at 6 days post inoculation (p.i.). Gross and microscopic lesions were similar to those reported in human infants, as was the clinical expiratory effort seen in the infected lambs.

Materials and Methods

Experimental design

Animal use and experimental procedures were approved by Iowa State University's Animal Care and Use Committee.

Pilot study. A pilot study was completed in order to determine the potential susceptibility of young lambs to hRSV M37. The pilot study included four lambs (2–5 days of age). One lamb was inoculated intranasally (2 mL/nostril, 1×10^6 focus-forming units [FFU]), and three were inoculated with a fiberoptic bronchoscope. The bronchoscope was inserted to the level of the mainstem bronchus just distal to the tracheal bifurcation in two lambs, which received 8 or 17 mL; the third lamb received 10 mL in the deep airway of the right cranial lobe. Clinical features, including heart rate, respiratory rate

and character, attitude, ocular or nasal discharge, body weight, and body condition, were recorded daily, and lung tissues were collected 6 days post-inoculation.

Intranasal study. Neonatal lambs (2–3 days of age) were randomly assigned to three groups: a control group ($n=2$), a low-dose RSV inoculated group ($n=2$), and a high-dose RSV inoculated group ($n=3$). Each lamb received 2 mL/nostril of inoculum administered via a syringe attached to a mucosal atomization device (Wolfe Tory Medical, Inc., Salt Lake City, UT). Control lambs were inoculated with cell growth media; low-dose lambs were inoculated with M37 hRSV grown in HEp-2 cells at 1×10^5 FFU/mL; high-dose lambs were inoculated with the same virus (M37 hRSV in HEp-2 cells) at 2.1×10^8 FFU/mL. Viral inocula were provided by Meridian Life Science (Memphis, TN), originally isolated from a severe clinical case of RSV in an infant and maintained at low passage. Virus was obtained from Meridian Life Science at passage 5, and a seed stock of passage 6 was made and frozen in aliquots. Passage 8 was used for inoculation of lambs. Lambs were given daily antibiotics (Ceftiofur, Pfizer, New York, NY; 1–2 mg/kg, intramuscular) to prevent secondary bacterial infection. Lambs were monitored daily for clinical signs of respiratory disease and overall health, including heart rate, respiratory rate and character, rectal temperature, attitude, ocular or nasal discharge, body weight, and body condition. Animals were euthanized by sodium pentobarbital (Beuthanasia, Schering Plough Animal Health, Union, NJ) overdose on day 6 p.i.

Postmortem

After euthanasia the thorax was opened, lungs were removed, and gross lesions were scored as described previously[20] and photographed *ex vivo*. Tissue samples were collected from each lung lobe of all animals in the same manner, with uniform sampling of each lobe. Briefly, three samples from each lobe were snap frozen in liquid nitrogen for reverse transcription quantitative polymerase chain reaction (RT-qPCR), two samples from each lobe were placed in tissue cassettes and put in 10% neutral-buffered formalin for histological and immunohistochemical analysis.

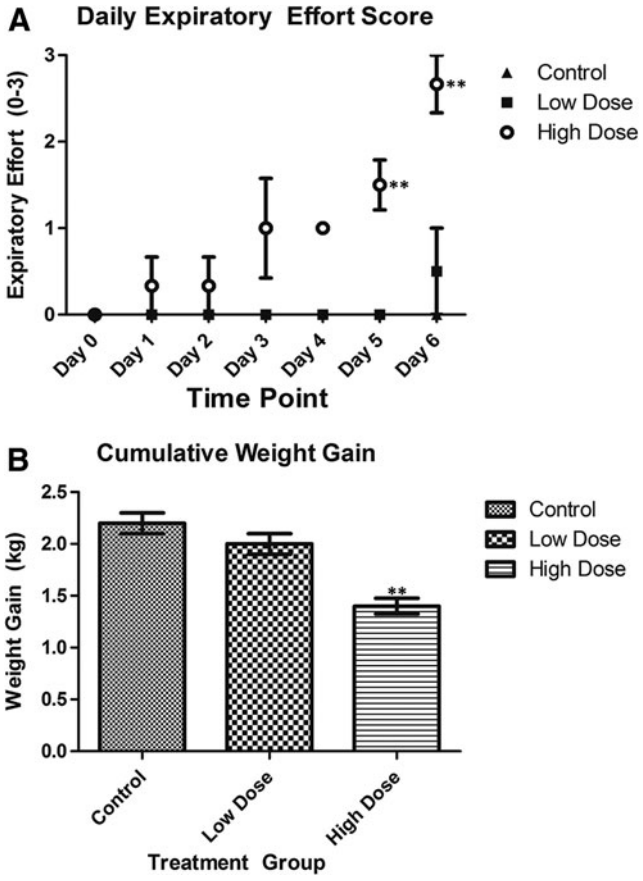

FIG. 1. Clinical data of lambs infected with human respiratory syncytial virus (hRSV) strain Memphis 37 (M37). **(A)** Lambs receiving the high-dose (4.2×10^8 focus-forming units [FFU]) of virus inoculum had a significantly higher clinical expiratory effort score than control or low-dose lambs on days 5 and 6 by two-way ANOVA (**). Expiratory effort score was assessed as follows: 0 = no effort; 1 = first detectable prolonged exhale; 2 = prolonged >1 sec, slight abdominal effort; 3 = prolonged to >3 sec, hard abdominal effort. **(B)** Cumulative weight gain over the study period was significantly lower for the high-dose group than either the control or low-dose group.

RT-qPCR

Whole vials of right and left cranial, right and left middle, and accessory lobes were homogenized in TRIzol (Invitrogen, Carlsbad, CA), then pooled for each animal to create a composite slurry. RNA isolation continued per manufacturer's instructions (Invitrogen), followed by DNase treatment (Ambion, TURBO DNase, Austin, TX), then diluted 1:10 with a combination of RNaseOUT (Invitrogen) and nuclease-free water (Invitrogen). Spectrophotometry (NanoDrop, Thermo Scientific, Waltham, MA) was used to assess each sample for purity and quantity. RNA integrity numbers >7.5 were routinely obtained by Bioanalyzer 2100 for all RNA samples. Hydrolysis probe-based RT-qPCR was carried out using One-Step Fast qRT-PCR Kit with ROX master mix (Quanta, BioScience, Gaithersburg, MD) in a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Carlsbad, CA) employing PREXCEL-Q for all set-up calculations.[21] Primer and probe sequences for all targets (Table 1) have been previously used in our lab. All sequences were generated using ABI Primer Express 2.0 software. All samples were diluted to achieve a final RT-qPCR concentration of 0.7844 ng/μL. Each sample was assessed in duplicate and each target gene amplification Cq converted to a relative quantity (rQ) based on the following equation: $rQ=10^{[(Cq\ -\ b)/m]}$, where Cq is the target quantification cycle, and b and m are the y-intercept and slope, respectively, from the Stock I (sample mixture)–derived standard curve for each target.[21] Additionally, the RSV Cq values were used to estimate viral copy number per milligram of lung tissue using a virtual absolute plasmid standard curve. This standard curve was estimated four times with plasmid constructs on a GeneAmp 5700 SDS using qScript One-Step Fast qRT-PCR kit with ROX mastermix; establishing an N_{Cq} value of 1.4516×10^{11} amplicons generated at Cq at a fixed ΔR_n RFU threshold of 0.04. The Stock I–derived standard curve for RSV M37 indicated an amplification efficiency of 99.61%, while the virtual plasmid absolute standard curve was assigned an amplification efficiency of 100%.

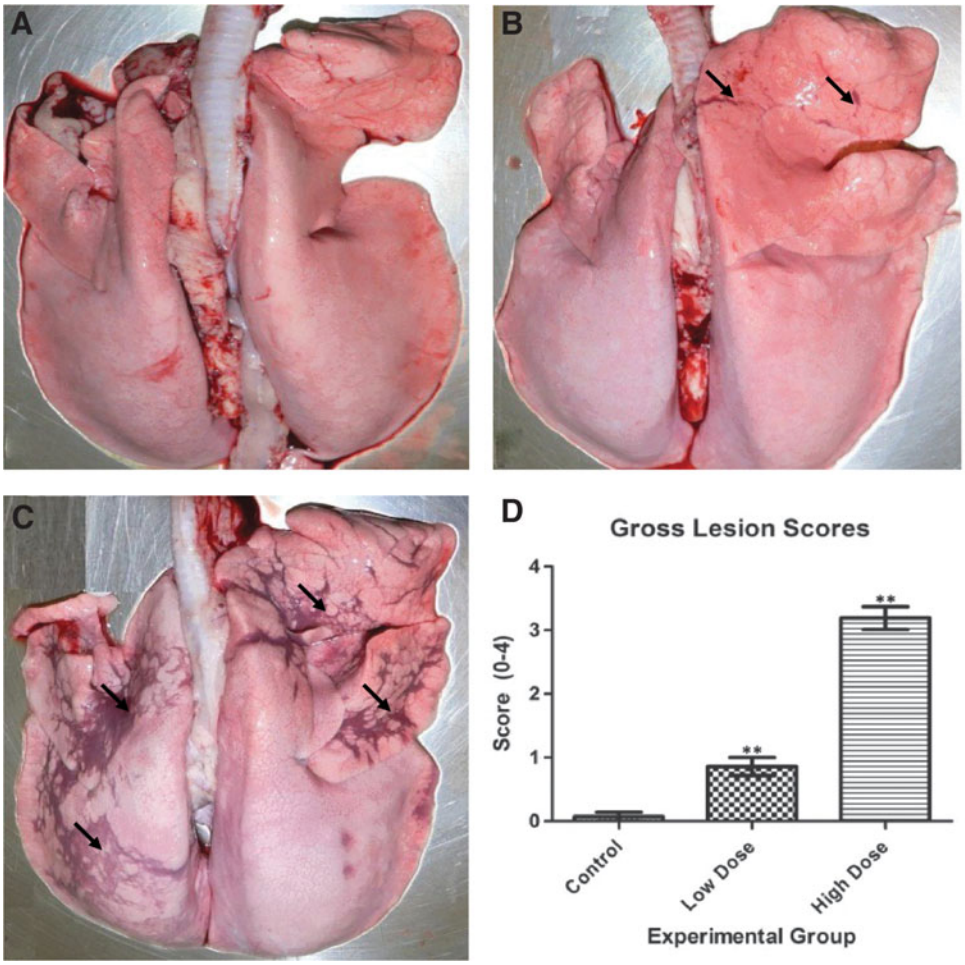

FIG. 2. Representative gross lesions and scoring of lesions caused by M37 hRSV in lambs. **(A)** Control lamb lung. **(B)** Low-dose (2×10^5 FFU) lamb lung. **(C)** High-dose (4.2×10^8 FFU) (arrows indicate lesions). **(D)** Lesion scores. The scoring scale is based on percent lung involvement for each lobe: 0% = 0; 1%–9% = 1; 10%–39% = 2; 40%–69% = 3; 70%–100% = 4. Lesion score in the high-dose group ($n=3$) is significantly higher than either control group ($n=2$) or low-dose group ($n=2$), low-dose score is significantly higher than control group; ANOVA, followed by Tukey's, $p<0.05$.

Gross lesion evaluation and scoring

After removal, percentage parenchymal involvement was estimated for each lung lobe. Percentages were converted to a scale using the following formula: 0% = 0; 1%–9% = 1; 10%–39% = 2; 40%–69% = 3; 70%–100% = 4. Group averages were calculated for the gross lesion score.

Histologic evaluation and scoring

A histologic score was given by determining percent consolidation followed by conversion to a consolidation scale used by our laboratory previously[20]: 0% consolidation = 0; 1%–9% consolidation = 1; 10%–39% consolidation = 2; 40%–69% consolidation = 3; 70%–100% consolidation = 4. Group averages were calculated for the alveolar consolidation score.

Immunohistochemistry

Immunohistochemistry for RSV antigen was performed on paraffin-embedded tissue as described previously,[19] with the following variations: instead of Pronase E antigen retrieval, heated buffer antigen retrieval was performed in pH 9.0 Tris-EDTA buffer containing 0.05% Tween 20, with microwave heating just to boiling followed by 30 min in a prewarmed steamer and cooling for 5 min on ice. Primary polyclonal goat anti-RSV antibody (BioDesign/Meridian, San Ramon, CA) was applied for 2 h at room temperature (~20°C) at a concentration of 1:300 instead of 1:50 as previously described. Color was developed using Nova Red (Vector, Burlingame, CA), counterstaining with Harris' hematoxylin, after which slides were dehydrated and cover-slipped. Slides were then scored by the following method: twenty 10× fields on each slide containing two pieces of lung were assessed for antigen staining. Immunoreactive (IR) cells were counted, then converted to a score where 0 (IR) = 0; 1–10 (IR) = 1; 11–39 (IR) = 2; 40–99 (IR) = 3; and ≥100 (IR) = 4. The number of fields per slide with antigen staining was also recorded. Composite numbers for both number of fields with antigen staining and score were assessed.

Statistical analysis

All analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., LaJolla, CA). All postmortem data were assessed with one-way ANOVA followed by Tukey's post-test. All clinical data were assessed with two-way ANOVA, and cumulative weight change was additionally assessed with one-way ANOVA followed by Tukey's post-test.

Results

Pilot study

At day 6 p.i., all four lambs had gross and microscopic lung pathology (epithelial degeneration and syncytia; neutrophils and debris in bronchioles/alveoli; lymphocytic infiltrates). Inoculation intranasally (2 mL/nostril) produced bilateral lung

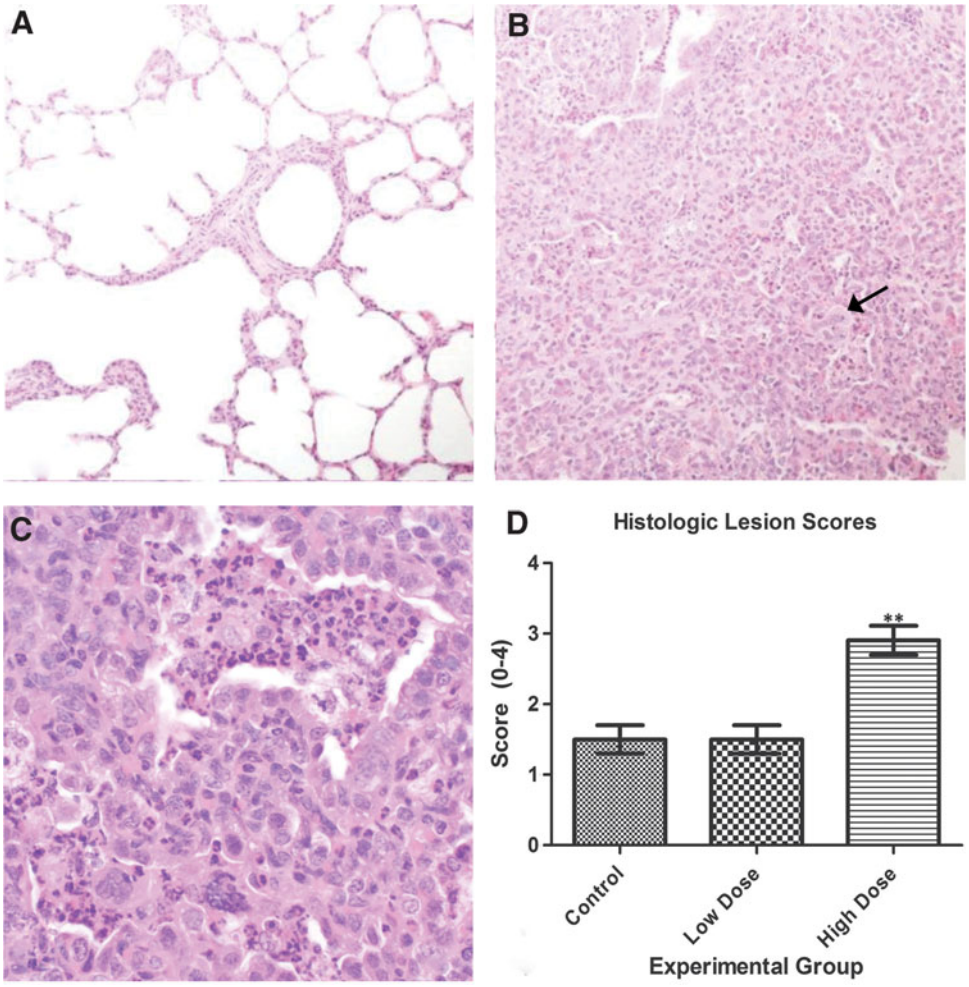

FIG. 3. Representative histologic lesions and scoring of lesions caused by M37 hRSV in lambs. **(A)** Control lamb lung with normal alveoli and bronchioles, 20× magnification. **(B)** High-dose lamb with characteristic inflammation including neutrophils and sloughed epithelia cells in bronchioles and alveolar septa infiltrated by macrophages, lymphocytes, and fewer neutrophils with syncytial cells (arrow), 20× magnification. **(C)** 40× view of characteristic histopathology. **(D)** Histologic lesion scores; the histologic score of the high-dose group is significantly higher than either control group or low-dose group; ANOVA, followed by Tukey's, $p < 0.05$.

lesions. Lambs inoculated directly into mainstem bronchus (8 or 17 mL) or into deep airway (10 mL) produced local lesions that were intense, occupying 60%–90% of the lung lobe. Daily body temperature was significantly higher (39.0°C vs. 39.8°C) at day 4 p.i. than at day 0. One lamb had mildly increased expiratory effort on days 4–6 p.i. Histologic lesions were present in all infected lambs and were similar to those described previously for RSV in lambs.[13,15] Briefly, lambs had bronchiolitis with multifocal bronchiolar epithelial cell necrosis and mild to moderate hyperplasia of nearby epithelial cells; intraluminal infiltrates of neutrophils, the presence of occasional syncytial cells, and accumulation of cell debris within bronchioles; mild to moderate peribronchiolar infiltrates of lymphocytes and plasma cells in the adventitia; mild to moderate alveolar type II cell hyperplasia; and minimal intraseptal infiltrates of lymphocytes.

Intranasal study

Clinical and postmortem findings. In the group infected with a high-dose inoculum, all three lambs had increased expiratory effort (i.e., forced expiration, abdominal breathing), first observed on day 1 in one lamb, and then noted in all three subjects by day 4. Group score averages for the high-dose group were significantly higher than control (0) on days 5 and 6 p.i. (Fig. 1). While there was no difference in weight gain on any given day, total weight gain in the high-dose group was significantly lower than either the control or low-dose group. No significant difference in weight gain was noted between the control and low-dose groups (Fig. 1). There were no significant changes in any other clinical parameters. All five infected lambs had multifocally to locally extensive dark red, well-demarcated foci of pulmonary consolidation, which varied in severity from moderate to severe (Fig. 2). These were slightly firm to rubbery on palpation. The three high-dose lambs had more extensive lesions than the two low-dose lambs on a basis of percentage tissue involvement. Gross lesion scores (based on percentage of lung parenchyma involved) of the high-dose group were significantly higher than either the control or low-dose group (Fig. 2).

Histopathology. Infected lambs had multifocal to confluent foci of cellular infiltrate that filled bronchioles and alveoli and expanded the alveolar interstitium consistent with RSV infection reported previously in lambs.[13,20] This infiltrate was composed of lymphocytes, neutrophils, macrophages, and plasma cells. Bronchioles and alveoli contained moderate to abundant amounts of sloughed, degenerate epithelial cells and neutrophils. Large epithelial syncytial cells were numerous within bronchioles. Pulmonary epithelium

lining affected airways was thickened in areas by plump cuboidal cells with oval, vesiculate nuclei (type II pneumocyte hyperplasia). Scoring of the histologic sections yielded a significantly higher score ($p < 0.05$) in high-dose lambs (3.19) than either control (0.07) or low-dose group (0.86) (Fig. 3).

Immunohistochemistry. Control lambs lacked staining for RSV antigen, while all low-dose and high-dose lambs had cells with immunoreactivity for RSV antigen present in areas of consolidation (Fig. 4). Antigen was present in epithelial cells lining alveoli and bronchioles and within the cytoplasm of occasional macrophages. Scoring of the histologic sections yielded both a higher overall score per animal as well as a greater number of total fields with immunoreactive cells per animal in the high-dose group.

Quantification of virus. Lung homogenates from each animal were assessed by RT-qPCR for relative mRNA levels. As expected, control animals lacked expression of RSV RNA. In contrast, the presence of RSV RNA was detected in lung homogenates of all infected animals. The mean RSV RNA level in lungs of the high-dose animals was 2.9-fold higher than that of the low-dose animals (Fig. 5a). The difference in the RSV RNA levels between the low- and high-dose animals was not statistically significant ($p > 0.05$). Lung samples from each animal were also assessed for the presence of bRSV by the same RT-qPCR method as for other targets and primer and probe sequence used in previous studies on bRSV in lambs.[18,22] The analysis demonstrated a lack of signal in all animal groups (not shown), indicating that the lambs did not acquire bRSV infection naturally.

Lung cytokine gene expression by RT-qPCR. The quantitative PCR analysis demonstrated that the high-dose group had significantly higher level of expression of macrophage inflammatory protein (MIP)-1α in lung tissue compared to the uninfected control group or low-dose group (Fig. 5b).

Discussion

Intranasal inoculation with the M37 strain of RSV yielded dose-dependent clinical expiratory effort scores, gross lesions, histologic lesions, and altered immune gene expression as assessed by RT-qPCR in infected lambs. Because this same viral strain causes sinusitis and clinical signs in normal human subjects following an experimental challenge,[17] infection in lambs allows mechanistic investigations that could improve understanding of RSV disease in humans. Also, the model could be used for the assessment of therapeutic antiviral compounds or vaccines developed specifically for human strains of RSV.

Previously, we have assessed a broad range of these genes in term lambs infected with RSV A2 strain and identified

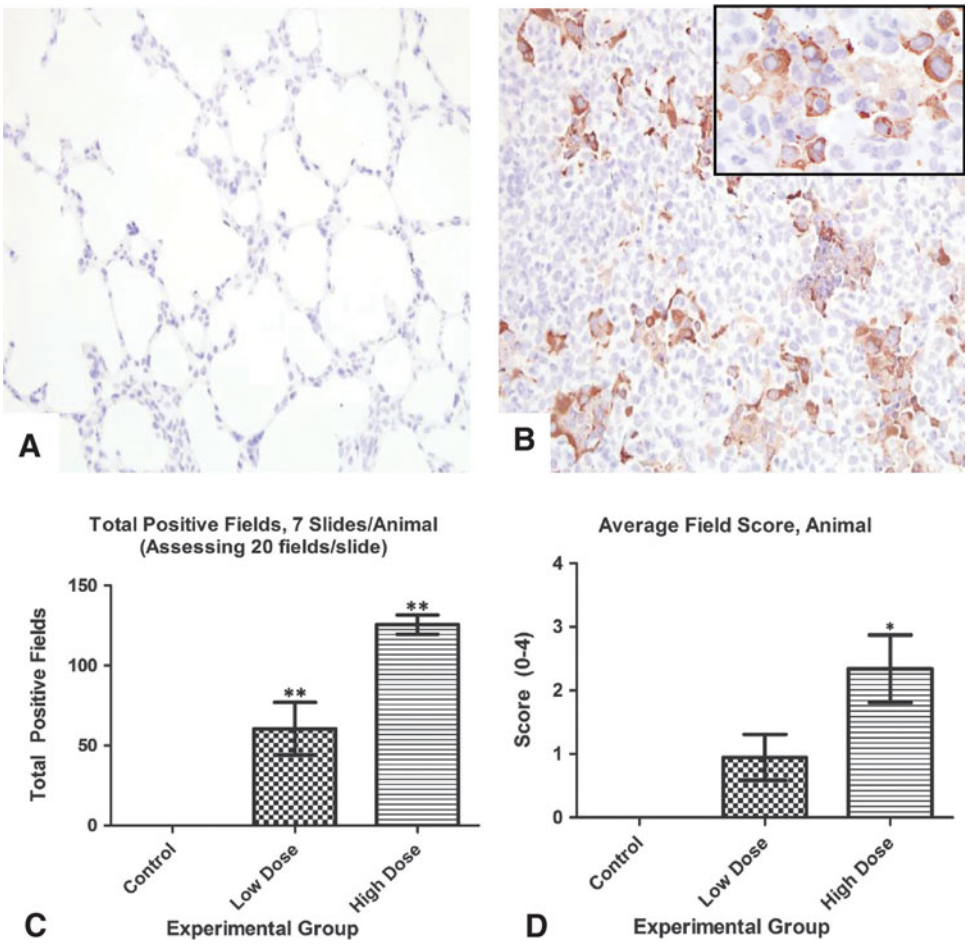

FIG. 4. Representative immunohistochemistry **(A, B)** and scoring of antigen immunoreactivity **(C, D)** in lambs infected with M37 hRSV. **(A)** Control lamb lung (20×). **(B)** Lung from a lamb receiving high-dose RSV with abundant intracellular staining (20×). Inset, 40× magnification. **(C)** Total number of fields with immunoreactivity for RSV antigen/animal for which 20 (10×) fields were assessed for each lung slide. Each slide contained two sections (minimum of 1 cm by 1 cm dimension) of lung from a lobe; all seven lobes of each animal were examined. The low-dose and high-dose groups were significantly different from the control and each other; ANOVA, followed by Tukey's, $p < 0.05$. **(D)** Slides scored by number of immunoreactive cells per field. The high-dose group had a significantly higher score than the control group; ANOVA, followed by Tukey's, $p < 0.05$.

mRNA increases in interferon (IFN)-γ, interleukin (IL)-8, monocyte chemotactic protein (MCP)-1α, and programmed cell death 1 ligand 1 (PD-L1), and decreases in IFN-β, IL-10, and tumor growth factor (TGF)-β.[23] While immune and inflammatory genes explored were not exhaustive in the present study, those analyzed have been reported to be altered in human disease and include TNF-α, IL-6, IL-8, MIP-1α, RANTES, MCP-1α, and limited IFN-γ.[24–26] The results of this study showed an increase in MIP-1α in animals infected with the high dose of M37. Additionally, while IL-8 was not statistically elevated in infected animals, control animals completely lacked signal, while high-dose animals all had detectable signal. There was a trend of increased expression of RANTES, MCP-1α, and IFN-γ in the high-dose group, though statistical significance at $p < 0.05$ was not achieved. Previous studies in lambs have shown limited changes in these chemokines, while marked changes in these chemokines have been reported in RSV-infected infants.[23,24] Limited sample sizes (two or three animals) in this study may underestimate significant differences between groups. Also, a lack of increased TNF-α or IL-6 in this study could be due to the sampling time point. We chose day 6 p.i. because previous studies have shown this to be a good time point for peak gross and histologic lesions, while increases in TNF-α or IL-6 tend to occur early in the course of disease (day 3 p.i.) in lambs infected with hRSV A2 strain.[23] Measurement of cytokine expression during the course of infection could be valuable in understanding the immune response with an eventual goal of altering that response to abrogate infection and improve clinical outcome.

Viral load as assessed in postmortem lung tissue by RT-qPCR was shown to peak at day 6 p.i. in our previous studies in lambs.[20] By RT-qPCR we had no detectable RSV RNA in control lambs, while all infected lambs showed significant RSV RNA levels. Day 6 p.i. termination point was selected because it provides time to collect additional clinical data and allows for the development of peak gross and histologic lesions as well as increased levels of viral RNA. For example, increased expiratory effort was first detected at day 1 and worsened by days 5 and 6. Earlier time points may be better for M37 virus isolation as shown in studies in mouse and cotton rat models in which RSV replication often peaks at day 3 and 4 p.i.[27,28] Additional information on the correlation between viral shedding and clinical symptoms would aid in understanding the kinetics of RSV infection. Nasal swabs were taken daily and stored in saline with sucrose but did not yield virus upon culture (data not shown). In subsequent experiments, our protocol for postmortem broncho alveolar lavage fluid (BALF) viral titers has been modified with great success; thus, lack of viral titers in the nasal swabs of these animals may be due to method and not lack of shedding. Future studies may use this modified protocol on daily nasal swabs. Additional procedures such as antemortem BALF collection may be of value as well.

Previous work in our lab with the RSV A2 strain yielded significant changes in body temperature but did not show markedly increased expiratory effort or as extensive gross lesions as observed in this study.[20] Differences between this study with RSV strain M37 and the previous study using infection with RSV strain A2 include a 10-fold higher inoculation viral titer (in the high-dose group) and intranasal versus intrabronchial inoculation. The intranasal route of inoculation used with M37 in this study may be more reflective of natural infection than intrabronchial inoculation as used in our A2 studies. Also, intranasal inoculation avoids sedation, which alters airflow and breathing as well as the inherent risk, albeit small, involved in any sedation technique. The drawback is that even small changes in head or body position, overall animal sizes, and natural variations in upper respiratory physiology can yield a more variable distribution of virus to the lung, and thus a very different pattern of gross and histologic lesion distribution. Overall, in this study there was a more diffuse pattern of lesions as compared to previous studies employing intrabronchial inoculations with a fiberoptic bronchoscope or intratracheal inoculation.[15,20] Studies to verify the greater virulence of M37 versus A2 among different inoculation methods would strengthen this model.

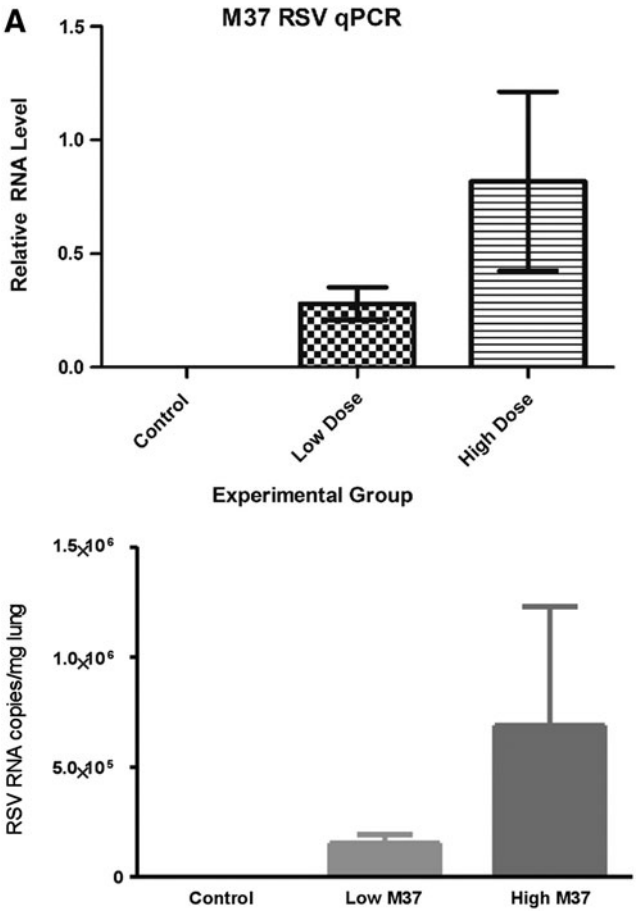

FIG. 5. **(A)** M37 gene expression analysis in postmortem lung tissue by quantitative polymerase chain reaction (PCR). M37 RNA was present in all infected animals and none of the controls. The data are presented as RNA relative to the standard curve. **(B)** Cytokine gene expression analysis in postmortem lung tissue by quantitative PCR mRNA levels were significantly higher in lambs receiving the high dose of M37 than either control or low-dose group for macrophage inflammatory protein (MIP-1α), as indicated by *. The data are presented as estimated copy number based on a virtual absolute plasmid standard curve. One-way ANOVA, followed by Tukey's method, $p < 0.05$. Data are presented as relative to high-dose lambs.

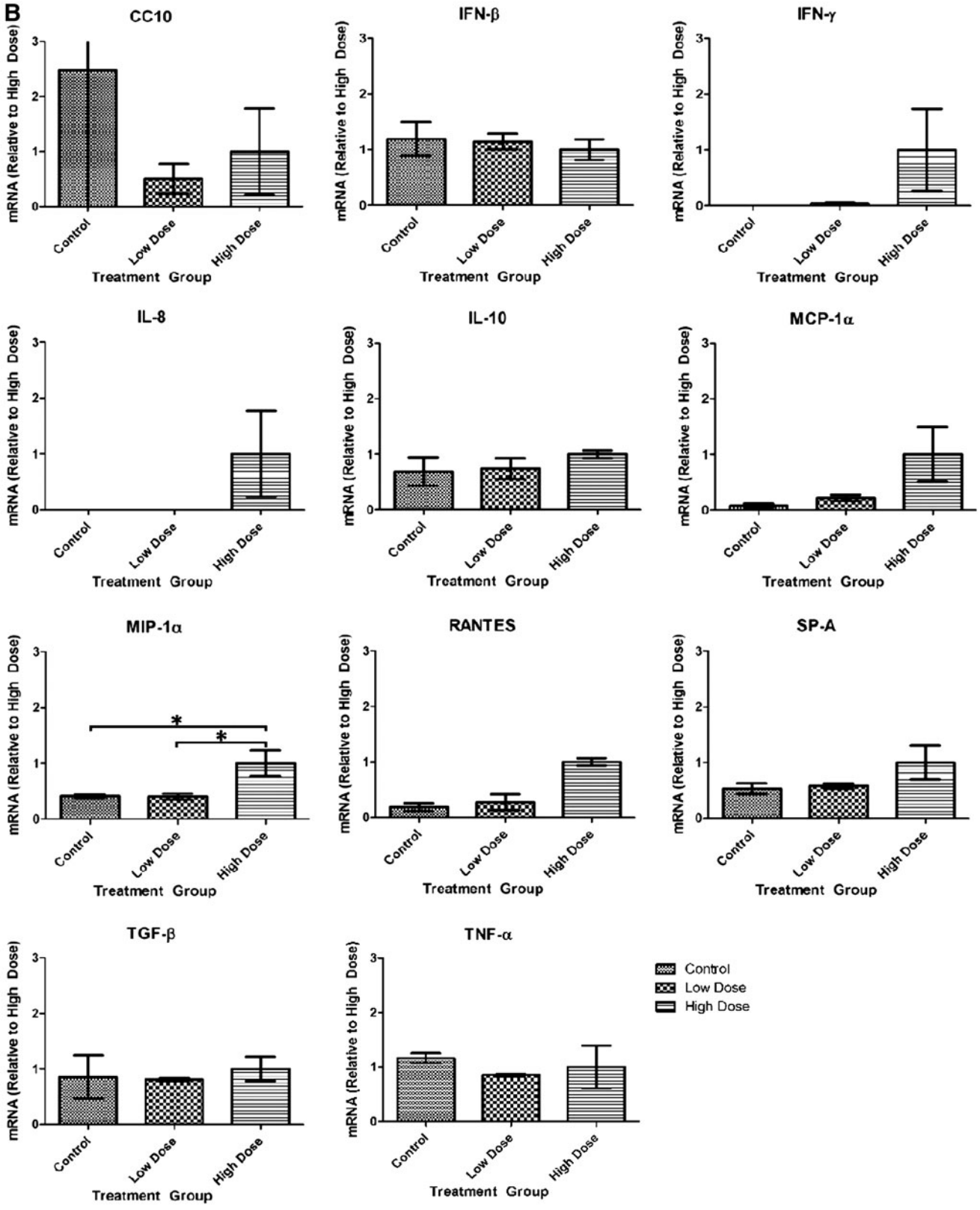

FIG. 5. (Continued).

Route of inoculation (nebulized, intranasal, intratracheal, intrabronchial), viral dose and dilution, as well as lamb factors such as vendor source, colostrum deprivation, preterm birth, or age at inoculation can all be adjusted with the goal to create low-cost, manageable, and reproducible models of moderate and severe RSV disease. Similar to our previous studies using RSV A2 strain, lambs in this study received colostrum and therefore may have had maternally derived bRSV-specific serum antibodies. Colostrum deprivation was not attempted due to the potentially higher health risk of these animals and also because RSV A2 strains caused significant disease without colostrum deprivation.[20] However, subsequent studies in our laboratory have used colostrum-deprived lambs.[29,30] Ideally, a separate study would be performed comparing colostrum-fed and colostrum-deprived lambs' responses to RSV, with additional measure of serum antibody and overall health possibly being helpful in determining the extent to which these antibodies affect hRSV disease in lambs. Colostrum-deprived calves have lower viability and increased complications compared to colostrum-fed calves,[31] and colostrum-deprived lambs exhibit decreased growth.[32]

Similar to humans, natural disease in cattle is more common in young animals (1–6 months) than in neonates. Comparing disease between newborn and older (6-week-old) calves, neonates shed virus longer and had more severe pathology, while clinical disease was less severe and pro-inflammatory cytokine levels were lower.[33] In experimental infection of lambs with bRSV, greater lesion severity has been observed in 1-week-old compared to 6-month-old lambs.[13,14] In a preterm lamb model, lesions were more severe in preterm compared to full-term lambs.[15] While the majority of cases of RSV occur in children 6 months to 2 years of age, infants <6 months of age are at increased risk of severe disease, and prematurity is an additional, independent risk factor.[34] A neonatal model is necessary to understand why clinical disease is less common, but severe disease more prevalent in this unique population. Acquiring lambs at a very young age provides insight to RSV in a specific, at-risk demographic and potentially limits their exposure to pathogens that could interfere with infection. A comparison of preterm, full-term, and older-age lambs has great potential to better understand the implications of age on disease outcome. Infection of healthy, full-term lambs presented an ideal starting point for these studies with a balance of potential for severe disease and applicability of design.

The data here demonstrate the capacity of a human clinical isolate of RSV used for human experimental infection to replicate and cause disease in lambs. The M37 strain of RSV can be used in lambs to produce moderate respiratory disease and has the potential to be a model of more severe disease than the A2 strain. Further investigation of these viruses in direct comparison is needed to discern differences in disease dynamics between the two strains.

Overall, lambs closely parallel human infant disease characteristics in clinical course, gross and histologic lesions, and inflammatory cytokine profile at key time points in disease. Also, lambs can be born preterm (90% gestation) and survive for experimentation, which allows assessment of RSV infection in preterm lung. Further exploration at multiple time points and expansion of data points can be pursued not only to better understand the disease, but also to assess response to novel therapeutic or prophylactic agents as well as vaccines in this model of disease. This study strengthens the lamb model by establishing infection and disease progression with a new human strain of RSV that is expected to be extensively used in the human experimental infection to obtain clinical proof of concepts for novel treatment approaches. Therefore, the lamb RSV M37 model holds great promise to facilitate the optimization, preclinical development, and selection of novel agents for the advancement into clinical studies.

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Author Disclosure Statement

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Abbreviations Used

BALF = broncho alveolar lavage fluid
bRSV = bovine respiratory syncytial virus
FFU = focus-forming units
hRSV = human respiratory syncytial virus
IFN = interferon
IL = interleukin
IR = immunoreactive
M37 = Memphis strain 37
MCP = monocyte chemotactic protein
MIP = macrophage inflammatory protein
p.i. = post inoculation
rQ = relative quantity
RSV = respiratory syncytial virus
RT-qPCR = reverse transcription quantitative polymerase chain reaction