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Abstract

The lung microenvironment is constantly exposed to microorganisms and particulate matter. Lung dendritic cells (DCs) play a crucial role in the uptake and processing of antigens found within the respiratory tract. Respiratory syncytial virus (RSV) is a common respiratory tract pathogen in children that induces an influx of DCs to the mucosal surfaces of the lung. Using a neonatal lamb model, we examined the in vivo permissiveness of DCs to RSV infection, as well as overall cell surface changes and cytokine responses of isolated lung DCs after bovine RSV (BRSV) infection. We report that isolated lung DCs and alveolar macrophages support BRSV replication. Isolated lung DCs were determined to be susceptible to BRSV infection as demonstrated by quantification of BRSV non-structural protein 2 mRNA. BRSV infection induced an initial upregulation of CD14 expression on lung DCs, but by 5 d postinfection expression was similar to that on control cells. No significant changes in CD80/86 or MHC class I expression were seen on lung DCs after BRSV infection. Low to moderate expression of MHC class II and DEC-205 was detected by day 5 postinfection. Initially, on day 3 postinfection, lung DCs from BRSV-infected lambs had decreased endocytosis of fluorescein isothiocyanate (FITC)–ovalbumin (OVA). The amount of FITC–OVA endocytosed by lung DCs isolated on day 5 postinfection was similar to that of controls. The most interesting observation was the induction of immunomodulatory interleukin (IL)-4 and IL-10 cytokine gene transcription in lung DCs and alveolar macrophages after in vivo infection with BRSV. Overall, these findings are the first to demonstrate that neonatal lung DCs support in vivo BRSV replication and produce type II cytokines after viral infection.

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Neonatal Ovine Pulmonary Dendritic Cells Support Bovine Respiratory Syncytial Virus Replication with Enhanced Interleukin (IL)-4 and IL-10 Gene Transcripts

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ABSTRACT

The lung microenvironment is constantly exposed to microorganisms and particulate matter. Lung dendritic cells (DCs) play a crucial role in the uptake and processing of antigens found within the respiratory tract. Respiratory syncytial virus (RSV) is a common respiratory tract pathogen in children that induces an influx of DCs to the mucosal surfaces of the lung. Using a neonatal lamb model, we examined the *in vivo* permissiveness of DCs to RSV infection, as well as overall cell surface changes and cytokine responses of isolated lung DCs after bovine RSV (BRSV) infection. We report that isolated lung DCs and alveolar macrophages support BRSV replication. Isolated lung DCs were determined to be susceptible to BRSV infection as demonstrated by quantification of BRSV nonstructural protein 2 mRNA. BRSV infection induced an initial upregulation of CD14 expression on lung DCs, but by 5 d postinfection expression was similar to that on control cells. No significant changes in CD80/86 or MHC class I expression were seen on lung DCs after BRSV infection. Low to moderate expression of MHC class II and DEC-205 was detected by day 5 postinfection. Initially, on day 3 postinfection, lung DCs from BRSV-infected lambs had decreased endocytosis of fluorescein isothiocyanate (FITC)–ovalbumin (OVA). The amount of FITC–OVA endocytosed by lung DCs isolated on day 5 postinfection was similar to that of controls. The most interesting observation was the induction of immunomodulatory interleukin (IL)-4 and IL-10 cytokine gene transcription in lung DCs and alveolar macrophages after *in vivo* infection with BRSV. Overall, these findings are the first to demonstrate that neonatal lung DCs support *in vivo* BRSV replication and produce type II cytokines after viral infection.

INTRODUCTION

RESPIRATORY SYNCYTIAL VIRUS (RSV) is a prominent viral pathogen whose tropism for the lower respiratory tract causes severe bronchiolitis and pneumonia. In-

fants and young children are most susceptible to the virus and reinfections are common because of an ineffective immune response to the virus (17,18,43). The pathogenesis of RSV is believed to be a combination of host proinflammatory cytokine production and the cytopathic ef-

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fects of viral replication (40). Lung epithelial cells are a primary site for RSV replication, but data from studies with monocyte-derived dendritic cells (MDDCs) and alveolar macrophages (AMs) indicate that *in vitro* replication of the virus does occur in other cell types (6,14,21,32).

An influx of DCs after RSV infection has been documented within the lungs of infected mice and by isolation of DCs from nasal washes of RSV-infected children (3,13). Dendritic cells are important sentinels of the immune system and play a crucial role in antigen capture, processing, and presentation to T cells. As the most potent antigen-presenting cells (APCs), DCs link the innate and adaptive immune responses after microbial infection. On viral infection, DCs are well equipped to prime T cells for primary and secondary antiviral responses. Therefore, viruses have been found to target DCs and modulate surface antigen expression, maturation, and cytokine production (33). A prominent respiratory pathogen belonging to the same *Paramyxoviridae* family as RSV is measlesvirus (MV), whose immunosuppression of DCs has been well documented using *in vitro*-derived DCs (CD34$^+$ precursors or monocytes) or Langerhans cells (16,35,36). MV modulates infected DCs through inhibition of maturation, interleukin (IL)-12 production, and CD80 and CD86 expression. In addition, herpesvirus (23), poliovirus (42), and vaccinia virus (8) have been shown to infect DCs and suppress their maturation, antigen presentation, and cytokine production.

To date, only *in vivo* adult lung DCs or *in vitro*-derived DCs have been examined after RSV infection (1,3,14,45). Neonatal, tissue-isolated lung DCs have not been examined *ex vivo* after RSV infection. In the present studies, we used a neonatal lamb model of experimental bovine RSV (BRSV) infection as previously described (22,30). Neonatal lambs have been used in pulmonary studies of naturally occurring respiratory diseases (26,27,30). The first aim of these studies was to isolate neonatal lung DCs after *in vivo* BRSV infection to determine whether these cells would support viral replication. Second, lung DCs were examined to determine the effects of viral infection on surface antigen expression and cytokine mRNA induction. We previously developed a procedure for the isolation and enrichment of CD11c$^+$ lung DCs from neonatal lambs (9). In the present study, we report that BRSV replication occurs within isolated lung DCs and AMs *in vivo*. Phenotypic changes due to the effects of viral infection were examined. Functional characteristics of lung DCs were determined by antigen uptake assays and cytokine gene transcription. Interestingly, BRSV induced IL-4 and IL-10 gene transcription in lung DCs and AMs. Results indicate that isolated lung APCs can support BRSV replication and that immunomodulatory cytokines are upregulated early in infection.

MATERIALS AND METHODS

Animals and experimental procedure

Healthy 2- to 4-d-old lambs of mixed Rambouillet and Polypay breeds were used for the experiments. Lambs received ceftiofur sodium (Naxcel, 2.2 mg/kg once per day, intramuscular) to prevent secondary bacterial complications (41) as previously done in a premature lamb model (30). Lambs were bottle fed four times per day with commercial milk replacer. Lambs received an intratracheal inoculation of either 22 mL of BRSV 375 inoculum or sterile medium with a 30-cm^3 sterile syringe and 20-gauge needle. Necropsies of day 3 postinoculation lambs ($n = 6$), day 5 postinoculation lambs ($n = 6$), or control lambs ($n = 6$) were conducted in two separate experiments according to the following procedure. Lambs were killed with an intravenous injection of sodium pentobarbital. During necropsy, the abdominal cavity was opened and the abdominal aorta was severed. Lungs were perfused by a similar procedure to that previously described (9,38). Briefly, the pulmonary artery was clamped as it exits the heart and catheterized with an 18-gauge needle distal to the clamp. The vasculature of the lungs was perfused with sterile phosphate-buffered saline (PBS) until the lungs were cleared of peripheral blood and were pale pink in color. The lungs were aseptically removed, lavaged with sterile PBS to remove alveolar macrophages, and placed in sterile PBS containing 1% antibiotic–antimycotic (Invitrogen, Carlsbad, CA). Accepted principles of animal care were followed and experiments were approved by the National Animal Disease Center (NADC, Ames, IA) Animal Care and Use Committee.

Viral inoculum

Bovine RSV (BRSV) 375 was propagated on adherent bovine turbinate cells and the viral inoculum was prepared as previously described (22,30). Briefly, by 7 d of incubation, or when more than 90% of virally induced cytopathic effect was observed, flasks were frozen at −80°C. Within 2 d, the flasks were thawed and the media were pooled and centrifuged to remove cellular debris. After thorough mixing and sterile filtration, viral inoculum was aliquoted and stored at −80°C. A standard plaque assay was used to determine the tissue culture infectious dose (TCID$_{50}$), $10^{4.9}$ TCID$_{50}$/mL (3.97×10^4 plaque-forming units [PFU]/mL). The viral inoculum was BVDV free as tested by polymerase chain reaction (PCR) (data not shown).

Pulmonary DC isolation and enrichment

Neonatal lamb pulmonary DCs were isolated as previously described (9). Briefly, the entire lung was cut into

small pieces with sterile scissors and forceps and then digested in RPMI 1640 (Invitrogen) containing Liberase Blendzyme 3 (7 mg/mL; Roche Applied Science, Indianapolis, IN) and DNase I, type IV from bovine pancreas (50 μg/mL; Sigma, St. Louis, MO) for 1 h at 37°C. The enzymatic digestion was stopped by the addition of 10% fetal bovine serum (FBS) to the decanted supernatant and the entire digestion step was repeated on the remaining tissue pieces. The cell suspension was filtered through sterile cheesecloth and washed with PBS. Contaminating red blood cells (RBCs) were removed from cell preparations by hypotonic lysis. Cells were resuspended in supplemented RPMI 1640 (cRPMI) containing 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 10% lamb serum, 2 mM L-glutamine, and 1% antibiotic–antimycotic (all from Invitrogen GIBCO, Grand Island, NY) and incubated for 12 h at 37°C in an atmosphere of 5% CO_2. Adherent and nonadherent cells were collected, washed, and incubated for 10 min at room temperature with 10% normal goat serum (Invitrogen) to block nonspecific binding. Cells were washed and stained with monoclonal antibody BAQ153A (anti-ovine CD11c; VMRD, Pullman, WA) followed by incubation with magnetic MicroBeads (Miltenyi Biotec, Auburn, CA). CD11c$^+$ DCs (lung DCs) were obtained by twice positively sorting with a magnetically activated cell sorter (Miltenyi Biotec).

Immunohistochemistry

Samples of PBS-perfused lung were frozen in Tissue-Tek O.C.T. compound (Sakura Finetek USA, Torrance, CA) in an ethanol–dry ice bath. Sections were cut onto silanated glass slides, fixed in acetone for 5 min, and stored at −80°C. Slides were rehydrated in 1× Tris buffer and blocked with normal goat serum (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 30 min at room temperature. The slides were washed in 1× Tris buffer and incubated with primary antibody BAQ153A (anti-ovine CD11c; VMRD) diluted in 1× Tris buffer, PBS, and 3% bovine serum albumin (BSA) overnight at 4°C. Slides were rinsed and polyclonal BRSV–fluorescein isothiocyanate (FITC) (50 μL; VMRD) was added for 30 min at 37°C. Slides were washed with 1× Tris buffer and incubated for 30 min at 37°C with isotype-specific secondary antibodies conjugated to Alexa Fluor 633 (Invitrogen Molecular Probes, Eugene, OR). To visualize nuclei, slides were washed and incubated with 4′,6-diamidino-2-phenylindole, dilactate (DAPI, 300 nM; Invitrogen Molecular Probes) for 5 min at room temperature. The slides were washed with 1× Tris buffer and coverslips were mounted with a 50:50 glycerin–PBS solution. Tissue sections were examined with a Leica TCS-NT confocal scanning laser microscope (Leica Microsystems, Exton, PA). Images were prepared with Adobe Photoshop version CS (Adobe, San Jose, CA) and InDesign version 3 (Adobe).

Flow cytometry

Isolated CD11c$^+$ cells were analyzed for cell surface antigens by three-color flow cytometry. Cell concentrations were adjusted to 1×10^5 cells/mL and stained with different combinations of primary and secondary antibodies diluted in FACS buffer (balanced salt solution with 1% FBS and 0.1% sodium azide). Ovine-specific monoclonal antibodies obtained from VMRD were used for detection of CD14 (CAM36A), CD11c (BAQ153A), MHC class I (BC5), and MHC class II (TH14B). Cross-reactive CD11b (CC126) and DEC-205 (CC98) monoclonal antibodies were obtained from Serotec (Raleigh, NC). CD80/86 was detected with human CD152-muIg/R-phycoerythrin (PE) fusion protein (Ancell, Bayport, MN). Isotype-specific primary antibodies used were anti-mouse IgG1, IgG2b, IgG2a, and IgM (Serotec). Secondary antibodies (SouthernBiotech, Birmingham, AL) included anti-IgM–PE, anti-IgG2b–PE, anti-IgG2b–FITC, anti-IgG2a–FITC, and anti-IgM–FITC. Anti-IgG1 Tricolor was obtained from Invitrogen Caltag Laboratories (Burlingame, CA). Cells were washed with FACS buffer and resuspended in 2% paraformaldehyde in PBS. For each sample 10,000 events were collected with a BD Biosciences Immunocytometry Systems LSR flow cytometer (San Jose, CA). Analysis of cell surface marker expression was done with FloJo software (Tree Star, Ashland, OR).

Tracer endocytosis

FITC-conjugated ovalbumin (FITC–OVA; Invitrogen Molecular Probes) and FITC-conjugated dextran (FITC–dextran; Invitrogen Molecular Probes) were used to examine the endocytic abilities of the lung DC populations. CD11c$^+$ cell concentrations were adjusted to 1×10^5 cells/mL and incubated at 37 or 4°C for 1 h with FITC–OVA or FITC–dextran (1 mg/mL). Cells were washed with FACS buffer and resuspended in 2% paraformaldehyde in PBS. For each sample 10,000 events were acquired with a flow cytometer (Becton Dickinson LSR). Data were analyzed with FloJo software (Tree Star).

RNA extraction and cDNA synthesis

Sorted CD11c$^+$ pulmonary cells were pelleted in sterile 1.5-mL Eppendorf tubes. Cells were resuspended in 600 μL of RLT buffer plus 2-mercaptoethanol from the RNeasy Mini RNA isolation kit (Qiagen, Valencia, CA) and stored at −80°C. Total RNA was isolated using the RNeasy Mini RNA isolation kit (Qiagen) according to the manufacturer's instructions. Contaminating genomic DNA

was removed during RNA isolation with an on-column RNase-free DNase I digestion set (Qiagen) according to the manufacturer's instructions. Total RNA was eluted by the addition of 40 μL of DNase–RNase-free water. One microgram of total RNA from each sample was reverse transcribed with oligo(dT)$_{12-18}$ primers (Invitrogen).

Cytokine and BRSV nonstructural protein 2 mRNA quantification

Primers were designed specifically for SYBR green quantification, using the PrimerSelect program (DNAS-TAR, Madison, WI) with suggested parameters for SYBR green chemistry that included a product size no greater than 100–150 base pairs and a 50°C annealing temperature. Ovine ribosomal protein S15 was chosen as the endogenous control for the genes of interest, which are listed in Table 1. Oligo(dT) cDNA was diluted 1:10 in DNase–RNase-free water and 2 μL was used for quan-

tification. SYBR green PCR master mix (Applied Biosystems, Foster City, CA) was used according to the manufacturer's instructions. An Applied Biosystems 7300 Real-Time PCR System machine was used with the same amplification conditions for all genes of interest: 10 min at 95°C, 15 sec at 95°C, 40 cycles of 1 min at 50°C, with a dissociation step of 15 s at 95°C, 30 s at 50°C, 15 s at 95°C. Final relative quantification was calculated by the $2^{-\Delta\Delta C_T}$ method (28), where the amount of target gene is normalized to an endogenous control (ovine ribosomal protein S15) and expressed relative to control cells. Primers were validated on the Applied Biosystems 7300 machine by using serial dilutions of total RNA with ovine ribosomal protein S15 and target gene primers, whose values were plotted as the log input amount versus ΔC_T values (target gene C_T—endogenous control C_T) for relative efficiency. Only primers with a slope of less than 0.1 were used, because of similar amplification efficiencies as the endogenous control.

TABLE 1.   PRIMER SEQUENCES FOR OVINE TARGET AND ENDOGENOUS CONTROL GENES

Primer	Sequence (5' → 3')	Length (bp)	Accession no.
IL-1β Forward	ATGGGTGTTCTGCATGAG	63	X54796
IL-1β Reverse	AAGGCCACAGGAATCTTG		
IL-4 Forward	GGACTTGACAGGAATCTC	80	Z11897
IL-4 Reverse	CTCAGCGTCTTGTACTC		
IL-6 Forward	GAGTTGCAGAGCAGTATC	85	X62501
IL-6 Reverse	GGCTGGAGTGGTTATTAG		
IL-8 Forward	AAGCTGGCTGTTGCTCTC	94	X78306
IL-8 Reverse	GGCATCGAAGTTCTGTACTC		
IL-10 Forward	GATGCCACAGGCTGAGAACC	53	U11421
IL-10 Reverse	GCGAGTTCACGTGCTCCTTG		
IL-12p40 Forward	AAGTCACATGCCACAAGG	72	AF004024
IL-12p40 Reverse	CACTCCAGAATGAGCTGAG		
TNFα Forward	CTCATCTACTCGCAGGTCCTC	86	X56756
TNFα Reverse	ACTGCAATGCGGCTGATGG		
ovRPS15 Forward	TACAACGGCAAGACCTTCAACCAG	105	[a]
ovRPS15 Reverse	GGGCCGGCCATGCTTACG		
BRSV			
NS2 Forward	ACCACTGCTCAGAGATTG	155	NC_001989
NS2 Reverse	AATGTGGCCTGTCGTTCATCG		

[a]Ovine ribosomal protein S15 mRNA sequence provided by Dr. Sean Limesand, Dept. of Pediatrics, University of Colorado Health Sciences Center, Perinatal Research Center, Aurora, CO.

In sterile Eppendorf tubes, 20 μL of total RNA from BRSV 375 inoculum and BRSV oligo(dT) cDNA were treated with RNase I$_f$ (1 unit/μL; New England BioLabs, Ipswich, MA) or untreated and all four samples were incubated for 30 min at 37°C. The digestion of single-stranded RNA was stopped by heating the tubes at 70°C for 20 min. Removal of the RNase enzyme was performed by Microcon column exclusion (100U columns; Millipore, Billerica, MA) according to the manufacturer's instructions. Samples were quantified with BRSV nonstructural protein 2 (NS2) primers and SYBR green chemistry as previously described above. No amplification of genomic BRSV NS2 was observed (data not shown).

Statistical analysis

Flow cytometric data were expressed as mean fluorescence intensity ± SEM. Cytokine data were expressed as the fold change in expression relative to controls. Statistical analysis of the data was performed by unpaired two-tailed Student t test or analysis of variance (ANOVA) (cytokine real-time PCR) using GraphPad Prism software (GraphPad Software, San Diego, CA). Multiple means comparisons of cytokine data were performed by Bonferroni's test (Prism software). Significance was determined as $p < 0.05$.

RESULTS

Colocalization of BRSV and CD11c$^+$ DCs within lung parenchyma

Immunofluorescence staining was used to demonstrate the presence of BRSV antigen within lung CD11c$^+$ cells. Frozen lung sections from the upper right lobe of neonatal lamb lungs of control, day 3 postinoculation, and day 5 postinoculation lambs were stained for the presence of BRSV and CD11c. DAPI was used as a nuclear stain to enhance visualization of the overall cellular distribution within the tissue sections. Coexpression of BRSV antigen and CD11c was seen in the alveolar septa and subjacent to the bronchiolar epithelium (Fig. 1). No BRSV antigen staining was detected in control lamb lung cryosections (data not shown). Overall, analysis of the immunofluorescently stained frozen sections showed BRSV antigens colocalized within lung CD11c$^+$ cells. However, additional experiments were required to determine whether lung DCs or AMs support active viral replication.

BRSV replication within antigen-presenting cells

On initiation of replication, the RNA-dependent RNA polymerase transcribes the RSV negative-sense genome in the 3′-to-5′ direction. Large amounts of the mRNAs encoding nonstructural proteins NS1 and NS2 are produced during viral replication, a result of the genome's transcriptional polarity, in which the genes on the 3′ end are transcribed in greater amounts than the 5′-end genes (4,10). To detect BRSV replication within isolated lung DCs and AMs, primers were designed to the NS2 mRNA of BRSV and relative quantification was performed by SYBR green chemistry. Expression of BRSV mRNA was detected in both DCs and AMs, and the NS2 transcript levels in AMs exceeded the detected levels in the lung DCs (Fig. 2). Taken together, these results demonstrate that both lung DCs and AMs support BRSV replication *in vivo*.

Analysis of surface antigen expression

Isolated lung DCs were analyzed for surface antigen expression 3 and 5 d after inoculation of BRSV. More than 89% of all isolated CD11c$^+$ lung DCs coexpressed DEC-205 (Fig. 3A). A significant increase in CD14 expression was observed on lung DCs isolated from 3 d postinoculation lambs when compared with control lung DCs (Fig. 3B). However, CD14 expression on isolated lung DCs 5 d postinoculation was similar to that on control lung DCs. Expression of DEC-205, MHC class I, and CD80/86 increased from day 3 to day 5 postinoculation, but no significant differences relative to controls were noted (Fig. 3B). No significant changes in MHC class II expression during BRSV infection were observed. Overall, BRSV infection induced a notable change in CD14 expression, but no significant changes in expression of surface antigens necessary for presentation and stimulation of antiviral T cells responses were observed.

Antigen uptake after BRSV exposure

Functional characteristics of lung DCs isolated from BRSV-infected lambs were assayed by measuring their endocytic uptake of antigens. Several pathways exist for antigen uptake and FITC–OVA was used as a measure of receptor-mediated endocytosis via clathrin-coated pits. In addition, FITC–dextran uptake was used to measure mannose receptor-mediated endocytosis by lung DCs. Endocytosis of FITC–OVA by lung DCs isolated 3 d after inoculation of BRSV was significantly decreased compared with controls (Fig. 4). By day 5 postinoculation, lung DCs endocytosed significantly more FITC–OVA than on day 3 postinoculation ($p < 0.05$). Mannose receptor-mediated endocytosis of FITC–dextran was lower in the controls and in lung DCs from BRSV-infected lambs, and no significant differences were noted (Fig. 4). Therefore, lung DCs were still able to take up antigen after 5 d of BRSV infection.
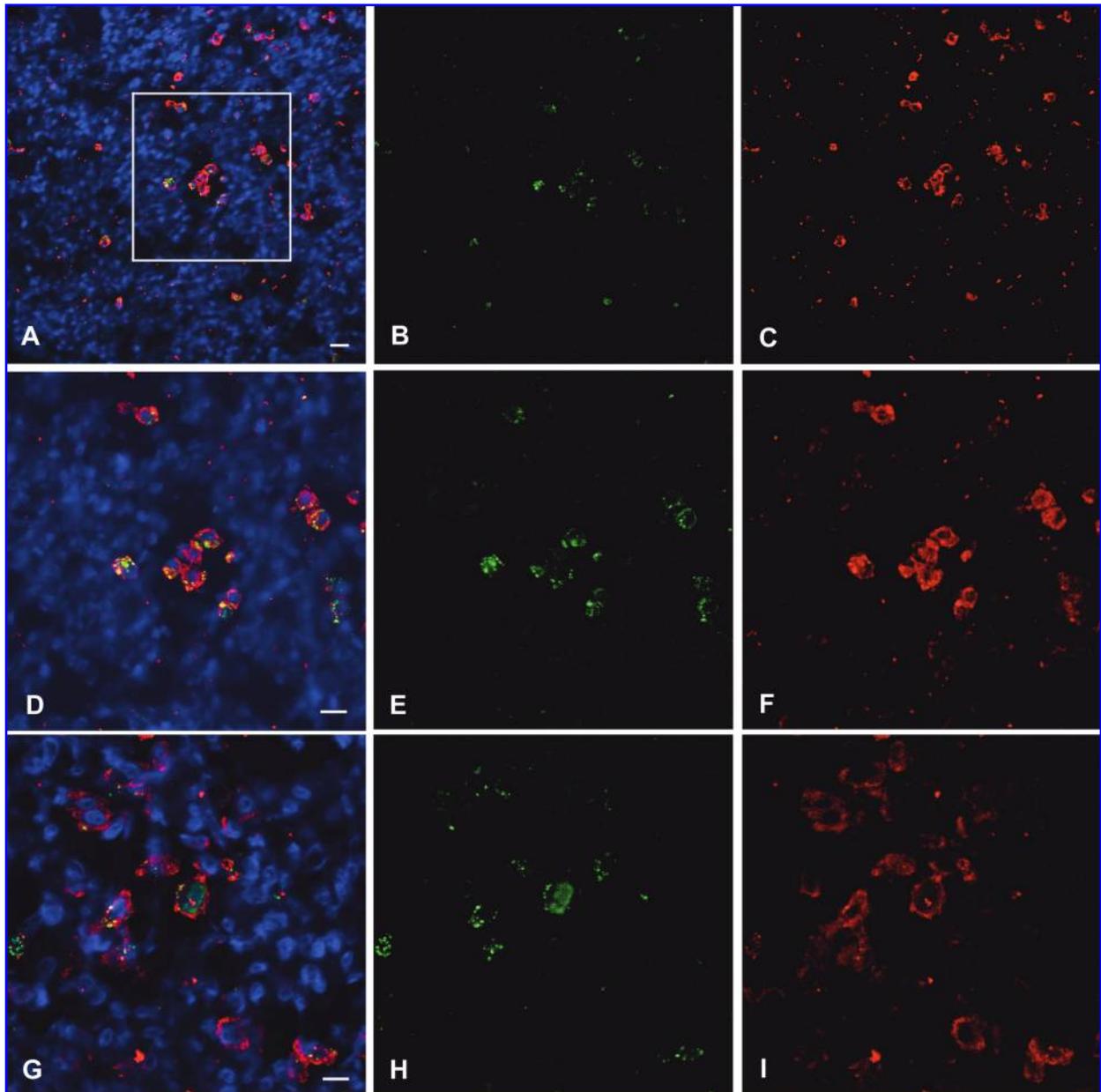
FIG. 1. BRSV antigen colocalizes with CD11c$^+$ cells in the lungs of neonatal lambs. Neonatal ovine lung cryosections were immunofluorescently stained with a primary antibody directed against CD11c (**C**, **F**, and **I**) followed by a secondary antibody fluorescently labeled with Alexa Fluor 633 (red). Directly conjugated BRSV–FITC (**B**, **E**, and **H**) antibody (green) was used to identify cells containing BRSV. Coexpression of CD11c and the virus is shown by yellow. Multicolor immunofluorescence staining shows dual-labeled DCs (**A**, **D**, and **G**) and nuclear staining with DAPI (blue). The enlarged view (**D–F**) is of the inset in (**A**) and an additional and different enlarged view (**G–I**) reveals more CD11c and BRSV colocalization. Data are representative of two experiments with 12 infected lambs. Scale bars: (**A–C**) 20 μm; (**D–I**) 10 μm.

Production of immunomodulatory IL-4 and IL-10 by lung DCs after BRSV infection

Cytokine gene expression was quantified on the basis of SYBR green chemistry, using primers specific for proinflammatory and immunomodulatory genes. A less than 2-fold increase in proinflammatory mediators IL-1β, tumor necrosis factor (TNF)-α, IL-6, IL-8, and IL-12p40 was detected in isolated lung DCs from day 3 and 5 postinoculation lambs (Fig. 5). In addition, similar results were seen in AMs, although expression of IL-6 and IL-12p40 mRNAs was higher than in lung DCs. Of note, a
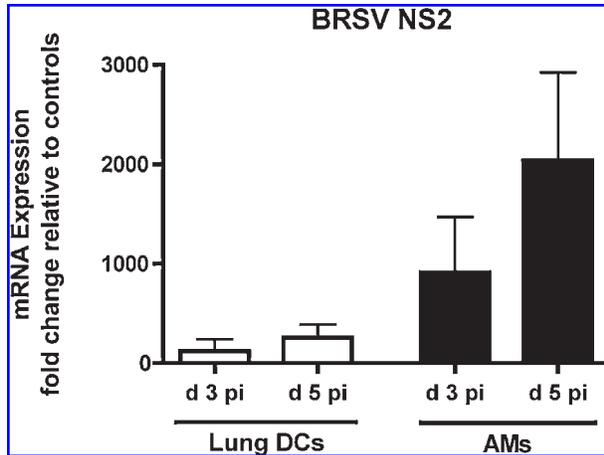
FIG. 2. BRSV replicates within lung dendritic cells (DCs) and alveolar macrophages (AMs). Isolated lung DCs and AMs were lysed in RLT buffer and total RNA was isolated. Oligo(dT) cDNA and BRSV NS2-specific primers with SYBR green chemistry were used for quantification of viral replication. Lung DCs supported BRSV replication, but less so than AMs. Data are representative of two experiments with six lambs per treatment group. Pi, Postinoculation.

striking induction of IL-4 mRNA transcripts was detected in lung DCs at both 3 and 5 d postinoculation. Moreover, a 10- to 15-fold increase in AM IL-4 mRNA transcription was observed, but this was lower than the levels in the lung DCs. Finally, we observed a 2- to 4-fold increase in IL-10 mRNA transcripts in lung DCs. Of note, no significant increases in cytokine or chemokine mRNA expression were observed in samples recovered from DCs or AMs on day 3 compared with day 5 postinoculation. Taken together, these results show that there is an early bias toward the production of immunomodulatory IL-4 and IL-10 gene transcripts in lung DCs and AMs isolated from BRSV-infected lambs.

DISCUSSION

In the present studies, we used a previously described neonatal lamb model of RSV infection (22,24,25,29–31) in which the pathogenesis and lesions observed (bronchiolitis, inflammatory cell infiltrates, and goblet cell hyperplasia) are similar to RSV infection in children. Interestingly, alveolar development begins prenatally in both humans and lambs, whereas mice have postnatal alveolar development (11). Therefore, neonatal lamb lungs are similar developmentally to neonatal humans and represent a comparable model for RSV infection. When comparing species-specific RSV strains, BRSV and ovine RSV are phylogenetically the most closely related sequences to the human RSVs. These ruminant RSV strains are not completely different

viruses but rather represent separate antigenic subgroups of the human RSV strains (7,10). The conservation pattern of amino acids in the human RSV subgroups is similar to that seen in BRSV, but some differences are observed (10). Overall, molecular cloning and field studies have demonstrated that human and bovine RSV are closely related, share common antigenic epitopes, and induce similar pathogenesis (2,34,46). Lambs can be naturally infected with BRSV and are fully susceptible to experimental BRSV infections (2). Taken together, lambs are an excellent model for experimental RSV infection.

At present, there are no data available examining *in vivo*-derived lung DCs after RSV infection in a neonatal animal model (3). The results derived from neonatal lambs in the present study demonstrate that BRSV antigen and CD11c colocalize on cells within the lung parenchyma and alveolar spaces. Three and 5 d postinoculation with BRSV, isolated CD11c$^+$ lung DCs and AMs contained NS2 mRNA transcripts, indicative of BRSV replication within these cells. After *in vivo* BRSV infection, on day 3 postinoculation, lung DCs increased CD14 expression and exhibited decreased endocytosis of OVA–FITC. By day 5 postinoculation, lung DCs expressed low to moderate levels of DEC-205, CD14, and MHC class II, whereas higher expression of MHC class I and CD80/86 were detected. Inflammatory mediators IL-1β, IL-6, IL-8, IL-12p40, and TNF-α were transcribed in small amounts within lung DCs, whereas a 3.5- and 75-fold increase in gene transcripts of IL-10 and IL-4 were quantitated within these cells. Higher levels of IL-6 and IL-12p40 were transcribed in isolated AMs compared with lung DCs. Overall, BRSV leads to a type II skewing of the lung DC response by inducing IL-4 and IL-10 mRNAs. The virus may inhibit the initial maturation of the lung DCs, because CD80/86 and MHC class I expression is not increased until day 5 after infection. Overall, RSV may modulate lung DCs by inducing an immunomodulatory cytokine response.

Identification and magnetic sorting of DCs were performed with the CD11c monoclonal antibody (mAb) BAQ153A, which specifically labels a majority of afferent lymph DCs and few lung tissue macrophages in the ovine species (15). Our results demonstrate the colocalization of BRSV antigen and CD11c$^+$ cells within frozen lung tissue sections from BRSV-infected lambs. These results are consistent with other *in vivo* data based on the use of isolated cells from nasal washes of infected children and indicating that HLA-DR-positive cells coexpress RSV fusion (F) protein (13). In addition to staining for viral antigen, we used detection of viral mRNA to address whether BRSV was capable of replicating within lung DCs *in vivo*. Three and 5 d postinoculation of BRSV, our results indicate that both lung DCs and AMs are permissive to BRSV infection and support repli-
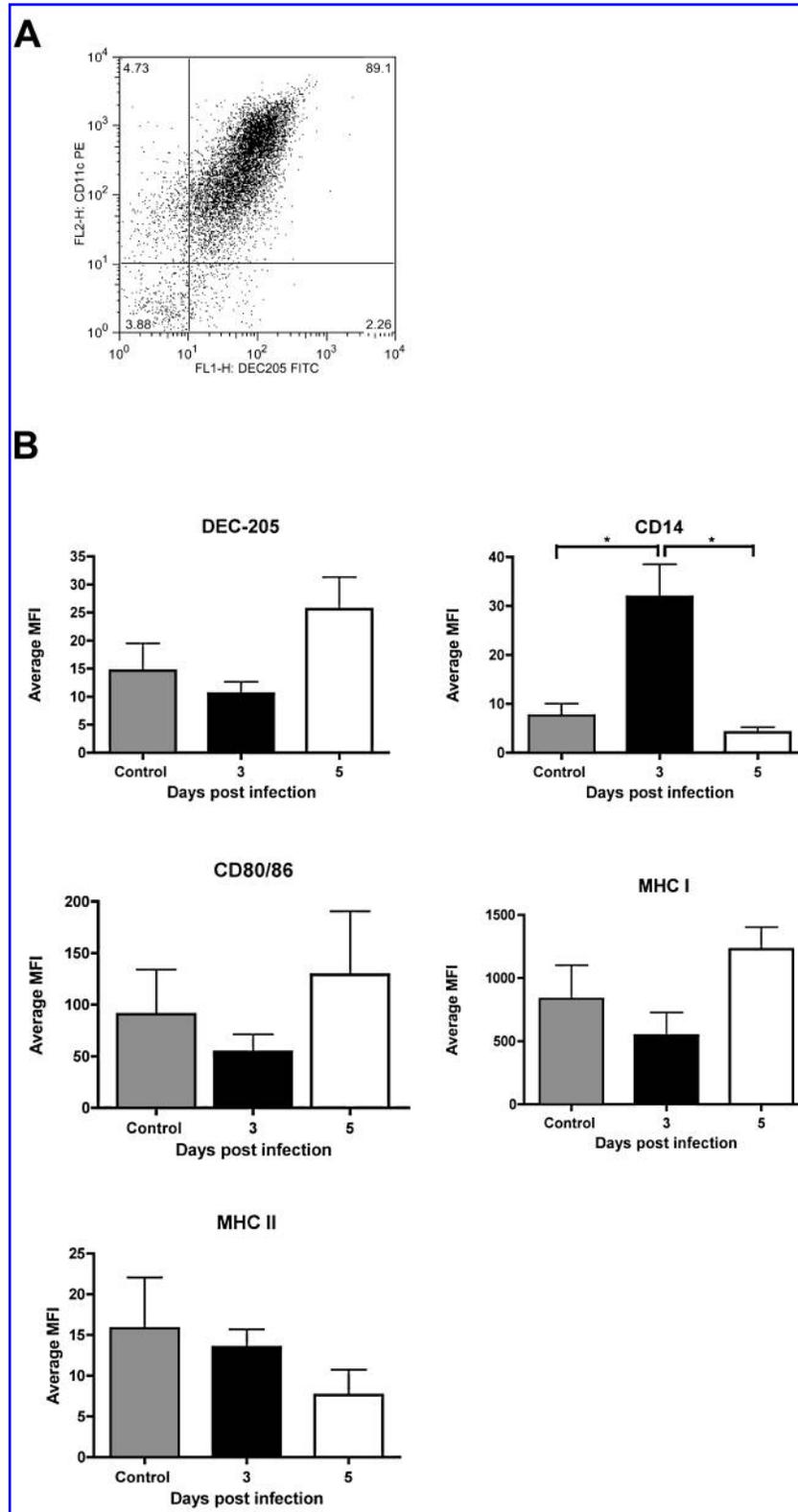
FIG. 3. BRSV infection of neonatal ovine lungs induces modulation of surface antigen expression on CD11c^{+} lung DCs. Lung DCs were sorted magnetically to purify CD11c^{+} cells from digested neonatal lung suspensions from controls, day 3 postinoculation lambs, and day 5 postinoculation lambs. Flow cytometric analysis of surface antigen expression revealed that 89% of the sorted lung DCs coexpressed CD11c and DEC-205 (**A**). Statistical analysis indicated a higher mean fluorescence intensity (MFI) expression of CD14 on lung DCs isolated on day 3 postinoculation when compared with controls or day 5 postinoculation lung DCs (**B**) (*$p < 0.05$). MHC class I and CD80/86 expression was higher on isolated lung DCs from day 5 postinoculation lambs. Each column represents the average of the MFI staining for the indicated cell surface antigen minus the MFI staining of the appropriate isotype control. Data are representative of two experiments with six lambs per experimental group.
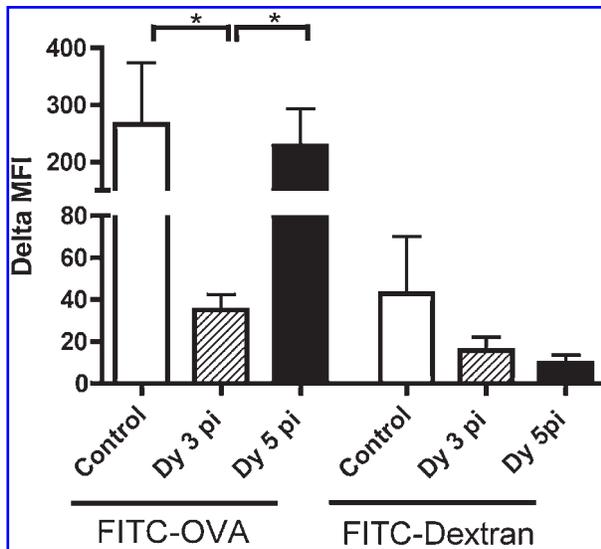
FIG. 4. Uptake of FITC–ovalbumin (FITC–OVA) and FITC–dextran by isolated lung DCs. Magnetically sorted CD11c$^+$ lung DC cell numbers were adjusted to 1×10^5 cells/mL and incubated with either FITC–OVA or FITC–dextran for 1 h at 37 or 4°C. Data are expressed as the change in mean fluorescence intensity (MFI) values, that is, the 37°C MFI values minus the 4°C MFI values. Statistical analyses indicate that lung DCs on day 3 postinoculation endocytosed less FITC–OVA than either the controls or day 5 postinoculation lung DCs (*$p < 0.05$). Data are representative of two experiments with six lambs per treatment group.

cation, as shown by an increase in NS2 mRNA transcripts. These results are in agreement with *in vitro* data demonstrating that monocyte-derived DCs (MDDCs) can be productively infected with RSV (14). In addition, *in vitro* data derived from monocyte- or cord blood-derived DCs indicate that these cells are permissive to RSV infection (1,21). Taken together, these results demonstrate that isolated lung DCs and AMs are permissive to RSV infection and support viral replication *in vivo*.

Previously, we have reported that neonatal MDDCs and lung tissue-derived DCs differ phenotypically. Lung DCs express higher levels of CD80/86, MHC class I, and MHC class II compared with mature MDDCs (9). In addition, levels of CD14 expressed on lung DCs are lower than levels expressed on macrophages (9). After *in vivo* BRSV infection, we report here a significant increase in CD14 expression on day 3 postinoculation. This observation is reminiscent of data demonstrating an increase in Toll-like receptor (TLR)4 expression on monocytes isolated from RSV-infected children during the acute phase of infection (12). Together, these data are supported by the known trimeric clustering of CD14, MD2, and TLR4 on the surface of cells (39). In contrast, it was noted that CD14 expression on lung DCs isolated 5 d postinoculation was similar to levels observed on control lung DCs. Given the dynamic nature of DCs, it is not clear at present whether DCs present in the lung on day 3 are the same population present on day 5. Expression of DEC-205, MHC class I, and CD80/86 was not significantly increased relative to controls, although a trend for increased expression of these molecules by day 5 postinoculation can be observed. MHC class II was expressed at low levels on lung DCs from BRSV-infected lambs and control lambs. There was no observed phenotypic modulation of bovine MDDCs exposed to BRSV (Snook strain) as evidenced by the lack of altered MHC class I, MHC class II, and CD80/86 expression (44). However, infection of human monocyte- or cord blood-derived DCs with human RSV resulted in increased expression of MHC class I and II, CD80, and CD86 (14,21). Further studies are warranted to investigate modulation of DC surface antigens after RSV infection.

Mature DCs are potent inducers of an adaptive immune response to microbial infection in the lung microenvironment. As a functional measure of maturation state, isolated lung DCs from control and BRSV-infected lambs were incubated with FITC–OVA and FITC–dextran. Immature DCs are known to actively take up antigen via macropinocytosis and receptor-mediated endocytosis (20). The findings indicate that isolated lung DCs from day 3 postinfection lambs phagocytosed significantly less FITC–OVA than did controls, with no difference on day 5. A potential explanation for these data is an initial inhibition of DC antigen uptake early in RSV infection, but recovery of DC tracer endocytosis by day 5. On the other hand, it is plausible that mature day 3 DCs have migrated to the draining lymph node and there are more immature DCs present in the lung on day 5. The observation of reduced tracer uptake is supported by work in mice infected with RSV, where isolated lung DCs were reported to have low FITC–dextran uptake after 10 d of viral infection (3). In contrast to FITC–OVA, uptake of FITC–dextran via mannose receptor-mediated endocytosis was lower and not significantly altered.

RSV infection is a known inducer of inflammatory and immunomodulatory mediators in APCs *in vitro* (1,14,44). In the present study, we quantitated mRNA levels present in isolated lung DCs and AMs after BRSV infection. Alveolar macrophages produced more IL-6 mRNA transcripts compared with lung DCs. In addition, the induction of IL-12p40 mRNA within AMs was greater than levels detected within lung DCs. In general, BRSV infection induced a 1.5- to 2-fold increase in the expression of IL-1β, IL-6, IL-8, IL-12p40, and TNF-α mRNA transcripts within lung DCs. Previous studies using human monocyte- or cord blood-derived DCs report similar cytokines and chemokines being produced after RSV infection (1,14). Our results are similar to the cytokine mRNA profile observed *in vitro* by BRSV infection of bovine MDDCs (44). Interestingly, we observed increases in both IL-4 and IL-10 gene transcription within
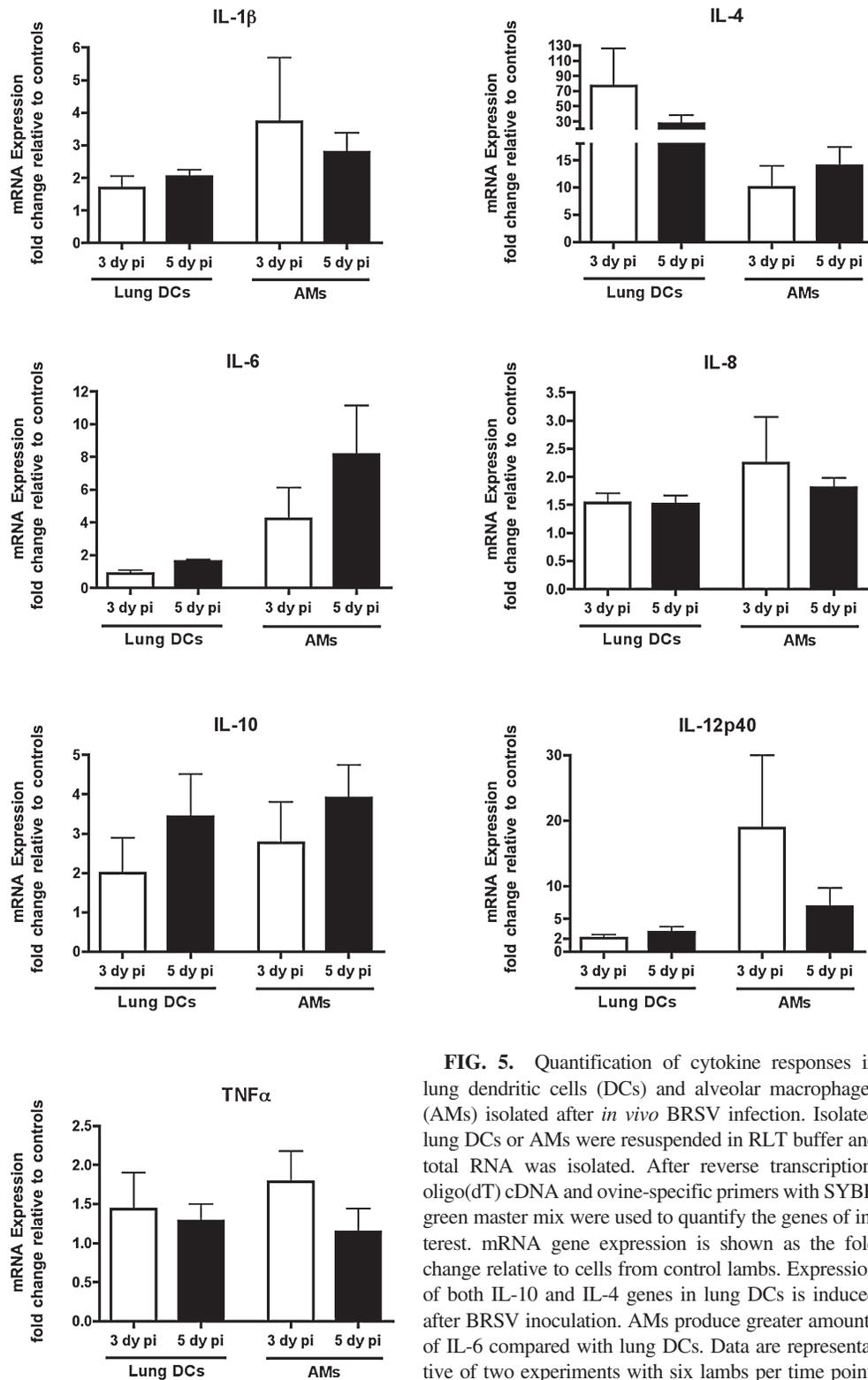
FIG. 5. Quantification of cytokine responses in lung dendritic cells (DCs) and alveolar macrophages (AMs) isolated after *in vivo* BRSV infection. Isolated lung DCs or AMs were resuspended in RLT buffer and total RNA was isolated. After reverse transcription, oligo(dT) cDNA and ovine-specific primers with SYBR green master mix were used to quantify the genes of interest. mRNA gene expression is shown as the fold change relative to cells from control lambs. Expression of both IL-10 and IL-4 genes in lung DCs is induced after BRSV inoculation. AMs produce greater amounts of IL-6 compared with lung DCs. Data are representative of two experiments with six lambs per time point.

lung DCs after BRSV infection. Increased levels of I L-4 protein in nasal washes from RSV-infected children have been reported although the exact cell source was not determined (13). Human plasmacytoid DCs isolated from peripheral blood mononuclear cells were infected with RSV and were reported to produce IL-4 after a 24-h exposure to the virus (14). Thus, it is plausible that DCs are a source of IL-4 during RSV infection in neonates.

Accumulating experimental data suggest that severe RSV infections early in childhood may predispose these children later on to develop recurrent wheezing, asthma, or other chronic pulmonary disorders (37). The progression of RSV disease is associated with a type II cytokine response and we report here that lung DCs are a likely source of IL-4 and IL-10 observed after infection. It has been suggested that the type II cytokine response after RSV infection predisposes these children to future pulmonary problems. Studies have associated severe RSV infections with IL-4 haplotypes and/or polymorphisms (5,19), further supporting the importance of IL-4 during RSV infections. Taken together, our results suggest that *in vivo* RSV infection may induce neonatal lung DCs to produce nonprotective cytokine responses that may result in enhanced host susceptibility to secondary infections or chronic pulmonary diseases such as allergies or asthma.

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