Differential Type I Interferon Induction by Respiratory Syncytial Virus and Influenza A Virus In Vivo[∇]

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Type I interferon (IFN) induction is an immediate response to virus infection, and very high levels of these cytokines are produced when the Toll-like receptors (TLRs) expressed at high levels by plasmacytoid dendritic cells (pDCs) are triggered by viral nucleic acids. Unlike many RNA viruses, respiratory syncytial virus (RSV) does not appear to activate pDCs through their TLRs and it is not clear how this difference affects IFN-α/β induction in vivo. In this study, we investigated type I IFN production triggered by RSV or influenza A virus infection of BALB/c mice and found that while both viruses induced IFN-α/β production by pDCs in vitro, only influenza virus infection could stimulate type I IFN synthesis by pDCs in vivo. In situ hybridization studies demonstrated that the infected respiratory epithelium was a major source of IFN-α/β in response to either infection, but in pDC-depleted animals only type I IFN induction by influenza virus was impaired.

Type I interferons (IFNs) were discovered because of their antiviral activity against influenza virus (34) and constitute the first line of antiviral defense. In all of the cell types that have been tested, virus infection results in transcriptional up-regulation, synthesis, and secretion of type I IFNs (IFN-α/βs). Once secreted, IFN-α/βs act in an autocrine or paracrine manner by binding the IFN-α/β receptor present on all cell types. Receptor binding stimulates the Jak-Stat signaling cascade leading to transcriptional up-regulation of the IFN-stimulated genes which mediate the biological effects of IFN (13). In addition to their direct antiviral effects, type I IFNs are known to be important for NK cell expansion and activation (5, 43), as well as promoting the activation and survival of virus-specific T cells (25, 32, 57). Given the major role that type I IFNs play in host defense against virus infection, it is not surprising that most viral pathogens of vertebrates have evolved mechanisms to inhibit the production and function of these cytokines (59).

IFN induction by influenza A virus involves the recognition of viral components by both cytoplasmic receptors and Toll-like receptor 7 (TLR7) (16), although the precise mechanism used depends upon the infected cell type. In fibroblasts and conventional dendritic cells (DCs), expression of the gene for IFN-β is largely dependent upon virus activation of the RNA helicases encoded by retinoic acid-induced gene I (*RIG-I*) (30) and melanoma differentiation-associated gene 5 (*mda-5*) (53), with the subsequent phosphorylation of IFN regulatory factor 3 (IRF3) by IKKε/TBK1. Once IFN-β (as well as IFN-α4 in the mouse) has been synthesized and secreted, signaling through the Jak-Stat pathway up-regulates the production of IRF7,

which then mediates the transcription of additional IFN-α genes (16, 38, 50). In this way, an amplification pathway is set up whereby early, IRF3-mediated production of IFN-β allows the subsequent synthesis of multiple IFN-α subtypes. An additional source of type I IFNs during virus infection in vivo is the plasmacytoid DC (pDC) (1, 52), which is triggered to produce very high levels of type I IFN when TLR7, -8, and -9, present in the endosomal compartment of these cells, encounter virus or viral components (16). Binding of TLR7, -8, and -9 by their ligands leads to MyD88-mediated activation of IRF7, which is constitutively expressed at high levels in the pDC. The mechanisms of IFN induction in the pDC are unique to this cell type; the need for IRF3 activation is bypassed (28), and pDCs rely upon TLRs for detection of viral pathogens (30). Influenza A virus is detected by TLR7 in mice (TLR8 in humans), which recognizes and binds to viral single-stranded RNA (14, 36).

The innate immune response to respiratory virus infection is best studied for influenza A virus, but observations from the clinical literature suggest that pathways induced by this virus may not be shared by other respiratory pathogens. Respiratory syncytial virus (RSV), a nonsegmented, negative-strand RNA virus belonging to the family *Paramyxoviridae*, is the major cause of bronchiolitis and pneumonia in infants, accounting for an estimated 86,000 pediatric hospitalizations in this country annually (22, 44). Reports in the pediatric literature state that while IFN-α/β is easily detected in blood and secretions from influenza virus-infected infants, only very low levels are present in RSV-infected patients (24, 40, 48). While RSV has been shown to induce type I IFNs both in vitro and in vivo (20, 27), there are few published studies which directly compare how specific viral pathogens differ with respect to the source and kinetics of IFN-α/β production in the course of an ongoing infection.

To determine whether RSV and influenza A virus do, in fact, regulate the production of these cytokines differently, we have

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used a mouse model to study the source and duration of IFN-α/β in vivo. In the BALB/c mouse, we have found that infected epithelial cells are a major source of type I IFNs in response to either infection but that only influenza virus is able to stimulate IFN-α/β production by pDCs. In mice lacking pDCs, type I IFN induction by influenza virus is impaired while induction by RSV is unchanged. Although we and others have demonstrated that, at high viral doses, live RSV will stimulate IFN synthesis by pDCs in vitro (21, 27), these findings suggest that pDCs play a minor role during RSV infection in the live animal.

MATERIALS AND METHODS

Mice. Wild-type BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN). IFNAR1⁻/⁻ animals lacking a functional IFN-α/β receptor were initially obtained from Michel Aguet (41) and backcrossed onto the BALB/c background for nine generations. These animals are maintained (20) under specific-pathogen-free conditions in the vivarium of the Columbus Children's Research Institute. All of the experiments described here were approved by the Columbus Children's Research Institute Committee on the Use and Care of Animals.

Virus, cells, and virus quantification. A laboratory stock of the human A2 strain of RSV (originally provided by R. Chanock, National Institutes of Health, Bethesda, MD) was propagated in HEp-2 cells (American Type Culture Collection, Manassas, VA) (19). Infectivity was determined by plaque assay as previously described (29). Influenza A/WSN/33 (H1N1) virus (WSN virus), provided by Adolfo Garcia-Sastre (Mount Sinai School of Medicine), was grown in Madin-Darby bovine kidney cells. The infectivity of the WSN virus was assayed in the same cell line used to analyze RSV infectivity, a fibroblast cell line derived from STAT1⁻/⁻ mice designated NY3. For the WSN virus, titers were determined 24 h after inoculation of indicator cells by fluorescent-focus assays using a polyclonal rabbit serum raised against whole WSN virus (7, 15) and a secondary fluorescein isothiocyanate-labeled goat anti-rabbit antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Propagation of the Hitchner strain of Newcastle disease virus (NDV) was done as previously described (42). In experiments with UV-inactivated viruses, the viruses were inactivated by exposure to UV light for 2 min. The human lung epithelium cell line A549 and the murine lung epithelial cell line LA4 were obtained from the American Type Culture Collection, Manassas, VA.

Virus infection. A549 or LA4 cells were plated at 5×10^5/well in a six-well plate. The cells were infected at a multiplicity of infection (MOI) of 1 with RSV, WSN virus, or NDV. Cell supernatants were harvested at 24, 48, and 72 h postinfection, and the IFN-α/β functional assay was performed as described below.

Mice were infected with various doses of RSV or influenza virus in a 50-μl volume intranasally (i.n.) and under light anesthesia. Groups of three to six animals, all 6 to 10 weeks of age, were used for each data point.

IFN-α/β assays. Type I IFN bioassay was carried out as previously described (39), by using lung homogenate supernatants, bronchoalveolar lavage (BAL) fluid samples, or DC supernatants. IFN-α and IFN-β protein concentrations in lung homogenates or BAL fluid samples were determined by enzyme-linked immunosorbent assay (ELISA; PBL Biomedical Laboratories, Piscataway, NJ).

Histology and immunohistochemistry. Six-micrometer sections were either stained with hematoxylin-eosin (H&E) for histological examination or deparaffinized for immunohistochemistry. Polyclonal goat anti-RSV (Biodesign; Saco, ME) serum at a 1:500 dilution was used as the primary antibody for detection of RSV antigen, and polyclonal rabbit serum raised against whole WSN virus was used (at a 1:1,000 dilution) as the primary antibody for detection of influenza virus antigen. Biotinylated anti-rabbit immunoglobulin (Vector Laboratories, Burlingame, CA) was used as the secondary antibody at a dilution of 1:1,000, followed by streptavidin-horseradish peroxidase (UltraTek HRP; ScyTek). Color was developed by using the aminoethylcarbazole substrate (AEC Substrate Kit; ScyTek) and a hematoxylin counterstain.

In situ hybridization. The murine *ifnα4* gene was PCR amplified from the129SvEvBAC clone RP23-360M23 with primers 5′ AGAGCGACCAGCAT CTAC 3′ and 5′ AGTCCTCTCCACACTTTG 3′. The PCR-amplified product was then cloned into the pCRII TOPO vector (Invitrogen, Carlsbad, CA), and a digoxigenin (DIG)-labeled riboprobe was synthesized with the DIG RNA labeling kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. Sections were incubated at 37°C in phosphate-buffered saline with 5 μg/ml

proteinase K, fixed in 4% paraformaldehyde, rinsed in phosphate-buffered saline, and immersed in 0.25% acetic anhydride–triethanolamine. Hybridization with the DIG-labeled probe (60 ng/sample) was carried out overnight at 42°C in a buffer containing 40% deionized formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10 mM dithiothreitol, 1 mg/ml yeast tRNA, and 1 mg/ml denatured salmon sperm DNA. Sections were washed at 37°C with 2×, 1×, and then 0.1× SSC; blocked with 2% normal goat serum; and then incubated with a 1:500 dilution of anti-DIG–alkaline phosphatase (Fab fragments; Roche, Indianapolis, IN). Signal was detected with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (BCIP; Roche, Indianapolis, IN). Stained sections were rinsed with water and counterstained with nuclear fast green (Fisher Scientific, Pittsburgh, PA). Care was taken to use identical incubation times and conditions for all probes and all samples.

DC culture and infection. Bone marrow-derived DCs (BMDCs) were isolated from the femurs and tibiae of wild-type BALB/c mice and cultured in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum, 1 mmol/liter penicillin-streptomycin, 50 μM 2-mercaptoethanol, and 100 ng/ml FLT-3 ligand (R&D). On day 9, individual wells were infected with either RSV or WSN virus at an MOI of 1 or left uninfected. Medium was harvested for determination of type I IFN levels 24 h after infection.

Depletion of pDCs. Male BALB/c mice received 500 μg of either rat immunoglobulin G (IgG; Jackson ImmunoResearch Laboratories, Inc.) or the monoclonal antibody PDCA-1 (Miltenyi) by intraperitoneal (i.p.) injection at 48 and 24 h prior to virus infection. Twenty-four hours following infection, the extent of depletion was monitored by staining blood and splenocytes from each animal with biotinylated antibody to CD11c plus allophycocyanin-conjugated streptavidin (eBioscience), PECy5-labeled antibody to B220 (eBioscience), and phycoerythrin-conjugated PDCA-1 (Miltenyi) and analyzing these samples by flow cytometry.

Flow cytometry. Cells harvested from BAL fluid were stained with biotinylated antibody to CD11c plus allophycocyanin-conjugated streptavidin, as well as fluorescein isothiocyanate-labeled antibodies to CD19, CD3, and NK1.1; phycoerythrin-labeled antibody to CD11b; PECy5-labeled antibody to B220; and Alexa 700-labeled antibody to CD8. All antibodies were purchased from eBioscience. Stained cells were analyzed with the Becton Dickinson LSRII flow cytometer and FlowJo software.

RESULTS

Infection of A549 cells by RSV or influenza A virus results in late induction of type I IFN. To determine whether reported differences in type I IFN production by RSV and influenza A virus could be understood at the level of the single cell, we compared cytokine induction by each virus in a human lung epithelial cell line, A549. Cells were infected at an MOI of 1 with RSV A2, the mouse-adapted A/WSN/33 strain of influenza virus, or NDV. NDV, an avian paramyxovirus known to be a potent viral inducer of type I IFNs in mammalian cells (6, 8), was included as a positive control. Cell supernatants were harvested at 24, 48, and 72 h postinfection; acid treated to inactivate input virus; and then assayed for antiviral activity against vesicular stomatitis virus. Type I IFN activity was found in the medium of NDV-infected cells at 24 h and in RSV-infected cultures by 48 h (Fig. 1A). In influenza virus-infected cultures, IFN-α/β activity was present only at the 72-h time point. To be certain that the relatively poor IFN production by influenza virus cultures did not reflect a difference in the numbers of infected cells, infected monolayers were fixed at each time point and stained with fluorescent antibodies. By this method, we determined that the majority of the cells were infected by 24 h with each virus tested (data not shown). These data are consistent with other reports showing ineffective induction of type I IFNs by influenza A virus in A549 cells (26, 49) but inconsistent with the notion that RSV is a poor inducer of type I IFNs. We also compared type I IFN induction by RSV
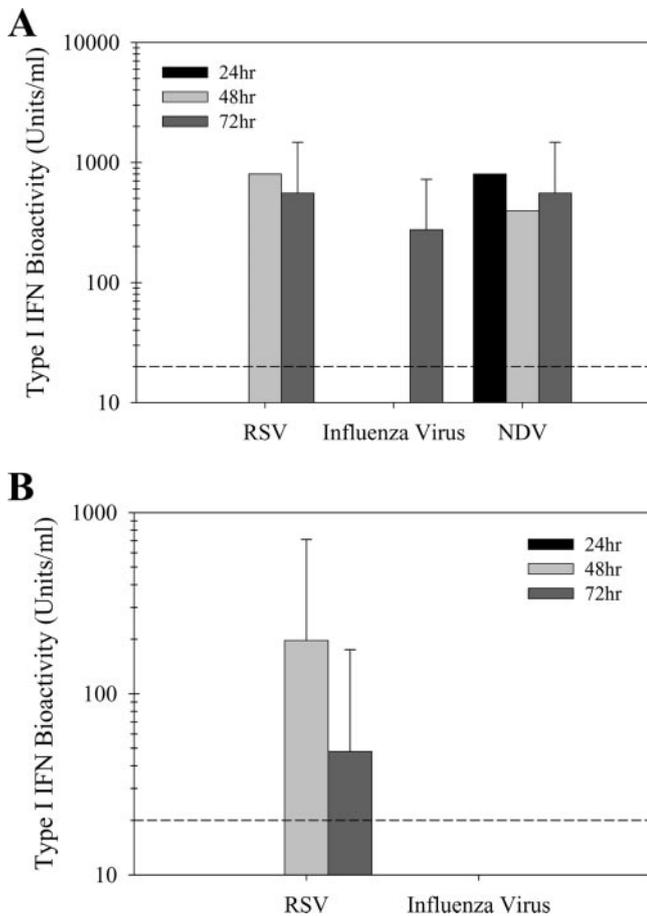
FIG. 1. Type I IFN induction in lung epithelial cell lines. (A) Levels of type I IFN bioactivity measured in supernatants from a human epithelial cell line, A549, infected with RSV, influenza virus, or NDV. Samples were taken at 24, 48, and 72 h after infection. NDV was included as a positive control. (B) Levels of type I IFN bioactivity in supernatants from a murine epithelial cell line, LA-4, infected with RSV and influenza virus. Samples were taken at 24, 48, and 72 h after infection. The limit of detection of IFN activity in the cells is indicated by the dashed line. Error bars are based upon duplicate experiments.

and WSN virus in the murine lung epithelial cell line LA-4 with similar results. In LA-4 cells, type I IFNs were detected only in the supernatants of RSV-infected cells.

Infection with influenza virus induces prolonged type I IFN production in vivo. As IFN-α/β induction by these viruses was similar in A549 cells, we looked to see whether the differences reported in samples from infected patients could be recapitulated in vivo with a mouse model. BALB/c mice were inoculated i.n. with either WSN virus or RSV at a dose of 10^6 PFU, and type I IFN activity was assayed in lung samples taken at 24, 48, and 72 h postinfection. Although the bioassay used for this determination does not discriminate among the various type I IFN species (IFN-α, -β, and -λ), we determined that the antiviral activity detected was mediated primarily by IFN-α and IFN-β as recombinant murine IFN-λ gave no signal in our bioassay (unpublished data). In this experiment (Fig. 2A), comparable levels of IFN-α/β were detected in the lung homogenates immediately following infection with either virus, but type I IFN activity decreased sharply in RSV-infected mice
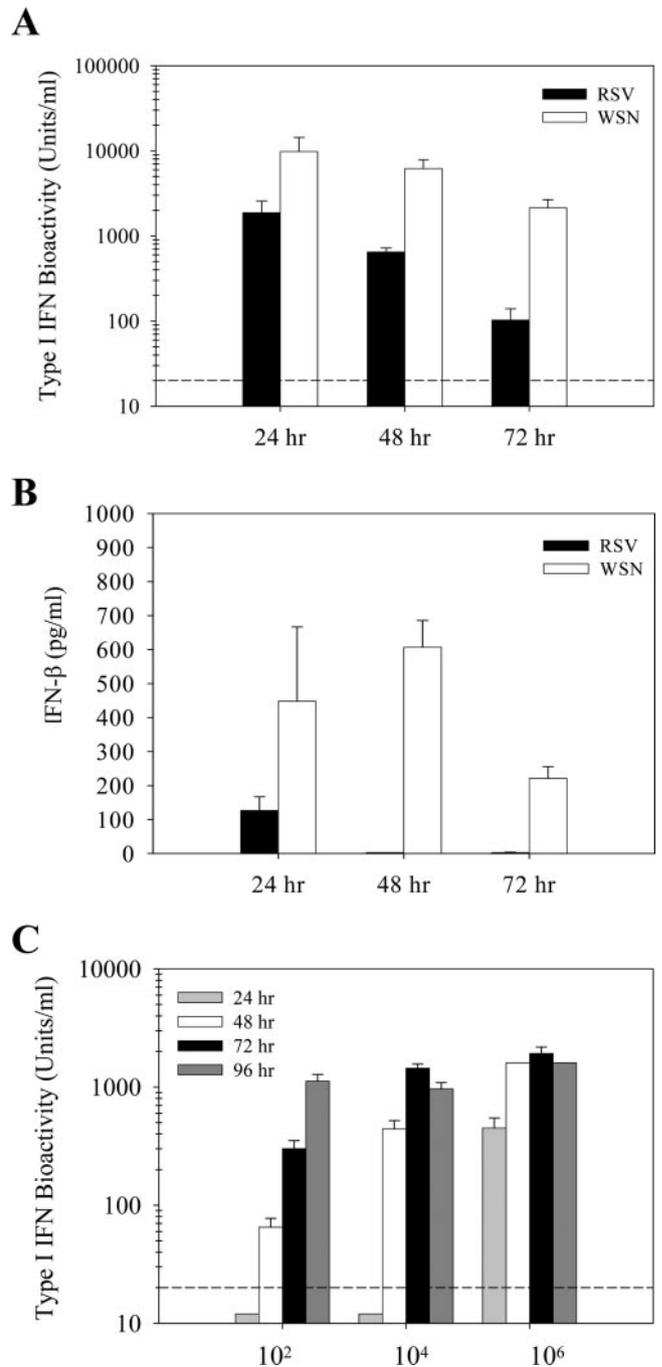

FIG. 2. Infection with influenza virus induced prolonged type I IFN production. (A) Mice were inoculated i.n. with 10^6 PFU of either RSV or WSN virus. Lungs were harvested 24, 48, or 72 h postinfection, and lung homogenates were assayed for antiviral activity against vesicular stomatitis virus. Samples from WSN virus- and RSV-infected animals are shown as white and black bars, respectively. The limit of detection of IFN activity in the lung homogenates is indicated by the dashed line. (B) Levels of IFN-β protein were determined by ELISA from BAL fluid samples in a repeat experiment. (C) The dose dependence of type I IFN bioactivity induced by WSN virus infection was determined by bioassay of BAL fluid samples. Error bars are based upon a sample size of three mice at each time point for each virus tested.

after 24 h while IFN levels in influenza virus-infected animals remained high for the duration of the experiment. The same pattern was seen when the same samples were assayed by ELISA for the presence of the IFN-β protein. IFN-β protein was present only at the 24-h time point in RSV-infected mice but was detected at all time points in influenza virus-infected mice (Fig. 2B). Additionally, the relationship between viral dose and levels of type I IFN activity in the lungs was explored at 24 h postinfection. We observed that lung IFN-α/β levels at 24 h were dose dependent following infection with either virus (data not shown), but these differences disappeared with time as influenza virus infection progressed (Fig. 2C). Altogether, these data demonstrate that both viruses can induce high levels of type I IFNs immediately upon infection, but there is a rapid decline in IFN-α/β production beginning 24 h after RSV, but not influenza virus, infection.

Prolonged IFN induction by influenza virus at a decreased virus dose. In the course of the experiments reported above, in which equivalent virus doses were used, we observed that significantly more virus was present in the influenza virus-infected lung by 24 h (data not shown). This was an expected finding due to the shorter replication cycle of influenza virus (8 to 10 h for influenza virus versus 24 h for RSV) (3, 11, 37), but we also wished to compare these infections at a dose of each virus that would result in roughly equivalent levels of viral antigen at 24 h postinfection. To determine the appropriate dose, we used immunohistochemistry to visualize viral antigen in lung tissue sections. Mice were infected i.n. with 10^4 PFU of WSN virus or 10^7 PFU of RSV, and at 24 h postinfection the lungs were harvested for histological analysis. H&E-stained lung sections from RSV- or influenza virus-infected animals are shown in Fig. 3. At this time point, a similar low level of viral antigen was present in cells lining bronchi and bronchioles of mice infected with either virus. At 96 h postinfection, when viral titers peak in the lungs of RSV-infected mice, viral antigen was present in a diffuse pattern throughout the lung but infecting primarily the pneumocytes that line the alveolar spaces. Diffuse staining for influenza virus was also found in influenza virus-infected animals; however, staining was more intense and not limited to a particular cell type. While RSV infection produced very little cytopathology, extensive cell damage was noted in lung sections of influenza virus-infected animals and a large amount of infected cell debris was present.

Having thus devised a protocol resulting in equivalent amounts of viral antigen at 24 h postinfection, we examined the effect of these adjusted doses on the levels of type I IFN activity induced by each virus over time. Mice were i.n. infected with 10^7 PFU of RSV or 10^4 PFU of WSN virus. A control group of animals was inoculated with cell lysate harvested from uninfected HEp-2 cells. BAL fluid supernatants collected from the infected mice at the 24-, 48-, and 72-h time points were assayed by ELISA for the presence of the IFN-α and IFN-β proteins (Fig. 4A and B). In RSV-infected animals, IFN-α production peaked at 24 h and quickly diminished over the next two time points, while IFN-β was detected only at 24 h. The influenza virus-infected mice had a very different pattern of type I IFN gene expression, with low initial levels of IFN-α which rose at each subsequent time point. IFN-β protein was undetectable until 48 h after influenza virus infection but had risen dramatically by 72 h. For each virus, the levels of IFN-β
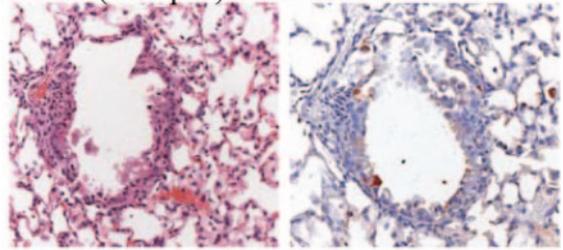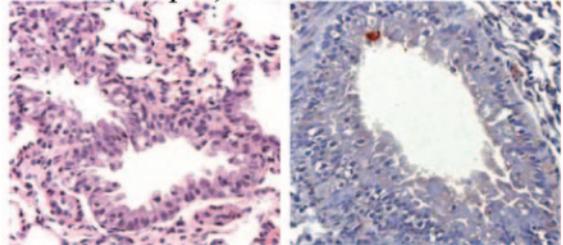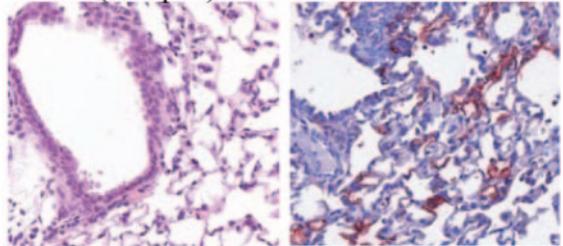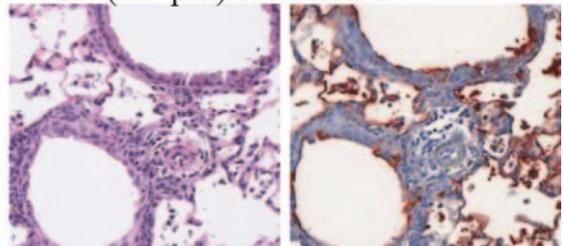

FIG. 3. Viral antigen loads in lung tissues at 24 and 96 h. Mice were infected i.n. with either 10^7 PFU of RSV (A and C) or 10^4 PFU of WSN virus (B and D). Representative lung sections from tissue harvested at 24 and 96 h postinfection are pictured, both H&E stained and immunostained. At 24 h (A and B), rare antigen-positive cells are present focally within bronchiolar epithelium following infection with either virus. By 96 h (C and D) postinfection with either pathogen, diffuse immunostaining is present. In RSV-infected mice (C), inflammation is sparse and infection is seen primarily in the cells lining the alveolar spaces. In WSN virus-infected animals (D), inflammatory cells surround bronchioles and vessels and there is intense immunostaining of both bronchiolar and alveolar epithelia. In addition, a large amount of antigen-positive cell debris is present in the alveolar spaces.

were consistently lower than those of IFN-α. Therefore, although RSV and WSN virus induce comparable levels of type I IFN, the shutoff of the synthesis of both IFN-α and IFN-β is very rapid in RSV-infected mice despite continuing virus production and spread.
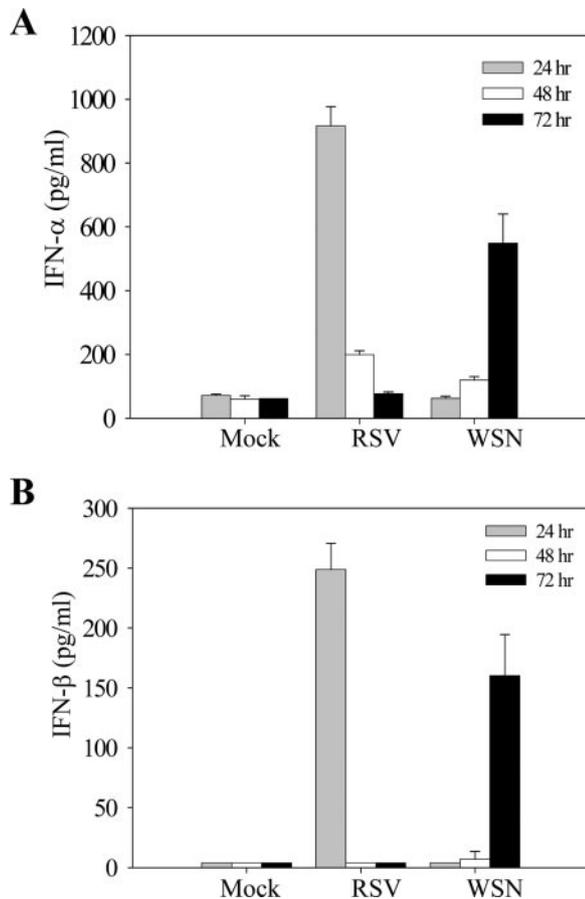
FIG. 4. Type I IFN induced by RSV infection declines after 24 h. Cohorts of mice ($n = 5$) were infected with 10^7 PFU of RSV or 10^4 PFU of WSN virus or mock infected with uninfected HEp2 cell lysate. BAL fluid samples from infected animals were harvested at 24, 48, or 72 h and assayed for IFN-α (A) and IFN-β (B) protein concentrations by ELISA.

Virus replication is necessary for the induction of type I IFN in the lung. To better understand the rapid shutoff of type I IFN synthesis during RSV infection, we sought to determine the relationship between virus replication and type I IFN induction in vivo. As DCs and macrophages can be stimulated through their TLRs, as well as by direct infection, we looked to see whether live virus is required for IFN-α/β induction in the lung. We inoculated cohorts of mice i.n. with either live or UV-inactivated virus (10^7 PFU of RSV or 10^5 PFU of influenza virus). A larger dose of influenza A virus was used in this study to guard against the possibility that a low-level response would not be detected. Only very low levels of IFN activity (less than 50 U/ml) were detected at any time point in the lungs of mice receiving UV-inactivated virus, whether RSV or influenza virus (Fig. 5). Figure 6 shows an example of a lung from an animal infected 96 h previously with 10^7 PFU of UV-inactivated RSV. In this control, as in all other animals receiving either mock- or UV-inactivated virus preparations, there is no evidence of infection or inflammation, consistent with the absence of detectable cytokine. Therefore, IFN-α/β induction in vivo requires virus infection and replication.

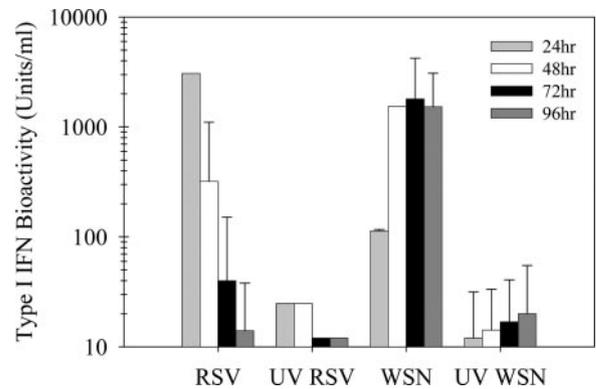Having seen that actively replicating virus was needed to

FIG. 5. Type I IFN induction requires virus replication. Mice were infected with equivalent doses of live and UV-inactivated virus (RSV, 10^7 PFU; influenza virus, 10^5 PFU), and lungs were harvested at 24, 48, 72, and 96 h postinfection. IFN activity was measured at 24 h (light gray bars), 48 h (white bars), 72 h (black bars), and 96 h (dark gray bars) postinfection. Error bars are based upon a sample size of five mice at each time point for each virus tested.

stimulate type I IFN production, we compared IFN induction as a function of virus replication. We infected mice i.n. with 10^7 PFU of RSV or 10^4 PFU of WSN virus and assayed lung homogenates for levels of both virus and IFN-α/β at various time points. For RSV, the initial IFN burst seen at 24 h postinfection declined over time as the virus continued to replicate (Fig. 7A). At 96 h postinfection, when RSV titers were maximal, type I IFN levels were very low, below 100 U/ml. This was in contrast to influenza virus infection, where the rise in type I IFN activity paralleled that of virus replication (Fig. 7B).

We conclude from these experiments that, in the lung, type I IFN production is replication dependent for either infection. However, and despite ongoing replication leading to diffuse pulmonary infection and peak virus titers around day 4, only RSV inhibits ongoing production of IFN-α/β after 24 h. This is consistent with the kinetics of type I IFN production by RSV A2 in mice observed by Guerrero-Plata et al. (20) and also with the clinical data comparing IFN production in RSV- or influenza virus-infected patients.
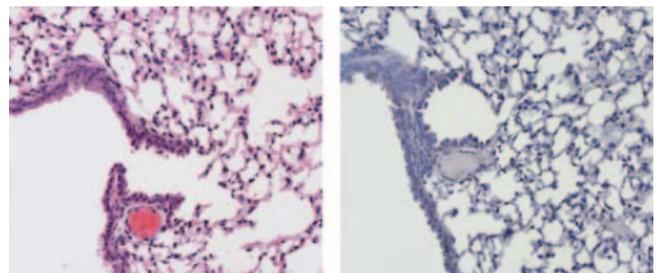

FIG. 6. No inflammation is triggered by i.n. instillation of UV-inactivated virus. Following instillation of live and UV-inactivated virus preparations, mice were monitored by histology for the presence of lung inflammation, epithelial cytopathology, or evidence of viral antigen. No evidence of infection was found in these mice or mock-infected controls. The left panel shows an H&E-stained section from a lung harvested 96 h following inoculation with UV-inactivated RSV. The right panel shows a serial section stained for the presence of RSV antigens. No viral proteins were detected by immunohistochemistry.
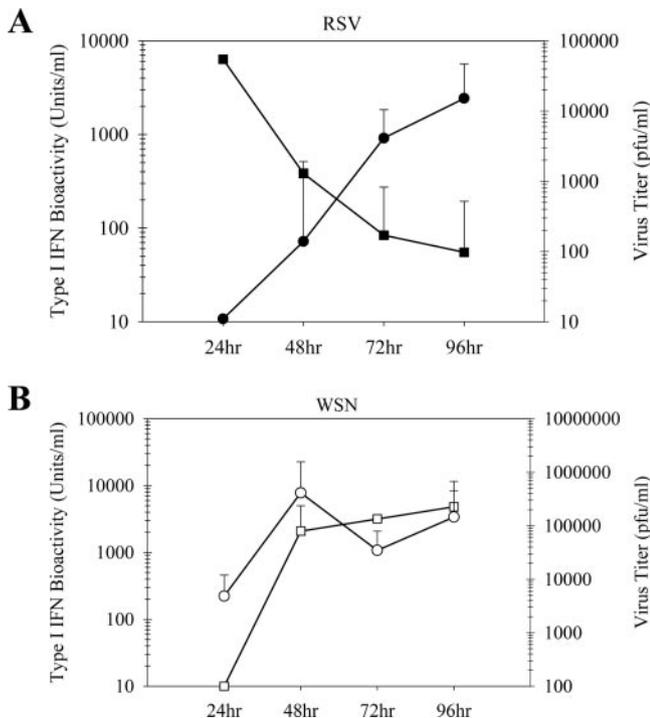
FIG. 7. RSV and influenza virus have distinct type I IFN induction profiles. Mice were infected i.n. with either 10^7 PFU of RSV (A) or 10^4 PFU of WSN virus (B), and their lungs were harvested at 24, 48, 72, or 96 h postinfection. Lung homogenates were assayed for type I IFN activity and for viral titers. Levels of type I IFN activity are plotted on the left *y* axis and are indicated by squares. Viral titers are plotted on the right *y* axis and are indicated by circles. Error bars are based upon a sample size of five mice at each time point. Black squares and circles, RSV (A). White squares and circles, WSN virus (B).

Lung epithelial cells appear to be the major source of type I IFN following RSV infection. pDCs are known to be an important source of type I IFN following virus infection in general, but this does not appear to be true for every viral pathogen (12, 33). In these experiments, we wished to determine which cell types contribute to type I IFN production following virus infection of the lung and whether these were the same for both RSV and WSN virus. To ask this question directly, we used in situ hybridization to visualize the presence of IFN-α transcripts (the IFN-α4 probe detects all of the alpha subtypes) in lung tissue sections and accompanying lymph nodes taken from infected mice at various time points. In the experiment shown in Fig. 8A, mice were infected with either 10^7 PFU of RSV or 10^4 PFU of WSN virus and tissues were harvested at 48 h, the earliest time point when mediastinal lymph nodes can readily be identified. The patterns of lung parenchymal IFN-α expression obtained with the two viruses were similar, with the strongest signal coming from the airway lining cells. However, and consistent with type I IFN bioactivity and ELISA data, the signal from RSV-infected tissue at 48 h was comparatively weak. Lymph nodes pictured in Fig. 8A were directly adjacent to the sections of lung shown in this figure. In the influenza virus-infected mice, many bright signals are visible within the lymph node, although the exact cell type producing the IFN-α transcripts cannot be determined by this method. Foci of IFN-α mRNA production were also apparent

in mediastinal lymph nodes taken from RSV-infected animals, but these were fewer and fainter than those seen in WSN virus-infected animals. Analysis of lung sections with an IFN-β riboprobe yielded patterns of staining for both RSV and influenza virus similar to those seen for IFN-α, but at diminished intensity.

Finally, to rule out the possibility that pDCs present within the airspace were not being detected in the course of our in situ hybridization studies, we sought to determine whether large numbers of these cells were present in BAL fluid samples at times corresponding to peak type I IFN production. Following i.n. infection with 10^4 PFU of WSN virus, BAL fluid cells harvested after 24, 48, 72, or 96 h were enumerated and phenotyped by flow cytometry. While the total number of lung DCs (all CD11c⁺) increased with time postinfection, as shown in Fig. 8B, the number of pDCs (CD11c, B220) present was very low at each of the time points tested, was undetectable at 24 h, and rose to a maximum of 2,000 to 2,500 cells/lung by day 4.

We therefore conclude from this set of experiments that (i) epithelial cells are a major source of IFN-α/β in both RSV- and influenza virus-infected lungs and (ii) that the relative contribution of type I IFN-producing cells within adjacent lymph nodes is much greater in the case of influenza virus.

IFN-α/β production by virus-inoculated DC cultures in vitro. While it is known that IFN-α/β is produced by virtually all virus-infected cells, activated pDCs that produce very high levels of these cytokines are thought to be a major source following virus infection (10). Given the very low levels of type I IFN transcripts detected in the lymph nodes of RSV-infected animals (Fig. 8), we wondered whether the relatively low levels of type I IFNs produced in response to RSV reflected the inability of this virus to activate pDCs. To directly compare the IFN-stimulating abilities of RSV and influenza virus in vitro, duplicate wells of BMDCs cultured with FLT-3 ligand (18) were infected with live or UV-inactivated RSV or WSN virus at an MOI of 1 and culture medium was harvested from each sample 24 h later. Figure 9 shows the quantities of IFN-α and IFN-β proteins detected in these supernatants. Only live RSV stimulated the production of type I IFNs by DCs, but importantly, even live RSV was a relatively ineffective inducer of type I IFNs in this cell type. IFN-α and -β protein levels were both increased in medium taken from influenza virus-infected cultures, and this difference was on the order of 5- to 10-fold compared to RSV-infected cultures. Interestingly, treatment with either virus led to the production of nearly equivalent levels of IFN-α and IFN-β proteins, as opposed to the predominance of IFN-α in infected-lung homogenates and BAL fluid samples. Therefore, despite the caveat that an in vitro experiment cannot model the dynamics of virus production and DC migration in the lung, these data demonstrate that under conditions where pDCs are exposed to equal doses of virus, influenza virus is a much more potent inducer of type I IFNs. This result is consistent with our in situ hybridization data that showed only low-intensity signals in lymph nodes from RSV-infected animals.

IFN-α/β production by pDCs in vivo. Direct determination of the contribution of pDCs to type I IFN levels in the infected lung was done by infecting pDC-depleted BALB/c mice with 10^6 PFU of influenza virus or 10^7 PFU of RSV. High-dose infection with both viruses was chosen for this experiment so
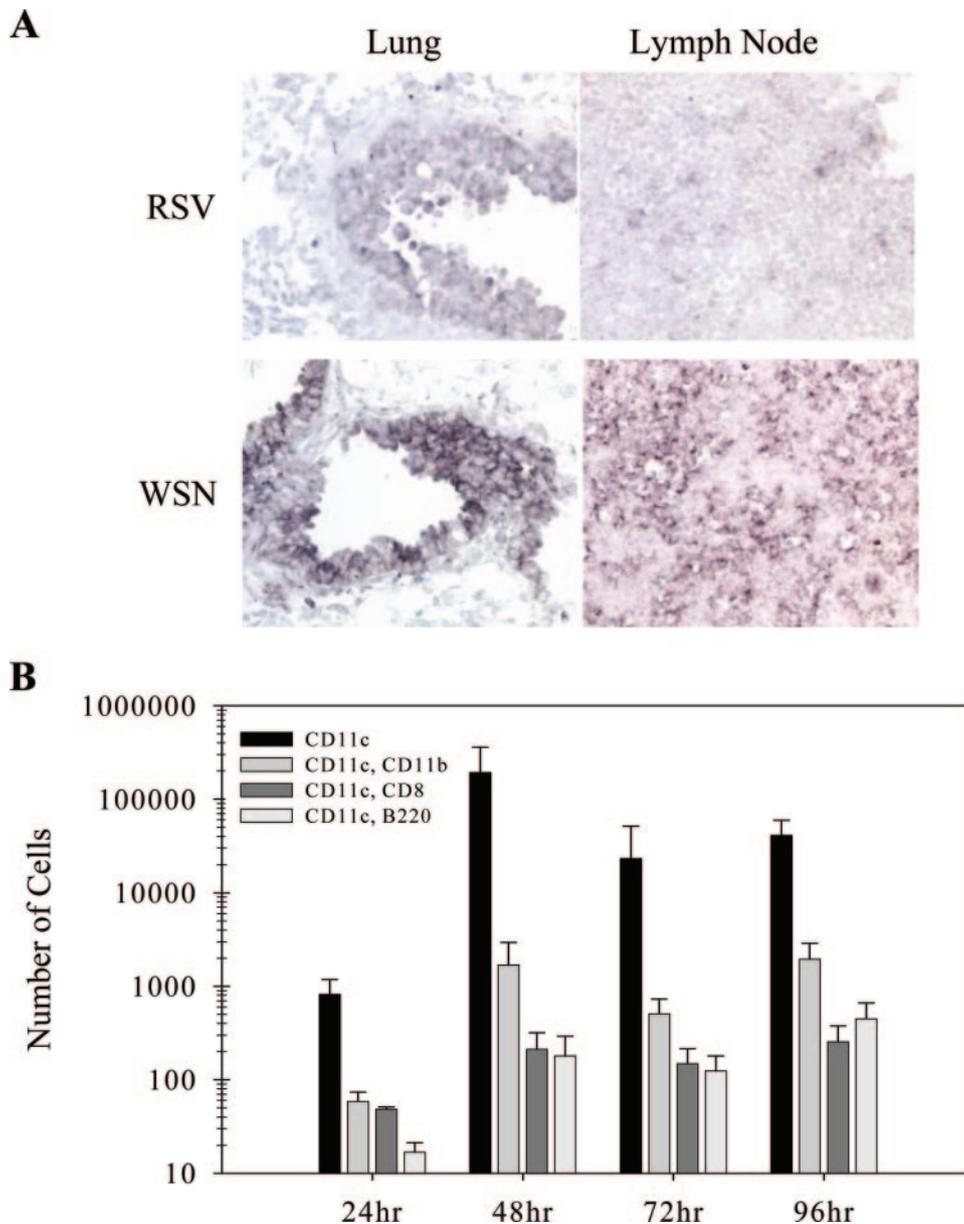
FIG. 8. Infected lung epithelial cells are a major source of type I IFNs. Mice were i.n. infected with either 10^7 PFU/ml RSV or 10^4 PFU/ml influenza virus, and tissues were harvested at 48 h postinfection. (A) In situ hybridization with a DIG-labeled murine IFN-α4 riboprobe was used to detect the presence of type I IFN transcripts in the lung tissue and adjacent lymph nodes. (B) Accumulation of the various DC subsets at 24-h intervals following i.n. infection of mice with 10^4 PFU of influenza A virus. CD11c = total DCs; CD11c, CD11b = myeloid DCs; CD11c, CD8 = antigen-presenting DCs; CD11c, B220 = pDCs. Error bars are based upon a sample size of five mice at each time point for each dose of virus tested.

that peak IFN-α/β levels could be measured simultaneously. Depletion was carried out by i.p. injection of 0.5 mg of the monoclonal antibody PDCA-1 at 48 h and again at 24 h prior to infection. Control groups were treated with the same dose of control rat IgG. Animals were sacrificed at 24 h postinfection and analyzed for the presence of pDCs in the spleen and blood, as well as lung IFN-α/β production. Antibody treatment resulted in the successful and specific depletion of pDCs, as shown in Fig. 10A, but this resulted in decreased lung IFN production only in the cohort of influenza virus-infected mice. Levels of lung IFN-α or -β were unchanged in RSV-infected

animals (Fig. 10B). This result demonstrated that pDCs do not contribute significantly to type I IFN production following RSV infection in vivo but do have a role in the IFN response to influenza virus. Nonetheless, even in WSN virus-infected lungs, the respiratory epithelium appears to be the major source of influenza virus-induced IFN-α/β in the lung, particularly at early time points.

This conclusion was strengthened by the converse experiment wherein type I IFN production was measured following infection of mice lacking the IFN-α/β receptor and thereby the ability to amplify IFN-α production via the IRF7 pathway in
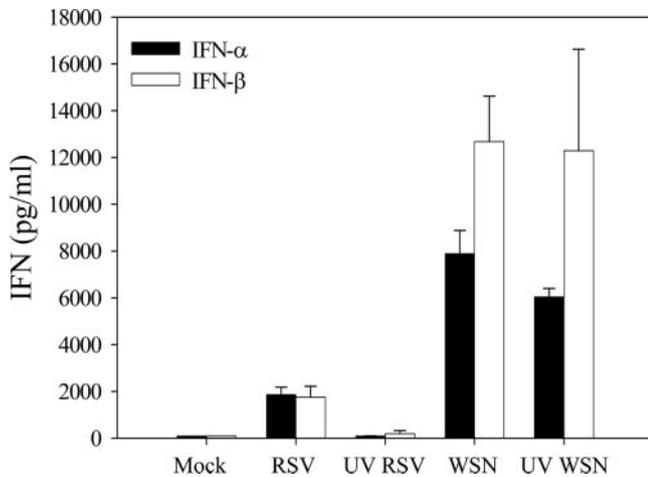
FIG. 9. Reduced IFN-α/β production by RSV-treated DCs in vitro. BMDCs cultured with FLT-3 ligand were mock infected or infected with either RSV (live or UV inactivated) or WSN virus (live or UV inactivated) at an MOI of 1. At 24 h postinfection, infected-DC supernatants were assayed for IFN-α and IFN-β protein concentrations by ELISA.

non-pDCs. For this study, mice were infected with 10^7 PFU of RSV or 10^4 PFU of WSN virus and lungs were harvested for measurement of type I IFN levels at 24-h intervals (Fig. 10C). If the infected epithelium is the major source of type I IFN in the lung, we would predict that IFN-β levels would be similar over time in both wild-type and IFNAR1$^{-/-}$ mice but that IFN-α levels would be markedly reduced in mice lacking the IFN-α/β signaling pathway. In fact, this was the case with normal IFN-β levels at each time point (compare Fig. 4 and 10), but levels of IFN-α were markedly decreased in the IFNAR1$^{-/-}$ animals. As shown in Fig. 10C, IFNAR1$^{-/-}$ mice produced no detectable IFN-α at early times following infection with either virus. In influenza virus-infected animals, a low level of IFN-α appeared at 72 h, which we presume is made by pDCs. In light of the type I IFN dependence of pDC migration (2), we would not expect a robust pDC response in IFNAR$^{-/-}$ mice even after WSN virus infection. We conclude from these experiments that i.n. infection with either virus leads to high levels of type I IFN production by the infected respiratory epithelium (α ≫ β) but that only influenza virus stimulates IFN production by pDCs.

DISCUSSION

We began these studies to determine the mechanism underlying the differential induction of type I IFN seen in influenza A virus or RSV-infected patients (23, 24, 40). This is an important question, as it has become increasingly clear that, in addition to mediating the up-regulation of antiviral protein genes, type I IFNs play an important role in the establishment of immune memory (57). As RSV is known for its inability to stimulate effective immunity and reinfection occurs throughout life, it is reasonable to ask whether this virus is uniquely able to avoid or suppress type I IFN production in vivo despite its demonstrated ability to induce IFN-α/β production in cultured cells.

The contribution of pDCs to total type I IFN synthesis in

mice has been studied for a small number of viral pathogens and appears to differ with each virus studied (1, 4, 12, 45). This is not surprising given that viruses differ with respect to the route of infection, tissues infected, and means of spread. However, both influenza virus and RSV are respiratory pathogens with their replication largely restricted to the epithelial lining of the pulmonary tree and occurring without an associated viremic phase. In addition to the clinical studies demonstrating robust type I IFN production in influenza virus-infected patients, the ability of influenza A virus to induce high levels of IFN-α production by pDCs via TLR7 has also been established (14, 36).

In published studies, it has been shown that RSV can trigger both type I IFN production and IRF7 synthesis in infected A549 cells (9, 17), as well as IFN synthesis by human pDCs ex vivo (21, 27). We have reproduced the epithelial cell data (Fig. 1) and have extended the ex vivo studies by comparing type I IFN induction by RSV and influenza A virus in the murine system. In A549 cells, RSV and WSN virus stimulated the synthesis of comparable levels of IFN-α/β but RSV was a relatively poor inducer in murine DCs cultured with FLT-3 ligand. In addition, live RSV was needed for cytokine induction, while both live and UV-inactivated influenza virus triggered high levels of type I IFN production. This requirement for active infection by RSV is consistent with results obtained by other groups with human pDCs (21, 27) and suggests that, unlike the response to influenza virus, the response to RSV is largely TLR independent. It is possible that this difference may be an important factor in the enhanced ability of influenza virus to induce type I IFNs in vivo despite inhibition of the IFN response by NS1 (31).

To determine whether the relative inability of RSV to stimulate high levels of IFN-α/β production by pDCs in vitro was reflected in vivo, we compared WSN virus and RSV infections in the BALB/c mouse. In the animal model, we observed that the major difference between the two infections was not the maximal level of type I IFN produced by the lung but rather the kinetics of its appearance and disappearance. At high virus doses, either pathogen induced the same initial IFN-α/β response, but in RSV-infected animals this burst was short-lived with a sharp decline in type I IFN production as the infection progressed. The opposite result was obtained in influenza virus-infected animals, where type I IFN synthesis was closely correlated with virus replication.

In support of our prediction that pDCs are not a significant source of IFN-α/β in RSV-infected animals, we found that type I IFN production following RSV infection of pDC-depleted animals was unchanged. On the other hand, IFN-α production was ablated in RSV-infected knockout mice lacking a functional IFN-α/β receptor and therefore the ability to stimulate epithelial IFN-α synthesis via the feedback loop. In WSN virus-infected animals, both the epithelial and pDC compartments contributed to IFN production, as evidenced by in situ hybridization, pDC depletion, and detection of virus-induced type I IFNs in IFNAR1$^{-/-}$ animals. Our findings differ from those of Smit et al. (54) and do not support their conclusion that pDCs are the sole source of IFN-α in the lungs of RSV-infected BALB/c mice. It is difficult to compare our studies directly as different methods of pDC depletion and a different strain of RSV were used by the other laboratory. In tissue sections,
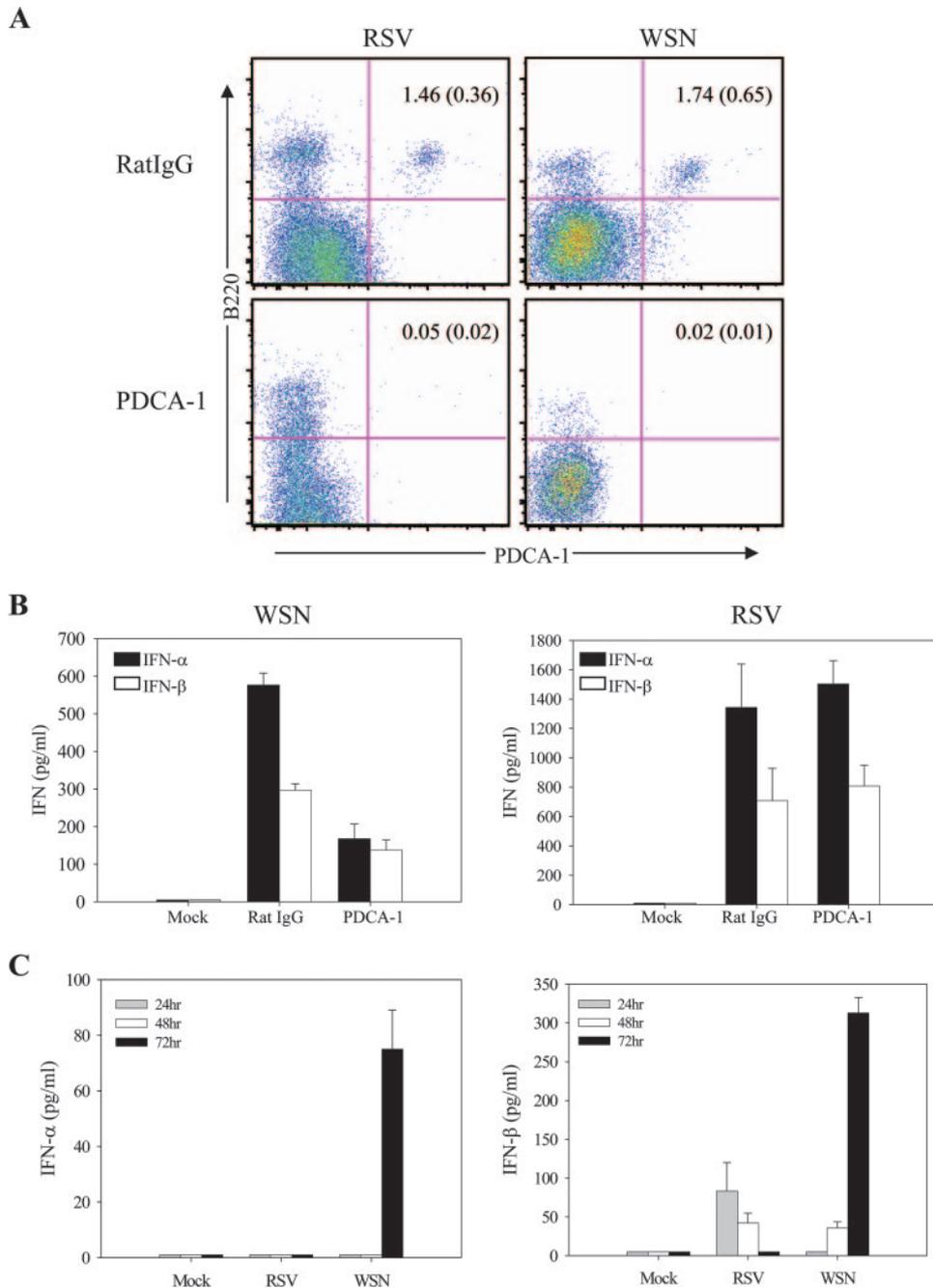
FIG. 10. pDC depletion does not alter type I IFN synthesis in RSV-infected mice. Mice were infected with virus (10^6 PFU of WSN virus or 10^7 PFU of RSV) following either pDC depletion by i.p. injection of monoclonal antibody PDCA-1 or i.p. injection of rat IgG, which was used as a control for antibody treatment. (A) Extent of pDC depletion was monitored by fluorescence-activated cell sorter analysis of blood taken from individual mice ($n = 4$ animals per group). (B) Type I IFN levels in lung homogenates were determined by ELISA 24 h after infection ($n = 4$ mice per group). Both IFN-α ($P = 0.0019$) and IFN-β ($P = 0.003$) were significantly lower in pDC-depleted, WSN virus-infected animals, but there were no significant differences in lung samples from RSV-infected mice with or without PDCA-1 treatment. (C) Lung IFN-α/β levels were measured at multiple time points following infection of IFNAR$^{-/-}$ mice with RSV (10^7 PFU) or WSN virus (10^4 PFU) ($n = 5$ animals per group).

nonepithelial IFN-producing cells were detectable only in lymph nodes, which do not appear until 48 h after the start of infection. The kinetics of type I IFN production stimulated by influenza virus are therefore best explained by a model in which early IFN-β and IFN-α are produced by the lung epithelium and the contribution from pDCs increases as they are drawn to the site of infection. If this model is correct, then the remarkably short burst of detectable IFN-α/β in the lung following inoculation with RSV is further evidence that the contribution of pDCs is limited in that infection.

The differential contribution of pDCs to the type I IFN response elicited by RSV and influenza virus is consistent with

the current understanding of virus-DC interactions. Whereas influenza virus enters the cell by means of the endosomal compartment, where it encounters and triggers TLR7 or -8 signaling (36), RSV fuses directly with the target cell's plasma membrane without this opportunity for interaction (11). This mechanism of cell entry, along with the requirement for live virus, implies that RSV recognition by the pDC depends upon intracytoplasmic receptors such as RIG-I and suggests that the ability of the viral nonstructural proteins to interfere with IRF3 activation could play a role even in pDCs, as well as epithelial cells (6, 35, 46, 47, 51, 55, 56). Therefore, while we and others (21, 27, 58) have seen that RSV can trigger type I IFN production by pDCs, it is also not surprising that this subset is a relatively inefficient producer of IFN-α/β in response to RSV infection in vivo.

In summary, our study shows a differential induction of type I IFNs by influenza virus and RSV in vivo and suggests that this difference is due, in part, to the limited contribution by pDCs in the RSV-infected animal. And while it does not appear that type I IFN is necessary for viral clearance in RSV infection (29), it is possible that poor induction of IFN-α/β synthesis by this virus contributes to its weak immunogenicity in the human host.

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