Respiratory Syncytial Virus–Induced Chemokine Production: Linking Viral Replication to Chemokine Production In Vitro and In Vivo

Allison L. Miller,1 Terry L. Bowlin,2 and Nicholas W. Lukacs1
1University of Michigan Medical School, Department of Pathology, Ann Arbor; 2Microbiotix, Worcester, Massachusetts

Respiratory syncytial virus (RSV) is a negative-sense, single-strand RNA virus that can initiate severe bronchiolitis in infants, as well as in elderly adults. Although RSV preferentially infects and replicates in the airway epithelium, studies have shown that RSV has the ability to infect and, to a limited extent, replicate in alveolar macrophages. In the present study, we sought to characterize the RSV-induced chemokine production in vitro and in vivo, because chemokines have been shown to contribute to both the inflammation and pathophysiology of disease. Our results show that RSV-infected airway epithelial cells and alveolar macrophages display differential profiles of chemokine production: airway epithelial cells produce CCL2/monocyte chemoattractant protein–1, CCL5/RANTES, CXCL10/gamma interferon inducible protein-10, and keratinocyte cytokine (KC); and alveolar macrophages up-regulate CCL5 and macrophage inflammatory protein (MIP)–2 after RSV infection. In vivo, we observed the induction of CCL2, CCL3/MIP–1alpha, CCL5, CXCL10, and KC after RSV infection. In the present study, we also addressed the necessity for viral infection and/or replication in chemokine induction by use of ultraviolet (UV)–inactivated RSV, as well as RSV inhibitors of binding/infection and replication, that is, NMSO3, a sulfated sialyl lipid compound, and ribavirin, respectively. Our results suggest that viral replication is necessary for optimal chemokine production.

Acute viral bronchiolitis is a significant health concern in both developing and industrialized countries. Although many viruses can precipitate bronchiolitis, studies have shown that respiratory syncytial virus (RSV) is the cause of ~70% of all cases of viral bronchiolitis [1]. Severe RSV infection is characterized by epithelial cell necrosis, lymphocytic inflammation, submucosal edema, and obstruction of the bronchioles by mucus, cellular debris, and fibrin [2]. Typically, viral infections that induce severe bronchiolitis occur as epidemics during the winter and present as an upper respiratory infection that is followed by wheezing, rapid respirations, and chest retractions [3, 4]. Other studies have shown that chemokines, such as CXCL8/interleukin (IL)–8, CCL2, CCL3, and CCL5 [5–8], are induced in respiratory secretions after RSV infection and, in certain cases, have been shown to correlate with disease [9–11]. To better understand the pathogenesis of RSV-induced disease, we characterized RSV-induced chemokine production in vitro and in vivo and related these findings to viral binding/infection and replication.

Chemokines are a family of chemotactic cytokines that are recognized for their role in leukocyte trafficking [12]. However, more recently, chemokines have been shown to participate in processes, such as cellular proliferation [13], angiogenesis [14], development [15], and disease pathobiology [16]. Chemokines can direct the migration of leukocytes from the vasculature into an affected area by forming a gradient. Once a gradient is established, cells that express chemokine receptors can move along the gradient to the source of chemokine production. Therefore, one can imagine that the chemokine profile that follows a particular stimulus will dictate the subsequent inflammatory response. Previous
studies have shown that RSV has the potential to up-regulate chemokine production in model systems [17–22]. Therefore, targeting RSV-induced chemokines may have the potential to prevent the excessive inflammation that leads to pathophysiological complications, such as mucus overproduction and airway hyperresponsiveness.

Efforts aimed at developing successful therapeutics to combat RSV infection have been in progress for decades. Despite attempts at vaccine and drug development and antiviral regimens, there are still no effective treatments for use in the clinic. Typically, infants hospitalized for RSV are given supplemental oxygen, and mucus is periodically suctioned to remove excess nasopharyngeal secretions. Many other treatment modalities have been tested in the clinic, including β2 agonists, racemic epinephrine, corticosteroids, anti–RSV antibody treatments, and ribavirin [3, 4, 23]. Of these, ribavirin has received the most attention. Ribavirin is a nucleoside analogue that has been shown to inhibit the synthesis of viral proteins, thus preventing viral replication. Although studies have shown that ribavirin can decrease the clinical severity of RSV disease, others have questioned the efficacy of this drug. In addition, the cost of ribavirin, coupled with the difficulty encountered with administration of the drug, has sustained the search for novel RSV treatments [4]. One such drug is NMSO3. NMSO3 is a sulfated sialyl lipid compound that has been shown to have antiviral activity against RSV, adenovirus, and rotavirus [24–26]. Kimura et al. [24] demonstrated that NMSO3 treatment prevented RSV infection both in vitro and in vivo and did not exhibit cellular toxicity. Their study suggested that NMSO3 prevented viral binding/adsorption to the cell surface and, thus, prevented RSV infection.

To gain insight into the spectrum of RSV-induced chemokine production and the potential cellular sources of chemokine production, we conducted in vitro studies in airway epithelial cells and alveolar macrophages. These 2 cell populations were chosen, because they are readily infected by RSV. In addition, we used a BALB/c mouse model of RSV infection to monitor chemokine production and relate it to viral replication in vivo. We also used the antiviral agents ribavirin and NMSO3 to sort the viral requirements necessary for chemokine induction.

**MATERIALS AND METHODS**

**RSV propagation.** Human RSV strain A2 was propagated in HepG2 cells (American Type Culture Collection). HepG2 cells were grown in a T-150 flask until they were ~40% confluent. The cells were inoculated with 2.5 × 10^3 pfu of RSV in 10 mL of Dulbecco’s minimal essential medium (DMEM)/10% fetal calf serum (FCS). Virus was adsorbed to the cells for 2 h at 37°C, and then an additional 10 mL of medium was added to the flask. After 3 days in culture at 37°C, 5 mL of the medium was pipetted from the flask and discarded. The flask was frozen at −80°C. The flask was subsequently thawed, and the cell lysate was clarified and aliquotted.

**RSV plaque assay.** A plaque assay was done to determine viral titers. Vero 81 cells were grown until they were ~90% confluent. The viral stock was serially diluted and added to each well in duplicate. After a incubation for 2 h at 35°C, virus was removed from the cells and was replaced with 0.9% methylocellulose/Eagle’s MEM (EMEM)/5% fetal bovine serum. The plates were incubated for 4–5 days at 37°C, during which syncytia were allowed to form. The methylcellulose solution was removed from each of the wells, and the cells were fixed with cold 80% methanol for 60 min at −80°C. The methanol was removed, and the plates were frozen for 1 h at −80°C, to lyse the cells before the plates were stained for RSV proteins. Blocking was done by use of 5% dry milk/PBS. The 1st antibody used was goat anti–RSV polyclonal antibody (Chemicon International), at a dilution of 1:500. The 2nd antibody used was rabbit anti–goat antibody conjugated to horseradish peroxidase (Sero-tec), at a dilution of 1:100. The antibody incubations were performed for 1 h at 37°C. One-step chloronapthol (Pierce) was added to each well, and the cells were incubated for 10 min at room temperature. Cells were washed with PBS, and plaque-forming units were counted.

**RSV infection.** Cultured cells were exposed to 1 × 10^3 pfu of human RSV A2 strain wild-type (RSV) or UV-inactivated RSV (UV-RSV) (MOI, ~0.1–0.2). UV inactivation was performed in a laminar flow hood for 30 min, with the virus being ~5 inches from the UV light source. RSV or UV-RSV (2.5–5 × 10^4 pfu) was administered via an intratracheal route to BALB/c mice. The mice were anesthetized with 0.3 mL of ketamine/xylazine (stock solution: ketamine, 0.75 mg/mL; xylazine, 0.05 mg/mL), which was injected intraperitoneally. Tracheotomy was performed, and viral infection was accomplished by delivering RSV or UV-RSV via a Hamilton syringe. The incision was closed with surgical staples, and mice were allowed to recover.

**Real-time polymerase chain reaction (PCR).** RNA was isolated from the upper-right lobes of the lung by use of Trizol (Invitrogen) and was reverse transcribed by use of a Moloney murine leukemia virus reverse-transcription kit (Invitrogen). Chemokine expression in lung homogenates was monitored by use of the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal positive control. The primer/probe sets for murine GAPDH, CCL2/monocyte chemoattractant protein (MCP)−1, and CCL5/RANTES were purchased as predeveloped kits from Applied Biosystems. Real-time PCR primers to detect RSV G protein and CXCL10/gamma interferon inducible protein (IP)−10 were custom designed. The primer/probe set used to detect RSV mRNA has been described elsewhere [16]. The primer/probe set used to detect CXCL10/
Figure 1. Respiratory syncytial virus (RSV) induces chemokine production in airway epithelial cells. LA4 airway epithelial cells were left uninfected (control) or were infected with human RSV A2 strain wild-type (RSV) or UV-inactivated RSV (UV-RSV). Tissue culture supernatants were collected 2, 9, 24, 48, and 72 h after infection, and RNA was harvested from the cells for analysis of viral gene transcription and chemokine production. Fold changes in RSV G protein expression were calculated relative to cells infected with RSV or UV-RSV, 2 h after infection. A statistically significant increase was observed in RSV G protein mRNA expression relative to RSV G protein mRNA expression 2 h after infection with RSV (A; *P < .05). CCL2, CCL5, and CXCL10 were transcriptionally up-regulated after infection with replication-competent RSV (B, D, and F). Fold differences in chemokine mRNA levels were calculated relative to uninfected cells at each time point analyzed. CCL2, CCL5, CXCL10, and keratinocyte cytokine (KC) protein levels were up-regulated after RSV, and not UV-RSV, infection (C, E, G, and H). A statistically significant increase in chemokine production relative to uninfected cultures at each time point analyzed was observed (*P < .05). Data are representative of 3 experiments (n = 4 mice/group/experiment). IP, gamma interferon inducible protein; MCP, monocyte chemotactic protein.
Figure 2. Respiratory syncytial virus (RSV) infection of alveolar macrophage results in viral gene expression and chemokine production. MHS cells were infected with human A2 strain wild-type RSV (RSV) or UV-inactivated RSV (UV-RSV), and RNA was harvested from the cell analysis of RSV G protein mRNA expression 2, 9, 24, 48, and 72 h after infection. Fold changes in RSV G protein mRNA expression were calculated relative to cells infected with RSV or UV-RSV 2 h after infection (A). A statistically significant increase was observed in RSV G protein mRNA expression, compared to RSV G protein mRNA expression, 2 h after infection with RSV \( (P < .05) \). Data are representative of 2 experiments \( (n = 4 \) mice/group/experiment). MHS cells were left uninfected (control) or were infected with RSV or UV-RSV. Tissue culture supernatants were collected 2, 9, 24, 48, and 72 h after infection, and RNA was harvested from the cells. CCL5 was transcriptionally up-regulated after infection with replication-competent RSV \( (B) \). Analysis of macrophage inflammatory protein (MIP)-2 mRNA after RSV or UV-RSV infection was not done (ND), because real-time polymerase chain reaction (PCR) primers/probe were not available. Fold differences in chemokine mRNA levels were calculated relative to uninfected cells at each time point analyzed. CCL5 \( (C) \) and MIP-2 \( (D) \) protein levels were up-regulated after RSV infection and, to a lesser extent, after UV-RSV infection. A statistically significant increase was observed in chemokine production in RSV-infected cultures relative to uninfected cultures at the time point being analyzed \( (P < .05) \). Data are representative of 3 experiments \( (n = 4 \) mice/group/experiment).

IP-10 was as follows: CXCL10/IP-10 forward primer, ACTGGA-GTGAAGCCACGCA; CXCL10/IP-10 reverse primer, TGATGGAGAGGCTCTCTGC; and CXCL10/IP-10 probe, 5'-FAM-CCCGTGTGCGATGGATGT-3' TAMARA. Gene expression was normalized to GAPDH before the fold change was calculated.

**RSV virion isolation from murine lungs.** To quantitate RSV virions in the lungs of RSV-infected mice, mice were killed, and their lungs were harvested. Lung specimens were resuspended in 1 mL of Dulbecco’s modified Eagle medium (DMEM)/15% FBS/0.1 g of tissue before being ground with a mortar and pestle. Sterile alundum (Fisher Scientific) was sprinkled onto the lungs before grinding to help break up lung tissue. Lung homogenates were clarified by use of centrifugation, diluted in EMEM/5% FCS, and used in a plaque assay, as described above.

**Chemokine ELISAs.** Chemokine-induced cell-culture supernatants or lung tissue were detected by ELISA, as described elsewhere \([27, 28]\). Supernatants from LA4 cells and MHS cells were subjected to ELISA for CCL2, CCL5, CXCL10, CXCL8, and keratinocyte cytokine (KC).
RSV induces CCL5 and macrophage inflammatory protein (MIP)–2 from alveolar macrophages in vitro. Although alveolar macrophages do not support the majority of viral replication during RSV infection, they are capable of being infected. Furthermore, several studies have suggested that these cells can be chronically infected, with a slow but steady level of continuous viral replication [29, 30]. MHS cells, a BALB/c-transformed alveolar macrophage cell line, were infected with 1 × 10^6 pfu of RSV, and, 24, 48, and 72 h after infection, cell-culture supernatants were harvested, and RNA was isolated from the cells. As described above, real-time PCR assays were used to measure viral mRNA and chemokine RNA levels, and ELISAs were used to measure protein levels. Viral G protein transcription was induced in MHS cells 24 h after infection and increased steadily up to 72 h after infection (figure 2A). There was no induction of RSV G protein mRNA observed after exposure to UV-RSV. The pattern of viral transcription in MHS cells was strikingly different, compared with that observed in airway epithelial cells, because the levels of RSV G protein expression were ~10–100 fold less in alveolar macrophages, and G protein levels peaked late after infection.

After infection with RSV, only 2 chemokines were induced in MHS cells, CCL5 (figure 2B and 2C) and MIP-2 (figure 2D). Optimal chemokine protein production was specific to fully competent RSV infection; however, UV-RSV did significantly induce, albeit to a much lesser extent, CCL5 protein production 48 h after infection. Chemokine production that resulted from UV-RSV administration was probably a result of a small population of virions that maintained their ability to infect and replicate, even after exposure to a UV light source. Chemokine production appeared to be significantly induced at time points that coincided with significant RSV G protein mRNA expression.

RSV induces biphasic chemokine production in BALB/c mice in vivo. BALB/c mice were intratracheally administered RSV or UV-RSV, and, 1–6, 8, and 12 days after infection, mice were killed, and their lungs were harvested for RNA and protein analyses. RSV G protein transcription steadily increased after viral inoculation and peaked 6 days after infection, before declining at 8 and 12 days after infection (figure 3). In separate experiments, mice were infected with RSV, and, 1, 4, 6, 8, and 12 days after infection, lungs were harvested to quantify RSV plaque-forming units. Results from this study paralleled the RSV G protein mRNA data, with peak numbers of RSV virions
in the lung 6 days after infection (figure 3). RSV virions were undetectable 12 days after infection, which suggests that the virus had been cleared.

To monitor chemokine protein levels after RSV infection, the left lobes of the lungs were isolated from uninfected mice and mice that had been exposed to RSV or UV-RSV. They were subsequently homogenized and used in ELISAs. We observed a significant induction of CCL2, CCL3, CCL5, CXCL10, and KC protein in the lungs of mice after RSV infection, compared with that in mice that received UV-RSV (figure 4A–E). Interestingly, these chemokines were produced in a biphasic pattern, with chemokines elevated at 1 day after initial infection and elevated again 6 and/or 8 days after infection. Exposure to UV-RSV resulted in the early induction of CCL2 and KC protein; however, it was significantly less than that observed with RSV. Although it appeared that infection with UV-RSV induced CCL5 protein 8 days after infection, levels of this chemokine were not elevated significantly above background levels of CCL5 in the lungs of uninfected mice. CCL6, CCL11, CCL22/MDC, and CXCL5/lipopolysaccharide-induced CXC chemokine (LIX) were significantly induced in the lungs of RSV-infected mice, compared with that in uninfected mice, but did not differ from chemokine levels in the lungs of mice receiving UV-RSV. In most cases, induction of these chemokines after RSV infection appeared biphasic, as described above.

Ribavirin and NMSO3 prevent RSV-induced chemokine production. Ribavirin is currently the only antiviral drug that is approved to treat infants hospitalized for severe RSV bronchiolitis. To determine whether ribavirin impacts RSV-induced chemokine production, we treated cells with concentrations of ribavirin (1–100 μmol/L) or NMSO3 (0.5–50 μmol/L) immediately before infecting LA4 cells with RSV. Twenty-four hours after

Figure 4. Biphasic chemokine production after in vivo respiratory syncytial virus (RSV) infection. Mice were left uninfected (control) or were infected with human A2 strain wild-type RSV (RSV) or UV-inactivated RSV (UV-RSV). Mice were killed 1–6, 8, and 12 days after infection, and the left lobes of the lungs were harvested for protein analysis. CCL2 (A), CCL3 (B), CCL5 (C), CXCL10 (D), and keratinocyte cytokine (KC) (E) were up-regulated after RSV infection. Data are the chemokine concentration levels relative to total protein content (ng/mL/mg total protein), as determined by use of a Bradford assay. Statistically significant increases were observed in chemokine levels in the lungs of RSV-infected mice, compared with those in both uninfected and UV-RSV–infected mice (*). IP, gamma interferon inducible protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein.
Figure 5. Prevention of respiratory syncytial virus (RSV)–induced chemokine production in airway epithelial cells after treatment with ribavirin. LA4 airway epithelial cells were treated with ribavirin and were left uninfected or were infected with RSV. Transcription of RSV G protein (A), CCL2 (B), CCL5 (D), and CXCL10 (F) was suppressed 24 h after infection, with ribavirin treatment administered in a dose-dependent fashion. RSV G protein mRNA fold increase/decrease levels were calculated relative to RSV-infected cells treated with 100 μmol/L ribavirin. A statistically significant decrease in RSV G protein transcripts relative to RSV-infected cells that were not treated with ribavirin was observed (*). Fold increase/decrease in chemokine transcription was calculated relative to uninfected cells that had not been treated with ribavirin. Asterisk indicates a statistically significant decrease in chemokine transcripts relative to RSV-infected cells that were not treated with ribavirin. ELISA analysis revealed that ribavirin also was able to inhibit CCL5 (E) and CXCL10 (G), but not CCL2 (C), protein production in a dose-dependent fashion 24 h after infection. Data are representative of 2 experiments (4 mice/group/experiment). IP, gamma interferon inducible protein; MCP, monocyte chemoattractant protein.
infection, cell-culture supernatants were harvested for ELISA analysis, and cells were used for RNA extraction. Ribavirin inhibited RSV G protein transcription in LA4 airway epithelial cells at all concentrations tested in a dose-dependent fashion (figure 5A). Fold change in RSV G protein transcription was calculated relative to the RSV-infected LA4 airway epithelial cells that were treated with 100 μmol/L ribavirin. Ribavirin had similar effects on the expression of CCL2, CCL5, and CXCL10, because almost all concentrations of the drug significantly reduced transcription of these gene products (figure 5B, 5D, and 5F). Fold change in chemokine levels was calculated relative to uninfected LA4 airway epithelial cells. Ribavirin-mediated decreases in CCL5 (figure 5E) and CXCL10 (figure 5G) mRNA, but not CCL2 (figure 5C) mRNA, were mirrored at the protein level. Ribavirin was ineffective at suppressing CCL2 protein levels 24 h after infection, because RSV does not induce CCL2 protein until 48 h after infection (as shown in figure 2).

NMSO3 is a newly developed antiviral compound with proven efficacy against RSV, rotavirus, and adenovirus infection in model systems [24–26]. We chose to examine the effects of this drug on chemokine production, because NMSO3 has been shown to prevent the binding/infection of RSV in cell culture. NMSO3 was tested at a lower concentration than ribavirin, because Kimura et al. [29] found that, at a lower concentration, NMSO3 was tested at a lower concentration than ribavirin, shown to prevent the binding/infection of RSV in cell culture. This drug on chemokine production, because NMSO3 has been proven efficacy against RSV, rotavirus, and adenovirus infection in model systems [24–26]. We chose to examine the effects of this drug on chemokine production, because NMSO3 has been shown to prevent the binding/infection of RSV in cell culture. NMSO3 was tested at a lower concentration than ribavirin, because Kimura et al. [29] found that, at a lower concentration, this compound was as effective as ribavirin in a plaque-reduction assay. NMSO3 effectively suppressed RSV G protein transcription in airway epithelial cells at all concentrations tested, with the exception of the 0.5-μmol/L dose (figure 6A). Fold change in RSV G protein transcription was calculated relative to the RSV-infected LA4 airway epithelial cells that were treated with 50 μmol/L NMSO3. Similar to ribavirin, NMSO3 significantly reduced the transcription of CCL2, CCL5, and CXCL10 (figure 6B, 6D, and 6F). Fold change in chemokine levels were calculated relative to those of uninfected LA4 airway epithelial cells. NMSO3-mediated decreases in CCL5 (figure 6E) and CXCL10 (figure 6G) mRNA, but not CCL2 (figure 6C) mRNA, were mirrored at the protein level. Similar to what we observed previously for ribavirin, NMSO3 was ineffective at suppressing CCL2 protein levels 24 h after infection, because RSV does not induce CCL2 protein until 48 h after infection (figure 2).

**Differential effects of ribavirin and NMSO3 on RSV G protein expression in alveolar macrophages.** MHS cells were treated with various concentrations of ribavirin or NMSO3 immediately before infection with RSV. At 24 h after infection, RNA was isolated from these cells, and RSV G protein expression was quantitated by use of real-time PCR assay. Ribavirin was unable to inhibit RSV G protein mRNA expression at all concentrations tested. Surprisingly, ribavirin treatment actually enhanced viral protein mRNA expression at the 100-, 50-, and 1-μmol/L doses (figure 7A). In contrast, NMSO3 was able to significantly decrease RSV G protein expression; however, it was only effective at the 50-μmol/L dose (figure 7B).

**DISCUSSION**

The cell-mediated immune response after RSV infection is primarily orchestrated by the airway epithelial cells and alveolar macrophages [31]. RSV infection of these cells induces the secretion of cytokines and chemokines [17–21] that can result in the further activation of the resident lung macrophages, the development of goblet cell hyperplasia, and the recruitment and activation of inflammatory cells. To further elucidate the innate immune response to primary RSV infection, we characterized viral gene expression and related it to chemokine production in airway epithelial and alveolar macrophage cell lines. Our results demonstrated that RSV infection resulted in a cell type–specific pattern of RSV G protein expression and chemokine production. Furthermore, significant induction of viral gene expression and chemokine production required infection with replication-competent virus, which suggests that viral replication is coupled to chemokine up-regulation.

RSV primarily infects and replicates in the airway epithelium that lines the nasal passages, as well as the large and small airways in the lung. After infection, RSV replicates, is packaged, and subsequently leaves the cell by budding from the surface of the infected cell or by fusing with the membrane of a neighboring cell. The latter results in the formation of syncytia, which are considered to be a hallmark of RSV-induced disease. Although the airway epithelial cell is often considered to be the sole target of RSV infection and replication, there is accumulating evidence that RSV also infects and replicates in alveolar macrophages [32, 33]. RSV RNA has even been shown to be present in peripheral blood mononuclear cells in RSV-infected patients [34–36]. In the present study, airway epithelial cells supported significantly more RSV G protein expression than did the alveolar macrophages. However, RSV G protein expression increased with time in the alveolar macrophage cell line. Previous studies have demonstrated that RSV can persist in vitro and in vivo in alveolar macrophages [29, 30], and that RSV persistence can lead to airway hyperresponsiveness and eosinophilia for a duration of 100 days after RSV infection in an animal model [37]. Therefore, it appears that the alveolar macrophages may contribute to RSV-induced disease by facilitating the production of RSV-encoded proteins and/or allowing for RSV antigens to persist after peak disease has subsided.

The profile of chemokines up-regulated in response to RSV infection of airway epithelial cells and alveolar macrophages, in vitro, mirrored the chemokine production profile, in vivo, with the exception of MIP-2. In addition, CCL3, CCL6/C10, CCL11, CCL22, and CXCL5 also were up-regulated after RSV infection in vivo. Of interest, viral replication was not required...
Figure 6. Prevention of respiratory syncytial virus (RSV)–induced chemokine production in airway epithelial cells after treatment with NMSO3. LA4 airway epithelial cells were treated with NMSO3 and then were left uninfected or infected with RSV. Transcription of RSV G protein (A), CCL2 (B), CCL5 (D), and CXCL10 (F) was suppressed with NMSO3 treatment 24 h after infection. For RSV G protein, fold increase/decrease was calculated relative to RSV-infected cells treated with 50 μmol/L NMSO3. Asterisk indicates a statistically significant decrease in RSV G protein transcripts relative to RSV-infected cells that were not treated with NMSO3. Fold increase/decrease in chemokine transcription was calculated relative to uninfected cells that had not been treated with NMSO3. Asterisk indicates a statistically significant decrease in chemokine transcripts relative to RSV-infected cells that were not treated with NMSO3. ELISA analysis revealed that NMSO3 was also able to inhibit CCL5 (E) and CXCL10 (G), but not CCL2 (C) protein production in a dose-dependent fashion 24 h after infection. Data are representative of 2 experiments (4 mice/group/experiment). IP, gamma interferon inducible protein; MCP, monocyte chemoattractant protein.
Figure 7. Differentially regulated respiratory syncytial virus (RSV) G protein expression in alveolar macrophages after treatment with ribavirin and NMSO3. MHS alveolar macrophages were treated with ribavirin or NMSO3 and were infected with RSV. Twenty-four hours after infection, RNA was isolated from the cells and analyzed for RSV G protein transcripts. Ribavirin did not suppress RSV G protein transcription but, instead, induced it (A). NMSO3 was able to decrease RSV G protein transcription, although only at the 50-μmol/L dose (B). Data are the percentage of increase/decrease in RSV G protein transcription, compared with that in control, with RSV G protein transcription in RSV-infected cells that were not treated, with an inhibitor set at 100%. Increase in RSV G protein transcription, compared with that in RSV-infected cells that were not treated with ribavirin or NMSO3, was statistically significant (*P ≤ .05). Decrease in RSV G protein transcription, compared with that in RSV-infected cells that were not treated with ribavirin or NMSO3, was statistically significant (*P ≤ .05) (4 mice/group).

for the induction of CCL6, CCL11, CCL22, and CXCL5 in the lung, because these chemokine levels were not statistically significantly different from chemokine levels in the lungs of mice that had received UV-RSV (authors’ unpublished data). Because most of these chemokines displayed a biphasic pattern of production, we can postulate that RSV infection and/or viral antigens are responsible for the early peak of chemokine production and that the immune response directed against RSV antigens is responsible for the later peak in chemokine levels.

Chemokine production that results from replication-competent RSV infection in vivo was biphasic, with the induction of chemokines occurring just after infection and then again during peak disease, when mice displayed airway hyperresponsiveness and mucus overproduction, as described elsewhere [16, 28]. These data parallel the pattern of CXCL8 production during RSV-induced disease in humans. CXCL8 levels were shown to increase early after infection and then decrease to baseline levels before increasing again during viral shedding and symptomatic illness, including rhinorrhea, headache, and pharyngitis [6]. Therefore, chemokines may either directly contribute to disease or be responsible for leukocyte recruitment and/or activation that exacerbate RSV-induced disease.

In addition to CXCL8 [5, 6, 10, 11], studies in humans have revealed that other chemokines, such as CCL2 [6], CCL3 [6–8, 11], and CCL5 [5, 7, 8, 11], are up-regulated in the nasal lavage fluids during RSV infection. Furthermore, Noah et al. [6] report that each of these chemokines is elevated during the period of viral shedding and illness. Our mouse model recapitulates these findings, because we found each of these chemokines to be elevated in the lungs 6–8 days after infection, when viral titers are at their peak and when pathophysiological changes are occurring within the airways. Clinical studies have revealed a connection between chemokine production and disease parameters. Specifically, CCL5 [5, 9] and CXCL8 [10] have been shown in separate studies to correlate with disease severity. Our laboratory has begun using a mouse model of RSV infection to dissect the role of particular chemokines and their receptors during RSV infection. Specifically, we have shown that both CCL5 and CXCR2 (the receptor for murine CXCL8 homologues) play a role in RSV-mediated mucus production and/or airway hyperresponsiveness [16, 38].

Despite the fact that RSV has been intensely studied, current treatment for infants hospitalized with RSV is not aimed at controlling virus infection. Instead, clinicians focus on relieving the symptoms of the disease by providing supplemental oxygen, fluids, and nasopharyngeal suctioning [4], while allowing for the patient’s immune system to clear the infection. Ribavirin is the only antiviral drug approved for treatment of infants hospitalized with severe RSV infections. Early studies suggested that ribavirin treatment was able to slow viral replication, reduce IgE synthesis [39], and improve clinical score [40, 41]. However, because more studies have been published, the results have proven somewhat contradictory, and the search for novel treatment strategies has continued. NMSO3 is a drug in development and has been shown to have antiviral properties against adenovirus, rotavirus, and RSV [24–26]. Although ribavirin prevents viral replication, NMSO3 has been shown to prevent the binding/infection of RSV into target cells.

Previous studies have demonstrated that ribavirin is effective at preventing viral replication [24, 42]. Furthermore, ribavirin treatment prevented RSV-induced CXCL8 production in human epithelial cells [43], and, more recently, ribavirin was
shown to prevent RSV-induced CCL3 and CCL4/MIP-1β up-regulation in human epithelial cells [44]. In the present study, both ribavirin and NMSO3 were effective at preventing RSV G protein transcription and chemokine production in airway epithelial cells. Of interest, only NMSO3 was effective at preventing RSV G protein transcription in alveolar macrophages.

As mentioned above, RSV can persist within macrophages, and RSV persistence has been linked to eosinophilia and airway hyperresponsiveness in a hamster model of RSV infection [29, 37, 45]. In the present study, chemokine production in alveolar macrophages was dependent on viral replication. However, there have been studies suggesting that RSV-induced NF-κB signaling that results in cytokine production can occur in alveolar macrophages independently of RSV replication [46–48]. Perhaps viral gene expression in alveolar macrophages is a component of RSV-induced disease that contributes to the detrimental effects of primary RSV infection, as well as the long-term sequelae resulting from the virus infection.

In conclusion, we have characterized chemokine production that results from RSV infection in airway epithelial cells and alveolar macrophages and have related it to viral gene expression. Our in vitro findings were validated in our mouse model of RSV infection, which we have shown to recapitulate the kinetics of RSV replication and chemokine production in humans. Studies that used UV-RSV or compounds that prevent RSV binding/infection or replication revealed that chemokine production appears to be triggered by replication-competent virus.

References