Respiratory Syncytial Virus Impairs Macrophage IFN- α/β - and IFN- γ -Stimulated Transcription by Distinct Mechanisms

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Macrophages are the primary lung phagocyte and are instrumental in maintenance of a sterile, noninflamed microenvironment. IFNs are produced in response to bacterial and viral infection, and activate the macrophage to efficiently counteract and remove pathogenic invaders. Respiratory syncytial virus (RSV) inhibits IFN-mediated signaling mechanisms in epithelial cells; however, the effects on IFN signaling in the macrophage are currently unknown. We investigated the effect of RSV infection on IFN-mediated signaling in macrophages. RSV infection inhibited IFN- β - and IFN- γ -activated transcriptional mechanisms in primary alveolar macrophages and macrophage cell lines, including the transactivation of important Nod-like receptor family genes, Nod1 and class II transactivator. RSV inhibited IFN- β - and IFN- γ -mediated transcriptional activation by two distinct mechanisms. RSV impaired IFN- β -mediated signal transducer and activator of transcription (STAT)-1 phosphorylation through a mechanism that involves inhibition of tyrosine kinase 2 phosphorylation. In contrast, RSV-impaired transcriptional activation after IFN- γ stimulation resulted from a reduction in the nuclear STAT1 interaction with the transcriptional coactivator, CBP, and was correlated with increased phosphorylation of STAT1 β , a dominant-negative STAT1 splice variant, in response to IFN- γ . In support of this concept, overexpression of STAT1 β was sufficient to repress the IFN- γ -mediated expression of class II transactivator. These results demonstrate that RSV inhibits IFN-mediated transcriptional activation in macrophages, and suggests that paramyxoviruses modulate an important regulatory mechanism that is critical in linking innate and adaptive immune mechanisms after infection.

Keywords: macrophages; IFN; signal transduction; transcriptional activation

Respiratory syncytial virus (RSV) is an important causative agent of severe respiratory tract infections in pediatric, immunocompromised, and elderly populations (1–5). RSV infection elicits a poor adaptive immune response; therefore, infections occur repeatedly throughout life (6, 7). This common paramyxovirus has also been associated with secondary bacterial infections of the lung (3, 8–10); however, as with other viral-induced secondary bacterial infections, the underlying mechanisms are not well understood.

IFN- α and IFN- β (type I) and IFN- γ (type II) are produced in the lung in response to microbial infection, and are potent activators of macrophage innate antimicrobial immunity; they also induce pathways that promote efficient antigen processing

and presentation to cells of the adaptive immune system (11, 12). The requirement of IFN for the efficient clearance of pathogens is evident in IFN- $\beta^{-/-}$, IFN- $\gamma^{-/-}$, IFN- α receptor $^{-/-}$, and IFN- γ receptor $^{-/-}$ mice, as these mice, lacking IFN signaling, display severe impairment in natural resistance to a variety of viral, bacterial, and parasitic infections (13–20). IFN- α or IFN- β ligation of the IFN- α/β receptor results in the phosphorylation and activation of the accessory protein kinases, Janus kinase (Jak)-1 and tyrosine kinase 2 (Tyk2) that subsequently phosphorylate signal transducer and activator of transcription (pSTAT)-2 and STAT1 (pSTAT1), leading to STAT2-STAT1 heterotrimerization with IFN regulatory factor 9 and nuclear localization (21). In the nucleus, this complex transactivates the IFN-stimulated response element (ISRE) found in the promoter of IFN-stimulated genes. Similarly, interaction of IFN- γ with the IFN- γ receptor initiates the Jak1/Jak2-dependent phosphorylation of STAT1 at tyrosine 701 (Y701). IFN- γ -mediated signaling and transcriptional activation also requires phosphorylation of STAT1 on serine 727 (S727) for transcriptional complex assembly (22, 23). pSTAT1 dimerizes, translocates to the nucleus, binds to the γ -activated sequence (GAS) DNA response element, and initiates transcription of IFN- γ -responsive genes (11, 12). Importantly, a protein-protein interaction between the coactivator, CBP/p300, and STAT1 is required for transcription of IFN- γ -responsive genes (24). STAT1 exists as both STAT1 α (91 kD) and STAT1 β (84 kD) isoforms through alternative splicing, with STAT1 β serving a dominant-negative function due, in part, to its inability to interact with the transcriptional coactivator, CBP/p300 (24, 25).

Viruses have evolved numerous unique mechanisms to inhibit the host type I and type II antiviral IFN signaling pathways (26). Although the type I and type II IFNs are structurally different molecules, they are functionally similar, and both transduce their signal through activation of the Jak/STAT signaling pathway. Numerous *Paramyxovirus* family members inhibit IFN signaling in epithelial cells through inhibition of STAT phosphorylation (26), proteasomal degradation of STAT proteins (27–30), sequestration of STAT proteins in high-molecular weight complexes (31, 32), and inhibition of nuclear localization of STAT proteins (33). Specifically, RSV inhibits type I IFN signaling in the respiratory epithelium through the proteasomal degradation of STAT2, a mechanism that is dependent on the expression of the RSV NS2 protein (27, 34). Although it is clear that RSV inhibits IFN signaling in the epithelium, it is currently unclear whether and by what mechanisms RSV inhibits the macrophage type I IFN and type II IFN response.

Herein, we demonstrate that RSV infection inhibits both IFN- α/β - and IFN- γ -mediated transcriptional activation in macrophages through distinct molecular mechanisms. The RSV impairment of transcriptional activation results in reduced expression of genes involved in the macrophage response to pathogen challenge. These findings provide increased understanding of the mechanisms by which RSV modulates immune function by altering the macrophage response to IFN.

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MATERIALS AND METHODS

Cell Culture

RAW 264.7 (RAW) macrophages were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin at 37°C and 5% CO₂. HEP2 cells were maintained in minimum essential medium (MEM) (Invitrogen) supplemented with 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, and 10% FBS (10% MEM). THP-1 cells, a human monocytic cell line, were grown in RPMI 1640 supplemented with 10% FBS and 100 U/ml penicillin. Differentiation of the THP-1 cells into macrophages was induced by culture in complete medium plus 160 nM phorbol 12-myristate 13-acetate (PMA) for 3 days (35).

Isolation of Alveolar Macrophages

Balb/c mice were exsanguinated after a lethal intraperitoneal injection of 468 mg/kg sodium pentobarbital and 60 mg/kg phenytoin sodium, and the lungs were lavaged three times with 1 ml PBS. Alveolar macrophages were recovered by centrifugation at 800 × *g*, and then cultured in RPMI 1640 supplemented with 10% FBS and 100 U/ml penicillin. Alveolar macrophage isolation was enriched by allowing cells to attach to the tissue culture plastic for 2 hours and then gently washing nonadherent cells from the culture. Greater than 99% of cells recovered were macrophages (data not shown). Experimental procedures were reviewed and approved by the Lovelace Respiratory Research Institute Institutional Animal Care and Use Committee. Male mice (56–70 d old) were used for this study.

RSV Preparation and Infection

The A2 strain of RSV was plaque purified three times under agarose. After selection, one plaque was used to inoculate a subconfluent HEP-2 cell monolayer. After adsorption for 1 hour at room temperature, 10% MEM was added, and the infection was allowed to proceed for 3 days at 37°C until the entire monolayer showed cytopathic effects. The contents of the flask were resuspended and distributed in 1-ml aliquots, snap frozen with alcohol/dry ice, and stored at –80°C. Virus was derived from this master stock by infecting subconfluent HEP-2 monolayer at multiplicity of infection (MOI) of 0.1, and harvesting the monolayer when it appeared to be completely infected. The cells and media were sonicated on ice and then the suspension was clarified by centrifugation at 1,000 × *g* for 10 minutes. The supernatant was frozen and stored at –80°C and thawed rapidly at 37°C for use. Viral titers were determined by plaque assay. Primary mouse alveolar macrophages, RAW macrophages, or PMA-differentiated THP-1 macrophages were infected with RSV at an MOI of 1 for 24 hours at 37°C in 5% CO₂. In some experiments, RSV was inactivated by exposure to 1,800 mJ of ultraviolet radiation before inoculation of the macrophages. Loss of viral infectivity was confirmed by plaque assay (data not shown).

Immunoprecipitation and Western Immunoblotting

RAW macrophage cell lysates were prepared in lysis buffer (10 mM Tris-HCl [pH 7.5], 15 mM NaCl, 0.5% Nonidet P-40, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA [pH 8.0], 0.2 mM sodium ortho-vanadate, and 0.4 mM PMSF). For nuclear lysates, cells were lysed in NE-PER extraction reagents according to the manufacturer's instructions (Pierce, Rockford, IL). Protein concentrations were measured using the BCA protein assay kit (Pierce) per the manufacturer's directions. Equal amounts of protein samples were resolved on 8 to 16% SDS-tris-glycine polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membrane (Invitrogen). The membrane was blocked with 5% powdered milk in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) and incubated overnight at 4°C with: rabbit anti-mouse STAT1 (Cell Signaling, Danvers, MA) diluted 1:1,000; rabbit anti-mouse STAT1α (Cell Signaling) diluted 1:1,000; rabbit anti-mouse STAT2 (Cell Signaling) diluted 1:1,000; rabbit anti-human Tyk2 (Abcam, Cambridge, MA) diluted 1:5,000; rabbit anti-mouse phospho-specific STAT1 (Y701) (Cell Signaling) diluted 1:1,000; rabbit anti-mouse phospho-specific STAT1 (S727) (Cell Signaling) diluted 1:1,000; rabbit anti-mouse phospho-specific STAT2 (Cell Signaling) diluted

1:1,000; rabbit anti-mouse phospho-specific Tyk2 (Cell Signaling) diluted 1:1,000; rabbit anti-human CBP antibody (R&D Systems, Minneapolis, MN) diluted 1:3,000; goat anti-mouse lamin B1 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1,000; rabbit anti-mouse inducible nitric oxide synthase (iNOS) (Upstate, Lake Placid, NY) diluted 1:1,000; or mouse anti-human β-actin (Sigma, St. Louis, MO) diluted 1:20,000 in TBST containing 5% powdered milk. Blots were washed with TBST and incubated with peroxidase-conjugated goat anti-mouse IgG, rabbit anti-goat IgG, or goat anti-rabbit IgG antibody (Pierce) diluted 1:10,000 in TBST containing 1% powdered milk. After washing, blots were developed with a chemiluminescence detection system (Millipore, Billerica, MA).

Immunoprecipitations were conducted using MagnaBind (Pierce) protein G magnetic beads, per the manufacturer's instructions. Briefly, lysates were precleared by incubation with bead slurry (1 h, 4°C). Simultaneously, antibody-protein G bead conjugates were formed by incubating 2 μg/ml rabbit anti-STAT1α (Chemicon, Temecula, CA) with the magnetic protein G beads for 2 hours at 4°C. The protein G-anti-STAT1α bead complexes were then added to precleared lysates and allowed to incubate for 2 hours at 4°C. Beads were collected with a magnet and washed four times with lysis buffer to remove unbound proteins. Laemmli buffer was added to the beads, and the samples were boiled to release the bound proteins, and samples were subjected to SDS-PAGE and Western blotting to detect CBP.

Flow Cytometric Analysis

RAW macrophages were resuspended in 200 μl of FACS buffer and incubated with Fc block (rat anti-mouse CD16/32; BD Biosciences, San Diego, CA) for 30 minutes at 4°C. Macrophages were fixed and permeabilized using the Cytoperm Cytofix kit (BD Biosciences), and dual stained with a goat anti-hRSV antibody (Biodesign International, Saco, ME) diluted 1:250, and rabbit anti-STAT2 antibody (Millipore) diluted 1:250 to determine per-cell STAT2 levels in macrophages staining positive and negative for RSV. After primary antibody staining, macrophages were stained with both Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and Alexa 488-conjugated rabbit anti-goat IgG (Invitrogen), analyzed by two-color flow cytometry using a FACScalibur flow cytometer (BD Biosciences) and results analyzed using FlowJo version 7.2.5 software (Tree Star Inc., Ashland, OR) on a personal computer.

Reporter Plasmid Transfection

Reporter gene assays were used to assess the IFN-β-mediated transactivation of the ISRE and the IFN-γ-mediated transactivation of the GAS. Luciferase levels were detected in RAW macrophages that were infected with RSV and transfected with 2 μg of either the IFN-β responsive plasmid containing ISRE (pISRE)-luciferase or IFN-γ responsive plasmid containing GAS (pGAS)-luciferase plasmid (Stratagene, La Jolla, CA) using the Lipofectamine 2,000 (Invitrogen) transfection reagent. RAW macrophages were infected with RSV and, after 4 hours, the cells were transfected with the pISRE-luciferase or pGAS-luciferase plasmid. After 18 hours of transfection, the RAW macrophages were stimulated with IFN-β (100 U/ml) or IFN-γ (10 ng/ml) for 6 hours. Cells were lysed in reporter lysis buffer (Promega, Madison, WI), and luciferase levels were measured using the Luciferase Assay System (Promega) in an LB953 AutoLumat Plus luminometer (Berthold, Oak Ridge, TN). All cells were transfected with a plasmid expressing β-galactosidase (β-gal) to normalize for transfection efficiency by dividing sample luciferase activity by the corresponding β-gal absorbance value.

Quantitative Real-Time RT-PCR

Total RNA was isolated from RAW macrophages using the acidified guanidinium method (36), treated with DNase I (DNA-free; Ambion, Austin, TX), and reverse transcribed using a cDNA cycle kit (Invitrogen). TaqMan RT-PCR was employed using reagents, primer, and probe sets (Applied Biosystems, Foster City, CA) following manufacturer's instructions to measure mRNA levels of class II transactivator (C2ta), Nod1, H2DMA, and GAPDH. The procedure was performed in an ABI Prism 7,300 Sequence Real-Time PCR System (Applied

Figure 1. Respiratory syncytial virus (RSV) inhibits IFN-mediated luciferase reporter assay transactivation and mRNA expression of Nod-like receptor (NLR) family genes. RAW 264.7 (RAW) macrophages were infected with RSV (multiplicity of infection [MOI] = 1; 24 h) or ultraviolet (UV)-inactivated virus and transfected with (A) the pISRE-luciferase or (B) pGAS-luciferase reporter plasmid. Cells were stimulated with either IFN- β (100 U/ml) or IFN- γ (10 ng/ml) for 6 hours and then harvested for luciferase activity assay. Multiplex real-time quantitative PCR analysis was performed to determine the mRNA levels of NLR family genes compared with the expression of GAPDH on mRNA harvested from macrophages stimulated with either IFN- β (100 U/ml) or IFN- γ (10 ng/ml) for 6 hours. RSV infection significantly inhibited the IFN- β -induced expression of Nod1 (C) and the IFN- γ -induced expression of C2ta (D). The expression of H2-DMA, a C2ta-regulated gene, was also reduced in the RSV-infected, IFN- γ -stimulated macrophages (E). The data represent three independent experiments run in triplicate, and are presented as means ($\pm$ SEM); * P < 0.05 compared with IFN- β and UV-RSV/IFN- β samples or IFN- γ and UV-RSV/IFN- γ samples in A and B; * P < 0.05 compared with IFN- β - or IFN- γ -stimulated samples in C–E.

Biosystems) using universal thermal cycling parameters. Each sample was run in triplicate, and each condition was performed in triplicate. Gene expression was analyzed using the comparative C_T method. GAPDH was set as the endogenous control, and the mock treatment group (no virus, no IFN) was used as the experimental control condition.

Generation and Transfection of STAT1 β -FLAG Expression Plasmid

A STAT1 β -FLAG expression vector was constructed by PCR amplification and cloning of the PCR product into a pTARGET (Promega) expression vector. First-strand cDNA of mouse STAT1 β was synthesized with Superscript First-Strand Synthesis System (Invitrogen) and amplified with AccuPrime Taq polymerase (Invitrogen) with primers mSTAT1 forward (AATGTCACAGTGGTTCGAGCTTC) plus 1 β reverse (ATTACTTATCGTCGTCATCCTTGTAATCGACTTCAGACACAGAAATCAAC) (the antisense of FLAG tag coding sequence is underlined). Sequence of the STAT1 β -FLAG plasmid was confirmed by DNA sequencing. RAW macrophages were transfected with 2 μ g of the STAT1 β -FLAG plasmid or pTARGET vector using Lipofectamine Plus (Invitrogen) according to manufacturer's instructions. After overnight incubation, cells were stimulated with IFN- γ (10 ng/ml) for 30 minutes and 6 hours. Western immunoblot analysis to detect pSTAT1 was performed on the lysates from macrophages treated with IFN- γ for 30 minutes. RNA was isolated from the 6-hour IFN- γ -treated macrophages, and C2ta gene induction by IFN- γ was determined by TaqMan quantitative real-time PCR analysis.

Statistical Analysis

Results were compared using Student's t test or ANOVA, followed by a *post hoc* Dunnett's test. Findings were considered statistically significant at probability levels less than 0.05. Results are presented as means ($\pm$ SEM).

RESULTS

RSV Inhibits Type I IFN- and Type II IFN-Inducible Transcriptional Activation

Although the capacity of RSV-encoded mechanisms to inhibit IFN-mediated signaling in epithelial cells is well documented (27, 30, 34, 37), the ability of RSV to infect macrophages and modulate their response to IFN- α/β or IFN- γ signaling has not been extensively investigated. To initially test the ability of RSV to subvert IFN-mediated responses, IFN- β -responsive pISRE-luciferase and IFN- γ -responsive pGAS-luciferase reporter assays were used to assess transactivation of ISRE- and GAS-modulated gene products. RAW macrophages were either mock- or RSV-infected at an MOI of 1 for 24 hours, and transfected with either the ISRE luciferase or GAS luciferase plasmid, along with a β -gal plasmid to normalize transfection efficiency. After RSV infection and reporter plasmid transfection, macrophages were stimulated with cell culture media or cell culture media containing IFN- β or IFN- γ for 6 hours. In mock-infected cells, IFN- β and IFN- γ treatment resulted in robust induction of pISRE-luciferase activity (Figure 1A) and pGAS-luciferase activity (Figure 1B), respectively. In contrast, RSV infection significantly reduced the IFN- β -stimulated pISRE-luciferase activity (Figure 1A) and IFN- γ -stimulated pGAS-luciferase activity (Figure 1B). Ultraviolet inactivation of RSV abrogated the inhibitory effect on IFN-dependent luciferase activity (Figures 1A and 1B), suggesting that live virus is required for the inhibition of IFN-mediated transcriptional activation.

IFN- α/β and IFN- γ modulate the expression of hundreds of genes that enhance macrophage antimicrobial and immunomodulatory function to promote pathogen elimination (38, 39).

Figure 2. RSV impairs IFN-mediated transcriptional activation in primary mouse alveolar macrophages. Western immunoblot analysis was performed to determine whether RSV infection (MOI = 1; 24 h) inhibited phosphorylation of STAT1 at Y701 in (A) IFN- β - (100 U/ml) or (C) IFN- γ -stimulated (10 ng/ml, 30-min treatment) primary mouse alveolar macrophages. RSV infection did not inhibit IFN- γ -stimulated STAT1 (Y701) phosphorylation; however, STAT1 phosphorylation in response to IFN- β was significantly impaired in RSV-infected alveolar macrophages. RSV infection increased the level of STAT1. The levels of β -actin were similar in all samples. Multiplex real-time quantitative PCR analysis was performed to determine the mRNA levels of IFN-responsive genes compared with the expression of GAPDH on mRNA harvested from alveolar macrophages stimulated with either IFN- β (100 U/ml) or IFN- γ (10 ng/ml) for 6 hours. RSV infection sig-

nificantly inhibited the IFN- β -induced expression of Nod1 (B), and the IFN- γ -induced expression of C2ta (D). The data represent three independent experiments run in triplicate, and are presented as means ($\pm$ SEM); * P < 0.05 compared with IFN- β - or IFN- γ -stimulated samples in B and D.

To further assess IFN-mediated transcriptional activation in RSV-infected macrophages, the ability of IFN- β and IFN- γ to up-regulate the expression of endogenous genes was analyzed by quantitative RT-PCR. In mock-infected RAW macrophages (Figure 1C), the mRNA expression of Nod1, a Nod-like receptor (NLR) family gene that recognizes bacterial peptidoglycan and activates pathways that are involved in cellular responses against bacteria, was markedly increased by IFN- β treatment. Conversely, in RSV-infected RAW macrophages (Figure 1C), the IFN- β -induced expression of Nod1 mRNA was significantly inhibited compared with the mock-infected, IFN- β -treated macrophages. Similarly, RSV infection inhibited the IFN- β -dependent induction of Nod1 mRNA expression in primary mouse alveolar macrophages (Figure 2B).

IFN- γ stimulation of macrophages induces the mRNA expression of C2ta, an NLR family gene and transcriptional enhancer that regulates the expression of major histocompatibility complex class II genes (12). C2ta mRNA levels were significantly reduced in both RSV-infected (24 h) primary mouse alveolar macrophages and RAW macrophages treated with IFN- γ (6 h) as compared with mock-infected, IFN- γ -treated alveolar (Figure 2D) and RAW macrophages (Figure 1D). Quantitative PCR analysis was performed to assess the level of the C2ta-regulated gene, H2-DMA. The expression of H2-DMA was down-regulated in the RSV-infected, IFN- γ -treated RAW macrophages when compared with mock-infected, IFN- γ -treated macrophages (Figure 1E). Together, these data suggest that RSV modulates a network of IFN-responsive genes involved in the macrophage immune response against pathogens.

RSV Inhibits IFN- α/β - but Not IFN- γ -Dependent STAT1 Phosphorylation

Phosphorylation of STAT1 at Y701 is required for the transcriptional regulation of both IFN- α/β - and IFN- γ -responsive genes. To determine whether RSV inhibited IFN transcriptional activation by modulating STAT1 phosphorylation, Western blot analysis for pSTAT1 (Y701) and STAT1 was performed on cell lysates from primary mouse alveolar macrophages and RAW macrophages infected with RSV (MOI = 1) for 24 hours, and then stimulated with IFN- α , IFN- β , or IFN- γ for 30 minutes. RSV infection increased the total cellular levels of STAT1 in primary alveolar macrophages, but impaired the IFN- β -dependent phosphorylation of STAT1 at Y701 (Figure 2A). Likewise, RSV infection increased STAT1 levels and impaired IFN- α - and IFN- β -stimulated STAT1 phosphorylation (Y701) in the RAW macrophages (Figure 3A). In contrast, RSV did not inhibit the IFN- γ -stimulated STAT1 (Y701) phosphorylation in either the primary alveolar macrophages (Figure 2C) or the RAW macrophages (Figure 3B). Together, these results suggest that RSV inhibits IFN- α/β - and IFN- γ -mediated transcriptional activation in both primary alveolar and RAW macrophages by distinct mechanisms, and indicates that RAW macrophages are a suitable model system to determine the underlying molecular mechanisms.

RSV Infection Inhibits Type I IFN-Mediated STAT2 Phosphorylation

STAT2 facilitates the IFN- α/β -stimulated phosphorylation of STAT1 by serving as a docking site for STAT1; STAT2 must be

Figure 3. RSV impairs IFN- α/β - but not IFN- γ -mediated signal transducer and activator of transcription (STAT)-1 phosphorylation. Western immunoblot analysis was performed to determine whether RSV infection (MOI = 1; 24 h) inhibited phosphorylation of STAT1 at Y701 in (A) IFN- α - (100 U/ml), IFN- β - (100 U/ml), or (B) IFN- γ -stimulated (10 ng/ml, 30-min treatment) RAW macrophages. RSV infection did not inhibit IFN- γ -stimulated STAT1 (Y701) phosphorylation; however, STAT1 phosphorylation in response to either IFN- α or IFN- β was significantly impaired in RSV-infected macrophages. RSV infection increased the level of STAT1. The levels of β -actin were similar in all samples. Each lane of the immunoblot was loaded with 20 μ g of protein, and the immunoblots shown are representative of three independent experiments.

phosphorylated in response to IFN- α or IFN- β before recruitment and subsequent phosphorylation of STAT1 (40). RSV prevents STAT1 phosphorylation in lung epithelial cells by targeting STAT2 for proteasomal degradation (27). Because RSV impaired the phosphorylation of STAT1 in response to IFN- α/β , we investigated whether RSV impairment of IFN- α/β -mediated STAT2 phosphorylation and transcriptional activation occurred by similar mechanisms in macrophages. STAT2 levels were increased, as assessed by Western blot analysis, suggesting that mouse macrophage STAT2 is not targeted for proteasomal degradation in response to RSV infection (Figure 4A). Flow cytometric analysis was performed to assess the effect of RSV on macrophage STAT2 protein levels on a per-cell basis. In agreement with our Western blot results in Figure 4A, the flow cytometric data demonstrate that RSV infection resulted in a 2.5-fold increase in the relative level of STAT2 in RAW macrophages (Figure 4B). Additional analysis was performed to understand if STAT2 levels were differentially modulated in macrophages that stained positive and negative for RSV after inoculation (Figure 4B, *hatched bars*). Interestingly, STAT2 was increased in both RSV-positive (30% of cells) and -negative (70% of cells) macrophages; however, macrophages staining positive for RSV

had the largest increase in STAT2 when compared with mock-infected macrophages (Figure 4B).

THP-1 cells, a human monocytic cell line, were used to determine whether the increase in STAT2 after RSV infection was due to species differences between mice and humans, or resulted from differences between macrophages and epithelial cells. THP-1 cells were differentiated to macrophages by treatment with PMA (160 nM, 72 h) and infected with RSV (MOI = 1; 24 h). RSV infection increased the protein levels of STAT2 in THP-1 macrophages (Figure 4C). This suggests that RSV differentially regulates STAT2 in macrophages and epithelial cells, and that the increase in STAT2 is not caused by species differences between mouse and human.

STAT2 phosphorylation was assessed using phospho-specific STAT2 antibodies after IFN- α or IFN- β stimulation of mock- and RSV-infected macrophages. As with phosphorylation of STAT1, RSV infection significantly inhibited both the IFN- α - and IFN- β -mediated phosphorylation of STAT2 in RAW macrophages (Figure 4A). RSV caused a similar inhibition of IFN- β -mediated STAT2 phosphorylation in human THP-1 macrophages (Figure 4C). These results suggest that the RSV-mediated impairment of type I IFN-stimulated transcriptional activation in both mouse and human macrophages is upstream of STAT phosphorylation.

RSV Impairs the Signaling Response to Type I IFN through Reduced Phosphorylation of Tyk2

The Jak kinases associate with intracellular domains of cytokine receptors, become phosphorylated after ligand binding and aggregation of the receptor chains, and are required for cellular signaling responses induced by IFN (41). Jak1 is a common signaling component for both the IFN- γ and IFN- α/β receptors; however, Jak2 and Tyk2 are specific to the IFN- γ and IFN- α/β receptors, respectively. The observation that RSV inhibited IFN- α - and IFN- β -, but not IFN- γ -mediated phosphorylation of STAT1 (Figure 2), suggests that the virus does not impair the function of Jak1. In addition, the mouse IL-10 receptor requires Jak1 for the phosphorylation of STAT3 in response to IL-10; RSV did not impair the IL-10-dependent phosphorylation of STAT3 (data not shown). Because Jak1 appears to be unaffected by RSV infection, we assessed whether RSV altered Tyk2 phosphorylation in response to IFN- β by Western blot analysis using phosphorylated Tyk2 (pTyk2)- and Tyk2-specific antibodies. In mock-infected RAW macrophages, IFN- β induced a rapid induction of Tyk2 phosphorylation; however, RSV significantly inhibited the IFN- β -dependent phosphorylation of Tyk2 at all time points examined (Figure 5A). The levels of Tyk2 were similar between all samples, suggesting that the RSV-dependent inhibition of Tyk2 phosphorylation was not due to a reduction in Tyk2 protein levels (Figure 5A). Similarly, RSV inhibited IFN- α -stimulated Tyk2 phosphorylation in PMA-differentiated human THP-1 macrophages (Figure 5B), and suggests a similar mechanism of inhibition in human and mouse macrophages.

Phosphatases are important cellular regulatory components that control the intensity and duration of signal transduction pathways. The tyrosine phosphatase inhibitor, sodium stibogluconate (42), was used to determine whether diminished Tyk2 phosphorylation resulted from elevated phosphatase activity, and whether phosphatase inhibition was sufficient to restore the downstream phosphorylation of STAT1 in response to IFN- β to the levels observed in the mock-infected macrophages. Before IFN- β stimulation, mock- or RSV-infected (MOI = 1; 24 h) macrophages were treated with 100 μ M sodium stibogluconate for 30 minutes. Western blot analysis for pTyk2 and pSTAT1 was performed, and the level of both pTyk2 and pSTAT1

(Y701) was similar in the IFN- β -stimulated mock- and RSV-infected macrophages pretreated with sodium stibogluconate (Figure 5C). Together, these results suggest that a phosphatase-mediated mechanism is likely involved in the reduced phosphorylation of Tyk2 and subsequent inhibition of STAT1 phosphorylation.

RSV Does Not Inhibit IFN- γ -Dependent STAT1 Nuclear Translocation

Translocation of pSTAT1 (Y701) to the nucleus is required for transcriptional activation of IFN- γ -responsive genes, and other paramyxoviruses have been shown to inhibit STAT nuclear localization (33); therefore, immunoblot analysis for pSTAT1 in the nuclear fraction was performed to determine whether RSV infection impaired the translocation of pSTAT1 to the nucleus. Nuclear proteins were isolated from RSV-infected (MOI = 1; 24 h) RAW macrophages that were stimulated with IFN- γ

(10 ng/ml, 30 min). Nuclear pSTAT1 levels after IFN- γ treatment were similar in nuclear lysates from RSV- and mock-infected RAW macrophages (Figure 6A). These results suggest that RSV infection does not inhibit macrophage IFN- γ signaling through a mechanism involving impairment of pSTAT1 nuclear translocation.

IFN- γ -Stimulated Association of STAT1 and CBP Is Inhibited by RSV Infection

Transcriptional activation of IFN- γ -activated genes requires the interaction of pSTAT1 with CBP/p300 (24). Nuclear lysates from IFN- γ -stimulated (10 ng/ml, 30 min) mock- and RSV-infected (MOI = 1; 24 h) RAW macrophages were immunoprecipitated with anti-STAT1 α antibody, followed by Western immunoblot analysis using an antibody against CBP. When compared with the mock-infected RAW macrophages, RSV-infected macrophages displayed reduced levels of STAT1-associated CBP after IFN- γ stimulation (Figure 6B). Impaired STAT1-CBP interaction was not due to reduced levels of CBP or sequestration of CBP outside of the nuclear compartment, because equivalent levels were observed regardless of RSV infection or IFN- γ treatment status (Figure 6C). These results indicate that RSV inhibits IFN- γ -induced gene expression by modulating STAT1-CBP protein-protein interactions and transcriptional complex assembly.

RSV Increases the Phosphorylation of the Dominant-Negative STAT1 β Isoform

Recruitment of the transcriptional coactivator, CBP/p300, also requires phosphorylation of STAT1 on S727 (22, 23). Because RSV potentially inhibited CBP-STAT1 interactions after IFN- γ treatment, Western blot analysis for phosphorylation of STAT1 at S727 on nuclear lysates was performed to determine whether infection impaired phosphorylation at this critical serine residue in response to IFN- γ . RSV did not inhibit the IFN- γ -stimulated STAT1 phosphorylation at S727 (Figure 7A).

To further detect mechanisms that might result in malfunctioning STAT1-CBP complex transcriptional complex, STAT1

Figure 5. RSV impairs IFN- β -mediated Tyk2 phosphorylation. (A) Western immunoblot analysis was performed to determine whether RSV infection (MOI = 1; 24 h) inhibited phosphorylation of Tyk2 in IFN- β -stimulated (100 U/ml) RAW macrophages. (B) Western immunoblot analysis was performed to determine whether RSV infection (MOI = 1; 24 h) inhibited phosphorylation of Tyk2 in IFN- β -stimulated (1,000 U/ml) THP-1 macrophages. (C) Western blot analysis for pSTAT1 and pTyk2 was performed on mock- or RSV-infected (MOI = 1; 24 h) macrophages that were treated with sodium stibogluconate (100 μ M) for 30 minutes before IFN- β (100 U/ml) stimulation to determine whether RSV-impaired Janus kinase-STAT phosphorylation resulted from elevated phosphatase activity. Each lane of the immunoblot was loaded with 20 μ g of protein, and the immunoblots shown are representative of three independent experiments.

isoforms were analyzed. STAT1 exists as both a STAT1 α (91 kD) and STAT1 β (84 kD) isoform through alternative splicing. Both STAT1 isoforms can be phosphorylated (at Y701; STAT1 β lacks the region containing S727), and can bind to DNA elements; however, STAT1 β functions as a dominant negative due to its inability to interact with the transcriptional coactivator, CBP/p300 (24, 25). Immunoblot analysis for pSTAT1 α and pSTAT1 β was performed on cell lysates from IFN- γ -stimulated RAW macrophages that were mock- or RSV-infected. The level of IFN- γ -stimulated pSTAT1 β was increased in RSV-infected RAW macrophages when compared with the mock-infected cells (Figure 7B). RSV infection increased the level of pSTAT1 β fourfold over mock-infected macrophages, and altered the pSTAT1 α :pSTAT1 β ratio from 2.5:1 in the mock-infected macrophages to 0.9:1 in the RSV-infected macrophages (Figure 7C), as determined by densitometry. The RSV alteration of the pSTAT1 α :pSTAT1 β ratio was also readily apparent in the Western blot from the primary alveolar macrophages (Figure 3C).

Previous reports indicate that overexpression of STAT1 β is sufficient to inhibit IFN- γ -dependent transcriptional activation (43). To determine if overexpression of STAT1 β and enhanced generation of pSTAT1 β were involved in the inhibition of IFN- γ -dependent C2ta mRNA expression, we transiently overexpressed STAT1 β -FLAG in RAW macrophages. Transfection with STAT1 β -FLAG expression vector, but not the empty vector, altered the pSTAT1 α :pSTAT1 β ratio to a 1:1 ratio (Figure 7D, *inset*), and significantly inhibited the IFN- γ -stimulated expression of C2ta mRNA (Figure 7D). Together, these results indicate that the overexpression of STAT1 β is sufficient to inhibit the IFN- γ -stimulated expression of C2ta, and suggest that RSV may inhibit macrophage IFN- γ transcriptional activation through

a mechanism that involves an increase in the abundance of the dominant-negative pSTAT1 β .

DISCUSSION

IFN- α , IFN- β , and IFN- γ are potent inducers of macrophage immune function in response to microbial infections. It is therefore not surprising that many pathogens have evolved sophisticated mechanisms to inhibit and evade the host IFN-induced immune response. Herein, we report that RSV infection inhibits both IFN- α/β - and IFN- γ -mediated transcriptional activation in macrophages through at least two distinct molecular mechanisms. RSV inhibits type I IFN-mediated transcriptional activation by disrupting the phosphorylation of STAT1 and STAT2 through a mechanism that involves the impairment of Tyk2 activation. In contrast, RSV did not inhibit the phosphorylation of STAT1 or the subsequent nuclear translocation of pSTAT1 in response to IFN- γ stimulation. Instead, RSV infection disrupted the nuclear protein-protein interaction of STAT1 with the transcriptional coactivator, CBP, after IFN- γ activation. Impaired STAT1-CBP interaction after RSV infection was associated with increased phosphorylation of STAT1 β , a dominant-negative splice variant that lacks the ability to interact with CBP, in response to IFN- γ . Overexpression of STAT1 β was sufficient to inhibit IFN- γ -mediated transcriptional activation in our model system.

Inhibition of host cell IFN signaling is a common immune system evasion strategy employed by many pathogens. RSV inhibits IFN- α and IFN- β signaling and transcriptional activation in human tracheobronchial epithelial cells through a mechanism that involves the proteasome-dependent destruction of STAT2, and requires the RSV NS1 and/or NS2 protein

Figure 6. RSV infection impairs nuclear pSTAT1-CBP protein–protein interactions. (A) Western immunoblot analysis of cytoplasmic and nuclear fractions for pSTAT1 and STAT1 was performed to determine if RSV infection (MOI = 1; 24 h) inhibited the nuclear translocation of pSTAT1 after IFN- γ treatment (10 ng/ml, 30 min). Nuclear and cytoplasmic fraction enrichment was assessed by Western blot analysis for lamin B and iNOS, respectively. Because interaction of pSTAT1 with CBP/p300 is required for transactivation of the IFN- γ response, the level of CBP/p300-STAT1 interaction in RSV-infected RAW macrophages was assessed. (B) Nuclear extracts from IFN- γ -stimulated (10 ng/ml, 30 min) mock- or RSV-infected (MOI = 1; 24 h) RAW macrophages were subjected to immunoprecipitation (IP) for STAT1 and then Western immunoblot analysis for CBP to determine whether CBP-STAT1 interactions were disrupted in RSV-infected RAW macrophages. (C) Western blot analysis for CBP was performed on nuclear lysates to determine whether diminution of nuclear STAT1-CBP interactions was due to an RSV-induced reduction in nuclear CBP levels. The immunoblots shown are representative of three independent experiments.

(27, 30, 34). Although we have found that RSV inhibits IFN- α - and IFN- β -driven transcriptional activation in macrophages, our results clearly indicate that RSV infection increases the cellular level of STAT2 in both human and mouse macrophages and, therefore, do not suggest that degradation of STAT2 serves as the inhibitory mechanism in macrophages as it does in human tracheobronchial epithelial cells (27). Instead, we have determined that RSV inhibits type I IFN-mediated signaling by reducing the phosphorylation and activation of Tyk2 in macrophages. Because treatment with the phosphatase inhibitor, sodium stibogluconate (42), ameliorated the RSV-dependent inhibition of Tyk2 and STAT1 phosphorylation, we speculate that RSV disrupts the type I IFN signaling cascade through a mechanism that promotes the dephosphorylation of Tyk2 and, as a result, reduces the Tyk2 kinase activity. Interestingly, Ramaswamy and colleagues (27) also reported that RSV infection of human tracheobronchial epithelial cells increased cellular STAT1 protein levels, and did not inhibit the IFN- γ -dependent phosphorylation of STAT1. Our data indicate that RSV impairs IFN- γ -inducible gene expression in macrophages; however, it is unclear if RSV impairs IFN- γ -inducible gene expression in epithelial cells by a similar mechanism.

Destruction of intermediate signaling molecules in the IFN- γ signal transduction pathway is a common inhibitory mechanism

used by many viruses. Varicella zoster virus inhibits the IFN- γ -dependent induction of C2ta mRNA expression by reducing the expression of STAT1 α and JAK2 (44), whereas the Epstein-Barr virus inhibits IFN- γ signaling by reducing the level of IFNGR on the cell surface (45). In contrast to Epstein-Barr virus and varicella zoster virus, the results herein indicate that RSV infection does not inhibit IFN- γ -stimulated phosphorylation of STAT1 or reduce the total cellular levels of STAT1. Instead, our results suggest that RSV inhibits macrophage IFN- γ signaling by impairing the interactions of proteins necessary for transcriptional complex formation. In addition, our results indicate that the RSV-mediated transcriptional complex inhibition is associated with an increase in the phosphorylation of the dominant-negative STAT1 β . Interestingly, *Leishmania mexicana* inhibits IFN- γ signaling through preferentially enhancing tyrosine phosphorylation of dominant-negative STAT1 β (46). Although inhibition of IFN- γ -dependent transcriptional activation through altering the levels of pSTAT1 β is a novel viral strategy for inhibition of IFN- γ -mediated transcriptional activation, the exact mechanisms need to be further defined, and will be the focus of future studies.

RSV is associated with secondary bacterial infections (3, 8–10); however, it is currently unclear how RSV alters the host response to bacterial pathogens. *In vivo* studies using a mouse

Figure 7. RSV infection does not inhibit IFN- γ -mediated STAT1 phosphorylation at serine 727 (S727), but does increase IFN- γ -stimulated pSTAT1 β . (A) Western immunoblot analysis was performed to determine whether RSV infection (MOI = 1; 24 h) inhibited phosphorylation of STAT1 at S727 in response to IFN- γ (10 ng/ml, 30-min treatment). (B) RAW macrophages infected with RSV were stimulated with IFN- γ (10 ng/ml, 30 min) and cell lysates were subjected to Western immunoblot analysis to determine the levels of pSTAT1 α (91 kD) and pSTAT1 β (84 kD). (C) Densitometry was used to quantify the levels of pSTAT1 β and the ratio of pSTAT1 α to pSTAT1 β detected by Western blot analysis. Each lane of the immunoblot was loaded with 20 μ g of protein, and the immunoblots shown are representative of three independent experiments. (D) RAW macrophages were transfected with 2 μ g of the STAT1 β -FLAG expression vector or empty vector. After overnight incubation, cells were stimulated with IFN- γ (10 ng/ml) for 6 hours, and mRNA was harvested for quantitative real-time PCR analysis. Multiplex real-time quantitative PCR analysis was performed to determine the mRNA levels of C2ta compared with those of GAPDH. RAW macrophages transfected with STAT1 β -FLAG expression vector or empty vector were stimulated with IFN- γ (10 ng/ml, 30 min), and cell lysates were subjected to Western immunoblot analysis to determine the levels of pSTAT1 α (91 kD) and pSTAT1 β (84 kD) (inset). The data represent three independent experiments run in triplicate. Data are presented as means ($\pm$ SEM); * P < 0.05 compared with RAW macrophages transfected with empty vector.

model of pulmonary viral-bacterial coinfection show that, as with humans, RSV predisposes the organisms to secondary bacterial infection by impairing the clearance of *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* as early as 24 hours after RSV infection, and as long as 7 days after viral infection (8). The inflammatory response after sequential infection of RSV and bacteria was enhanced over infection with either virus or bacteria, indicating that impaired bacterial clearance was not due to the inability of the host to mount an inflammatory response and recruit immune cells to clear the bacterial pathogen. Instead, our observations suggest that the alveolar macrophages from infected mice may be unable to integrate signals that facilitate efficient pathogen clearance. In this study, we have shown that RSV impairs the IFN-mediated

expression of the NLR family genes, Nod1 and C2ta. The NLRs are a family of cytosolic biosensors for intracellular and extracellular microbes that drive innate and adaptive immune responses through activating transcription factors and downstream effectors (47). Defective NLR signaling results in recurrent infection by failing to mount a response against pathogens and to instruct the adaptive response against microbes (47). Although further experimentation will be necessary to understand the biological consequences that inhibiting NLR gene expression has on macrophage immune function, these results suggest that RSV does disrupt activation of physiologic pathways that are involved in efficient sensing and clearance of microbial pathogens.

In summary, RSV impairs macrophage type I and type II IFN-mediated transcriptional activation by distinct mechanisms.

These inhibitory mechanisms likely have important consequences concerning the ability of the macrophage to function as an efficient component in the host innate and adaptive immune responses subsequent to paramyxovirus infection.

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