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IL-12p40 and IL-18 Modulate Inflammatory and Immune Responses to Respiratory Syncytial Virus Infection

Shan-Ze Wang, Yi-Xiao Bao, Cynthia L. Rosenberger, Yohannes Tesfaigzi, James M. Stark, and Kevin S. Harrod

Respiratory syncytial virus-induced bronchiolitis has been linked to the development of allergy and atopic asthma. IL-12 and possibly IL-18 are central mediators orchestrating Th1 and/or Th2 immune responses to infection. To determine a possible role for IL-12 in regulating the immune response to acute respiratory syncytial virus infection, IL-12p40 gene-targeted (IL-12p40−/−) and wild-type mice were intratracheally infected with respiratory syncytial virus, and lung inflammatory and immune responses were assessed. Lung inflammation and mucus production were increased in the airways of IL-12p40−/− mice as compared with those of wild-type mice, concurrent with increased levels of the Th2 effector cytokines IL-5 and IL-13. Respiratory syncytial virus clearance and levels of Th1 effector cytokine IFN-γ were not altered. Interestingly, IL-18, another mediator of IFN-γ production, was significantly increased in the lungs of IL-12p40−/− mice early during the course of infection. Abrogation of IL-18-mediated signaling in IL-12p40−/− mice further enhanced Th2 immune response and mucus production in the airways during respiratory syncytial virus infection but failed to modulate IFN-γ production or viral clearance. These findings implicate a role for IL-12 and IL-18 in modulating respiratory syncytial virus-induced airway inflammation distinct from that of viral clearance. The Journal of Immunology, 2004, 173: 4040–4049.

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2 S.-Z.W. and Y.-X.B. contributed to this article equally.
3 Address correspondence and reprint requests to Dr. Kevin S. Harrod, Asthma and Pulmonary Immunology Program, Lovelace Respiratory Research Institute, 2425 Ridgecrest Drive, SE, Albuquerque, NM 87108. E-mail address: kharrod@lrri.org
4 Abbreviations used in this paper: RSV, respiratory syncytial virus; i.t., intratracheal; BALF, bronchoalveolar lavage fluid; AB, Alcian blue; PAS, periodic acid-Schiff; MUC5AC, mucus 5 AC; AHR, airway hyperreactivity; MCM, mucus cell metaplasia; WT, wild type.

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Materials and Methods

Animals

Specific pathogen-free BALB/c mice were purchased from the National Cancer Institute and IL-12p40−/− mice with the BALB/c background were purchased from The Jackson Laboratory (Bar Harbor, ME) and kindly provided by Dr. M. Schuyler (University of New Mexico, Albuquerque, NM). The mice were housed in Lovelace Respiratory Research Institute (LRRI) animal facilities under pathogen-free conditions. The average age of the mice when infected was 7–9 wk. The animal protocol was approved by the LRRI Institutional Animal Care and Use Committee.

RSV stock and RSV plaque assay

A stock of RSV long-strain A2 was generated in Hep-2 cells, and viral titration was performed as described elsewhere (24, 25). Purified RSV titers or RSV infectivity in fresh lung tissues were determined in triplicate for each culture dilution using standard plaque assay procedures performed as previously described (24). Briefly, lung tissues were collected from both wild-type (WT) and IL-12p40−/− mice 7 days after RSV infection. The lung tissues were homogenized briefly for 10 s and centrifuged at 2000 rpm for 5 min at 4°C. The supernatants were collected and diluted serially in cold PBS. The diluted supernatants were inoculated onto Hep-2 cells in six-well plates in triplicates for each dilution. The inoculated plates were then incubated for 1 h at 37°C and in 5% CO2, with rocking of the plates every 15 min to ensure even distribution of the inoculants. After this incubation period, 2 ml of 0.75% methyl cellulose in Earle’s MEM containing 10% FBS was overlaid into each well, and the plates were incubated for 7 days. On day 7, the methyl cellulose was aspirated and the plates were stained with hematoxylin and the viral plaques were counted.

Mouse model of RSV infection

Intratracheal (i.t.) inoculation of 2 × 106 PFUs of RSV in 100 μl of culture medium was performed by insertion of a 27-gauge tube through the oral cavity of mice under anesthesia by inhalation of a halothane/nitrous oxide mixture. Intratracheal insertion was confirmed by the lack of negative pressure applied by retraction of a syringe plunger. A syringe containing inoculant was expelled into the trachea followed by multiple injections of air to facilitate dispersion of inoculant into the lung periphery. Mice usually recovered within 5 min and resumed normal eating and grooming activities (26, 27). Sterile medium (100 μl/mouse) was used as control.

Neutralization of endogenous IL-18 in IL-12p40−/− mice

Endogenous IL-18 was neutralized as described previously (28). Briefly, 30 μg/mouse of a rat IgG1 anti-murine IL-18 Ab (MBL, Nagoya, Japan) was instilled i.t. 2 h before RSV instillation in IL-12p40−/− mice. These mice were further injected i.p. with 20 μg/mouse of anti-IL-18 Ab on days 3 and 5 after infection. The mice were then sacrificed and samples were collected on day 7 after infection. Rat IgG1 isotype (R&D Systems, Minneapolis, MN) was used as a control.

Cell counts in BALF

Six to 10 mice for each group were used for all of the experiments. BALF was obtained by three serial i.t. instillations of 0.6 ml of PBS into the right lung using a fine catheter tied to the right main bronchus, and the samples were pooled for each animal. The BALF was processed, and the total and differential cells were counted as described elsewhere (26, 27). The remaining lung tissues were then used for histopathological and molecular studies as described below.

Pulmonary histopathology

Lung inflammation was assessed by histological staining of lung sections as described elsewhere (26, 27). Left lungs were inflated via a tracheal cannula at 20 cm of pressure with 4% paraformaldehyde and removed en bloc from the thorax. Inflation-fixed lungs were washed in PBS, 30, 50, and 70% ethanol, and bisected transversely in a dorsoventral direction just caudal to the entry of the mainstem bronchus for paraffin embedding. Lung sections (5 μm) were taken starting 100 μm from the designated reference point and collected at 100-μm intervals. Two sections of left lung for each animal were stained with H&E and graded on a minimal, mild, moderate, and marked scale corresponding to numbers 1 (minimal) to 4 (marked) according to the following criteria: septal, perivascular, peribronchial infiltrates, and epithelial cell hyperplasia/hyper trophy (27). All slides were scored by a blinded observer, and a score was determined for mean ± SEM of 6–10 animals per group.

Immunohistochemical staining for CD3 in the lungs

Lung sections were stained overnight with primary rabbit anti-mouse CD3 Ab (Sigma-Aldrich, St. Louis, MO). Slides were rinsed in PBS and incubated with secondary goat anti-rabbit Ab conjugated to biotin. A streptavidin-conjugated alkaline phosphatase detection system was used to visualize CD3 following incubation with Vector Red (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. The total number of labeled cells in tissue was counted manually in 10 fields with magnification of ×400.

Alcian blue-periodic acid-Schiff staining

Lung sections of the left lung lobes (6–10 mice/group) at 100 μm from the reference point were stained by Alcian blue-periodic acid-Schiff (AB/PAS) stain to identify mucus-secreting cells as described previously (26, 27). Briefly, the lung sections were deparaffinized in xylene and hydrated in decreasing concentrations of ethanol. The slides were then stained in AB for 30 min, washed in running water for 5 min, oxidized in 1% periodic acid for 10 min, and washed in running water for 5 min. After staining in Schiff’s reagent for 10 min, slides were rinsed three times in sodium metabisulfite, washed in running water for 10 min, and mounted following dehydration in ethanol and xylene. In all mice, only the mucus cells in epithelia lining the main intrapulmonary bronchi were analyzed. The length of basal lamina lining the large airways of WT and IL-12p40−/− mice and the volume density of stored AB/PAS-stained mucusubstances in the surface epithelium were determined using Scion Image Software (National Institutes of Health, Bethesda, MD) and calculated as described previously (27, 29).

Immunohistochemical staining for mucin 5AC (MUC5AC) in the lungs

Tissues were deparaffinized in xylene and hydrated in graded ethanol followed by deionized water, then washed in 0.05% 1/3 Brij-35 in Dulbecco’s PBS (pH 7.4). Endogenous peroxidase activity was blocked by incubating the sections in 2% hydrogen peroxide in methanol for 1 min. The MUC5AC protein was unmasked by treating the slides using the Digest-All kit (Zymed Laboratories, San Francisco, CA) at a 1/3 dilution of trypsin to dilute at 32°C for 10 min. After preincubation in 100 mM Tris (pH 7.7) containing 550 mM NaCl, 10 mM KCl, 1% normal goat serum, and 2% BSA, slides were incubated overnight at room temperature with the MUC5AC Ab, HO8, a gift from Dr. S. Ho (University of Minnesota Veterans Administration Medical Center, Minneapolis, MN) at a dilution of 1/4000. MUC5AC immunoreaction was detected using the Vectastain ABC kit (Vector Laboratories) and the peroxidase substrate diaminobenzidine (Vector Laboratories) according to the manufacturer’s directions; tissue sections were counterstained with hematoxylin and mucous cells were identified by staining with AB (0.05%) for 10 min.

RNA isolation and RT-PCR

Total RNA was isolated from the cardiac and diaphragmatic lobes of the right lungs using Tri-reagents (Molecular Research Center, Cincinnati, OH) per the manufacturer’s protocol. RT-PCR analysis was designed to detect nascent viral mRNA transcripts, but not to detect genomic or progeny RSV RNA, as a measure of viral transcriptional activity as described previously (26, 27). Virus-specific mRNA transcript for RSV gene F (designated RSV-F) was converted to cDNA by reverse transcription reaction using the following virus-specific primer sequence: RSV-F, 5′-CAATCTC CAACTTATATATGCC-3′. The RSV-specific sequence was amplified by PCR using the following primer set: RSV-F′ upper primer, 5′-CCAAG CTTGGCTAGATCCTCA-3′ and RSV-F′ lower primer, 5′-CAT CGACCCTGTTAGAAATG-3′. Primer sequences were identified using Lasergene software (DNASTAR, Madison, WI). Amplification assays were run over multiple cycles to define cycle numbers yielding linear production of cDNA products. Varying concentrations of input RNA for the reverse transcriptase reaction and varying concentrations of cDNA for the PCR were used and assure a semiquantitative analysis. The RSV-specific sequence was amplified for 30 cycles, and RT-PCR amplification was performed.
products were visualized by ethidium bromide-stained gel electrophoresis under UV light illumination. Gel images were captured and densitometric analysis was performed using the Gel-Doc documentation system and Quantity One software (Bio-Rad, Hercules, CA).

**Pulmonary cytokines**

Cytokine protein levels in the lung were measured as described elsewhere (27). Briefly, the apical and intermediate lobes of the right lungs were homogenized in 1 ml of PBS and centrifuged at 2000 rpm for 7 min at 4°C. The supernatants were collected for cytokine protein analysis. The levels of IL-4, IL-5, IL-13, IFN-γ, and IL-18 were analyzed using ELISA (R&D Systems). The sensitivity of the kits for IL-4, IL-5, IL-13, IFN-18, and IFN-γ are 2, 7, 1.5, 25, and 2 pg/ml, respectively. Data were normalized by total lung protein. Standard curves were calculated from known standards for calculation of cytokine.

**Statistical analysis**

Results from 6 to 10 different mice in each group were expressed as mean ± SEM unless otherwise indicated. Differences between two groups were assessed for significance by Student’s t test when data were available in only two groups. When data were available in more than two groups, ANOVA was used to perform pairwise comparisons, followed by Fisher’s least-significant difference test. When data sets failed to exhibit parametric distribution, data were transformed to achieve a parametric distribution and analyzed by ANOVA. Differences were considered significant at p < 0.05.

**Results**

**RSV clearance is not altered in IL-12p40−/− mice**

To assess the effect of IL-12 on viral clearance, IL-12p40−/− and WT mice were infected with RSV (2 × 10⁶ PFUs/mouse) i.t. for 3, 7, and 10 days, respectively. RT-PCR analysis of viral-specific gene expression of RSV-F was performed from the total RNA isolated from the lungs of mice at each time point following RSV infection. RT-PCR analysis of endogenous β-actin mRNA steady-state levels was used as loading assay controls. At 3 days after RSV infection, the RSV-F gene was expressed readily in the lungs of all IL-12p40−/− and WT mice. At 7 days after infection, the RSV-F gene was only detected at very low levels in the lungs of both types of mice. At 10 days after infection, RSV-F gene expression was undetectable in the lungs of all mice (Fig. 1a). No statistical difference in the relative levels of RSV gene expression between IL-12p40−/− and WT mice was observed at any time point (Fig. 1b). The viral infectivity was also assessed using the plaque assay. The numbers of viral plaques were similar in the WT and IL-12p40−/− mice 7 days after RSV infection (Fig. 1c), and this result is consistent with the data as assessed using RT-PCR.

**Inflammatory cell counts in BALF are increased in IL-12p40−/− mice after RSV infection**

To determine the effect of IL-12 deficiency on lung inflammation, inflammatory cell populations in BALF from IL-12p40−/− and WT mice were assessed following RSV infection. On day 3 after RSV infection, the numbers of total inflammatory cells, macrophages, and lymphocytes were increased in BALF from WT mice infected with RSV as compared with those of vehicle-control WT mice. Although the other cell types were similar in the IL-12p40−/− mice and WT mice infected with RSV, the number of lymphocytes was significantly increased in the BALF from IL-12p40−/− mice infected with RSV as compared with that of vehicle-control IL-12p40−/− or WT mice infected with RSV (Fig. 2a). On day 7 after RSV infection, the total inflammatory cell counts and the numbers of macrophages, lymphocytes, and neutrophils in infected WT mice were significantly higher than those of vehicle-control WT mice. The total inflammatory cell counts and the numbers of lymphocytes and neutrophils were further increased in the BALF from IL-12p40−/− mice as compared with those from WT mice infected with RSV (Fig. 2b). Minimal eosinophil numbers were observed in these mice and there was no statistical difference in eosinophil counts between the WT and IL-12p40−/− mice infected with RSV.

**CD3+ lymphocytes are increased in the lungs of IL-12p40−/− mice after RSV infection**

To further characterize the cell type of lung inflammation, CD3+ lymphocytes in the lungs of IL-12p40−/− and WT mice were assessed following RSV infection. At 3 days after RSV infection, the numbers of CD3+ lymphocytes were slightly increased in WT and IL-12p40−/− mice infected with RSV as compared with those of vehicle-control mice, respectively; however, there was no statistical difference between the WT and IL-12p40−/− mice infected with RSV (Fig. 3). At 7 days after RSV infection, the CD3+ lymphocytes in WT and IL-12p40−/− mice infected with RSV were increased significantly compared with those of vehicle-control mice, respectively (see Fig. 3a, representative photomicrographs from mice 7 days after infection). The CD3+ lymphocytes were
Airway mucus overproduction is a hallmark clinical feature of IL-12p40β/ mice infected with RSV (Fig. 3b). AB/PAS-positive mucous substances are increased in the airways of IL-12p40β/ mice as compared with those in WT mice infected with RSV (Fig. 3b).

**AB/PAS-positive mucosubstances are increased in the airways of IL-12p40β/ mice following RSV infection**

Airway mucus overproduction is a hallmark clinical feature of RSV bronchiolitis. The role of IL-12 in modulating RSV-induced mucous cell metaplasia was examined by AB/PAS staining of the airways in IL-12p40β/ and WT mice following RSV infection. Mucus staining was not apparent in the airway epithelium of vehicle-treated WT and IL-12p40β/ mice (data not shown). AB/PAS-positive cells were visible in the airways of WT and IL-12p40β/ mice after RSV infection for 3 days with no significant difference between the two infected groups (data not shown). The intensity and number of AB/PAS-stained cells were increased in the airways of both WT and IL-12p40β/ mice infected with RSV for 7 days as compared with WT and IL-12p40β/ mice infected with RSV for 3 days, respectively. Importantly, both the numbers of AB/PAS-positive cells and the intensity of the mucous staining were increased in the airways of IL-12p40β/ mice as compared with those of WT mice at 7 days following RSV infection (Fig. 4a, representative photomicrographs). The volume density of stored AB/PAS-positive material was also increased in the airways of IL-12p40β/ mice as compared with that of WT mice at 7 days after RSV infection (Fig. 4b).

**Th2 cytokines are increased in the lungs of IL-12p40β/ mice after RSV infection**

To determine the role of IL-12 in modulating Th2 immune responses to RSV infection, the levels of Th2 cytokines IL-4, IL-5, and IL-13 were assessed in the lung homogenates of IL-12p40β/ and WT mice at 3 or 7 days following RSV infection. The levels of IL-5 and IL-13 were higher in IL-12p40β/ mice as compared with those of WT mice infected with RSV for 3 days or WT mice infected with RSV for 7 days (Fig. 5a). The levels of IL-5 were markedly increased in the lungs of IL-12p40β/ mice at 7 days postinfection as compared with those of IL-12p40β/ mice infected with RSV for 3 days or WT mice infected with RSV for 7 days (Fig. 5a). The levels of IL-13 were also significantly increased in IL-12p40β/ mice as compared with those of WT mice infected with RSV for 7 days (Fig. 5b). IL-4 levels were very low in both IL-12p40β/ and WT mice postinfection, and there was no statistical difference in IL-4 levels between IL-12p40β/ and WT mice before or after infection (data not shown).

**FIGURE 2.** Total cell counts and lymphocyte numbers are increased in BALF from IL-12p40β/ mice after RSV infection. Total and differential cells were counted in BALF from WT and IL-12p40β/ mice treated with either vehicle (Veh) or RSV for 3 days (a) and 7 days (b) (n = 7–10/group). Data are expressed as mean cell counts ± SEM. *, p < 0.05, as compared with respective vehicle-control group; **, p < 0.01.

**FIGURE 3.** CD3+ lymphocytes were increased in IL-12p40β/ mice as compared with those of WT mice 7 days postinfection. WT and IL-12p40β/ mice were treated with vehicle (Veh) or RSV for 7 days (n = 7–10/group), and CD3+ lymphocytes were determined by immunohistochemical staining of lung sections (5 μm). a. Photomicrographs were taken from representative slides (original magnification, ×200). CD3+ lymphocytes (indicated by red staining) were increased in WT mice infected with RSV as compared with those of vehicle-control mice, and CD3+ lymphocytes were further increased in IL-12p40β/ mice as compared with those of WT mice after infection with RSV. b, CD3+ lymphocytes were counted in 10 fields (original magnification, ×400) and the data are expressed as mean cell counts ± SEM. *, p < 0.05, as compared with respective vehicle-control group; **, p < 0.01.
increased in the lungs of both IL-12p40−/− mice 7 days after RSV infection. The levels of IL-18 were significantly increased in IL-12p40−/− mice as compared with that of WT mice after RSV infection (n = 7–10/group; *, p < 0.05).

**FIGURE 4.** Mucus staining was increased in the airways of IL-12p40−/− mice 7 days after RSV infection. Mucus-producing cells in the airways were identified by AB/PAS staining. a, Photomicrographs were taken from representative slides of WT and IL-12p40−/− mice infected with RSV for 7 days (original magnification, ×200). b, The volume density (Vs) of stored AB/PAS-positive material in airway epithelia was significantly increased in IL-12p40−/− mice as compared with that of WT mice infected with RSV for 3 days or 7 days (original magnification, ×200). The sensitivity of the kits for IFN-γ and IL-18 is <2 and 25 pg/ml, respectively. Data were normalized by total lung protein. The data are expressed as mean levels ± SEM.

To elucidate the role of IL-12 in modulating a Th1 immune response to RSV infection, the levels of IFN-γ and IL-18 were assessed in the lung homogenate of IL-12p40−/− and WT mice infected with RSV for 3 and 7 days. IFN-γ levels were not changed in either IL-12p40−/− or WT mice 3 days after RSV infection (Fig. 6a). At 7 days postinfection, IFN-γ levels were significantly increased in the lungs of both WT and IL-12p40−/− mice as compared with those of vehicle-control mice (Fig. 6a). However, there was no significant difference between WT and IL-12p40−/− mice following 3 or 7 days of RSV infection. The levels of IL-18 were increased in the lungs of both IL-12p40−/− and WT mice at 3 days after RSV infection as compared with those of vehicle-control mice, respectively (Fig. 6b). IL-18 levels were further increased in IL-12p40−/− mice as compared with those of WT mice infected with RSV after 3 days. At 7 days postinfection, IL-18 levels were increased in IL-12p40−/− mice infected with RSV as compared with those of vehicle-control mice (Fig. 6b).

**FIGURE 5.** Increased IL-5 and IL-13 levels in the lungs of IL-12p40−/− mice after RSV infection. Cytokine levels were determined using ELISA in the lung homogenates from WT and IL-12p40−/− mice treated with RSV for 3 or 7 days (n = 7–10/group). The sensitivity of the kits for IL-5 and IL-13 is <7 and 2 pg/ml, respectively. Data were normalized by total lung protein. The data are expressed as mean levels ± SEM. a, The levels of IL-5 were significantly increased in IL-12p40−/− mice infected with RSV for 7 days as compared with those of vehicle-control mice (**, p < 0.01). b, The levels of IL-13 were also significantly increased in IL-12p40−/− mice as compared with those in WT mice infected with RSV for 7 days (*, p < 0.05).

**FIGURE 6.** IFN-γ and IL-18 levels in the lungs of WT and IL-12p40−/− mice after RSV infection. Cytokine levels were determined using ELISA in the lung homogenates from WT and IL-12p40−/− mice infected with RSV for 3 or 7 days (n = 7–10/group). The sensitivity of the kits for IFN-γ and IL-18 is <2 and 25 pg/ml, respectively. Data were normalized by total lung protein. The data are expressed as mean levels ± SEM. a, The levels of IFN-γ were significantly increased in both WT and IL-12p40−/− mice infected with RSV for 7 days as compared with those of vehicle (Veh)-control mice (*, p < 0.01). The IFN-γ levels were similar in WT and IL-12p40−/− mice after RSV infection for either 3 or 7 days. b, IL-18 levels were increased in WT mice 3 days after infection as compared with those of vehicle-control mice (*, p < 0.05) and were further increased in IL-12p40−/− mice as compared with those of RSV-infected WT mice or vehicle-control mice (**, p < 0.05). IL-18 levels were also increased in IL-12p40−/− mice infected with RSV for 7 days as compared with those of the vehicle-control group (*, p < 0.05).

**FIGURE 7.** Neutralization of IL-18 increased lung inflammation in IL-12p40−/− mice infected with RSV. IL-12p40−/− mice were pretreated with either isotype control Ab (IgG1) or monoclonal anti-IL-18 Ab and then infected with RSV for 7 days. a, Photomicrographs were taken from representative slides of the mice (original magnification, ×200). Leukocyte infiltration in the lung parenchyma, lung epithelial hyperplasia, and myofibroblast hyperplasia (arrows) was increased in the mice treated with anti-IL-18 Ab. b, Pathological scoring of lung inflammation and injury was assessed in these mice, and the data are expressed as mean scores ± SEM. The histopathological scores were significantly increased in the mice treated with anti-IL-18 Ab as compared with those of control IgG1 (**, p < 0.01).
with those of vehicle-control IL-12p40−/− mice, but not to WT mice infected with RSV (Fig. 6b).

**IL-18/IL-12-coupled regulation of inflammatory and Th2 immune responses to RSV infection**

To determine the role of IL-18 in regulating lung inflammation, mucus production, and Th2 immune response in IL-12p40−/− mice, an IL-18-neutralizing Ab was administered, and lung inflammatory and immune responses were assessed during RSV infection. Increased inflammatory cell infiltration was noted in proximal airways of IL-12p40−/− mice after IL-18 neutralization as compared with that of IL-12p40−/− mice treated with isotype control Ab IgG1 (Fig. 7a, representative photomicrographs). Epithelial histopathology was also increased by IL-18 depletion in IL-12p40−/− mice, including increased airway epithelial remodeling, increased myofibroblast hyperplasia (Fig. 7b, arrows), and increased inclusion bodies in secretory/goblet cells. Accordingly, the mean pathological score was significantly increased in the lungs of IL-12p40−/− mice treated with anti-IL-18 Ab as compared with IgG1 treatment (Fig. 7b).

Abrogation of IL-18 further increased IL-13 levels in the lung of IL-12p40−/− mice 7 days after RSV infection (Fig. 8a), although the levels of IL-4 and IL-5 were similar in the mice treated with either anti-IL-18 Ab or control IgG1 (data not shown). Consistent with the increase in IL-13 levels, IL-18 neutralization also further increased the numbers of AB/PAS-positive cells and the intensity of the mucous staining in large airways of IL-12p40−/− mice after RSV infection for 7 days (Fig. 8b, representative photomicrographs). The volume density of stored AB/PAS-positive mucosubstances was also increased in the airways of IL-12p40−/− mice after IL-18 neutralization (Fig. 8c). Immunohistochemical staining indicated that the main mucin that was synthesized in airway epithelia in response to RSV infection was MUC5AC (Fig. 8d). The increase in immunoreactivity for MUC5AC corresponds to the amount of quantified intraepithelial mucosubstances from AB/PAS-stained tissues (Fig. 8b).

**IL-18 neutralization did not alter viral clearance and IFN-γ production in the lungs of IL-12p40−/− mice infected with RSV**

To determine the effect of IL-18 on modulating viral clearance and Th1 immune response in the absence of IL-12, RSV-F gene expression and IFN-γ levels in the lungs of IL-12p40−/− mice were determined following neutralization of IL-18 signaling. At 7 days after RSV infection, the RSV-F gene was detected at very low levels in the lungs of IL-12p40−/− mice, but not to WT mice infected with RSV (Fig. 6b).

**FIGURE 8.** Neutralization of IL-18 increased IL-13 levels and mucus production in the lungs of IL-12p40−/− mice infected with RSV. IL-12p40−/− mice were pretreated with either isotype control Ab (IgG1) or monoclonal anti-IL-18 Ab and then infected with RSV for 7 days (n = 7/group). IL-13 levels in the lung homogenates were determined using ELISA. Mucus-producing cells in the airways were identified by AB/PAS staining and immunohistochemical staining for MUC5AC. a. IL-18 blocking further increased IL-13 levels in the lungs of IL-12p40−/− mice after RSV infection for 7 days. b. Photomicrographs were taken from representative slides of lung sections stained by AB/PAS staining (original magnification, ×200). c. The volume density (Vs) of stored AB/PAS-positive material in airway epithelia was significantly increased in IL-12p40−/− mice treated with anti-IL-18 Ab as compared with that of control IgG1. d. Increased immunoreactivity for MUC5AC is shown in mice pretreated with anti-IL-18-neutralizing Ab. The increase in immunoreactivity for MUC5AC corresponds to the amount of quantified intraepithelial mucosubstances from AB/PAS-stained tissues (original magnification, ×400). ***, p < 0.05 as compared with vehicle (Veh)-control group; ***, p < 0.05 as compared with both the vehicle-control group and IgG1-RSV group.
levels in the lungs of both groups, with no statistical significance between the mice pretreated with control or anti-IL-18 Abs (Fig. 9, a and b). RSV infection significantly increased IFN-γ production in IL-12p40−/− mice treated with either anti-IL-18 Ab or control IgG1, with no significant difference between these two groups (Fig. 9c).

Discussion

The present study shows that IL-12p40 deficiency increased RSV-induced lung inflammation and Th2 cytokine production and was associated with increase in mucus hypersecretion, a hallmark feature of severe RSV bronchiolitis in infants. Neutralization of IL-12 in IL-18 in IL-12p40−/− mice further increased the Th2 immune response, lung inflammation, and mucus production. Interestingly, the absence of IL-12 alone or both IL-12 and IL-18 did not affect IFN-γ production and viral clearance. These findings suggest that IL-12 and IL-18 inhibit the RSV-induced Th2 immune responses and lung inflammation independent of the mechanisms affecting viral load.

Lung inflammation is a crucial aspect for limiting respiratory infections and restoring lung function. However, aberrant regulation of lung inflammation can lead to severe or chronic lung disease. In the present study, lung inflammation was increased in IL-12p40−/− mice as compared with that of WT mice infected with RSV. Throughout the infection period, increased lymphocytes, as identified by CD3 staining, were apparent in the lungs of IL-12p40−/− mice. Increased lymphocytes were associated with bronchial and bronchiolar regions of the lungs in IL-12p40−/− mice, consistent with increased lymphocytes in BALF and the increased airway mucous response observed during RSV infection. Similar to the findings reported here, a recent publication demonstrates that the numbers of bronchoalveolar lavage cells in IL-12p40−/− mice are significantly increased as compared with those of WT mice after RSV infection, and, accordingly, CD8+ lymphocytes are also markedly increased in IL-12p40−/− mice (30). In the same study, it was shown that pulmonary NK cell recruitment is impaired in IL-12p40−/− BALB/c mice, indicating that IL-12p40 may play an important role in NK chemotaxis and/or migration during RSV infection. During RSV infection, lung DX5+/CD3+ NK cells, which produce significant amount of IFN-γ, peak on days 3 and 4 of primary infection and are virtually absent by days 7 and 8 (31). Interestingly, IL-12-activated NK cells express high levels of IFN-γ and reduce lung eosinophilia to the RSV G protein (32), suggesting that NK cells are an important component in IL-12-mediated immune response to RSV infection. Clinical studies have shown that CD19+ (33) or CD23+ B cells (34) are increased in the peripheral blood of infants with RSV bronchiolitis, and this group of infants with increased CD23 Ag has RSV-specific IgE and IgG4 Abs (34), indicating that the increased B cells may contribute to the Th2-predominant immune response to RSV infection. Importantly, IL-12 treatment attenuates Th2 and B cell responses to RSV infection (35) Taken together, IL-12 may play a key role in regulating lymphocyte, specifically, CD8+ lymphocyte, NK cell, and B cell, responses during RSV infection.

Cytokines play an important role in the pathophysiology of RSV-induced airway disease. In the present study, the Th2 cytokines IL-5 and IL-13 were significantly increased in the lungs of IL-12p40−/− mice after RSV infection. Clinical studies indicate that severe RSV infection in early life is associated with increased IL-13 production (9, 10). IL-13 can also induce lung inflammation, mucus production, and AHR in RSV infection in mice (27, 36–39). IL-13 is sufficient for the expression of pathophysiological features of asthma, including mucus cell metaplasia (MCM), in a manner that is independent of IgE and eosinophils (40, 41). Indeed, transgenic mice overexpressing IL-13 in their lungs show extensive MCM and AHR (42) through a STAT-6-dependent pathway (43). The direct effect of IL-13 on epithelial cells which causes MCM was also shown in human bronchial epithelial cells (44). Therefore, IL-13 may play a key role in the increased lung inflammation and MCM in IL-12p40−/− mice during RSV infection.

Mucus overproduction plays an important role in the development of AHR and wheezing in RSV-induced bronchiolitis and asthma. In the current study, the number of mucus-secreting cells
and the volume density of mucus production were significantly increased in the airways of IL-12p40−/− mice as compared with those of WT mice after RSV infection. This study suggests that decreased IL-12 levels in infants with RSV-induced bronchiolitis may cause mucus overproduction, leading to AHR and wheezing. The increased AB/PAS materials primarily consisted of MUC5AC as detected by this specific Ab. MUC5AC is the primary mucin protein expressed by airway epithelial cells in humans (45). The induction of mucus hypersecretion in this mouse model suggests its utility as a clinically related end point in the study of mechanisms of RSV-induced bronchiolitis in humans.

The present study showed only minimal numbers of eosinophils in the BALF or lungs of either IL-12p40−/− or WT mice during RSV infection, and there was no significant difference between the two groups. This result is consistent with the previous clinical studies on leukocyte analysis in infants with RSV-induced bronchiolitis (46, 47). However, IL-5 is a potent chemokine that attracts eosinophils (48, 49), and the mechanism for the lack of eosinophilia in our studies with RSV infection is unclear.

Viral clearance from the respiratory tract is mediated by both hemopoietic and nonhemopoietic cell lineages. The current findings suggest that RSV clearance was not altered in IL-12p40−/− mice, although lung inflammation, Th2 immune responses, and mucus production were increased, suggesting the notion that increased Th2-mediated mechanisms do not enhance or hinder RSV clearance. In light of the increase in mucus production, the changes in hemopoietic cell-mediated viral clearance may have been offset by increased nonhemopoietic viral clearance mechanisms encoded by lung epithelial cells, such as mucocilliary clearance. Multiple cytokines or proteins, such as IFN-γ, IFN-αβ, surfactant protein A (50), surfactant protein D (51), and Clara cell secretory protein (27), may be involved in viral clearance. Among the aforementioned cytokines or proteins, IFN-γ may play a key role in RSV clearance (52, 53). In the present study, IFN-γ levels were increased to a similar level in IL-12p40−/− and WT mice infected with RSV, indicating that there is an IL-12p40-independent pathway in regulating IFN-γ production and possibly viral clearance during RSV infection. This finding is consistent with those found in respiratory adenovirus infection (28). Similarly, patients with complete deficiency of the IL-12p40 chain or IL-12Rβ1 chain of its receptor complex demonstrate only a limited increased susceptibility to mycobacteria or Salmonella infections, not the expected complete lack of resistance to infections (54). In a mouse model of parasitic infection, IL-12 was not required for a Th1 response, although its expression was essential for optimal responses (55). Collectively, our findings are consistent with published results in other infection models, suggesting a role for IL-12 in coordinating immune responses to RSV infection but not essential for lung viral clearance mechanisms.

IL-18 is an IFN-γ-inducing cytokine and is localized primarily to airway epithelium and mononuclear cells in both mice and humans (56). Interestingly, neutralization of endogenous IL-18 in IL-12p40−/− mice did not alter IFN-γ production and viral clearance. Particularly noteworthy is the lack of regulation of IFN-γ production in the lung by either IL-12 or IL-18, indicating other mechanisms of IFN-γ induction in RSV infection. This finding is consistent with that found in genital herpes simplex type 2 infection (57). Similarly, a recent study also showed that IL-12 and IL-18 are not required for a pronounced Th1 response to RSV infection (58). However, in the above study, lung histopathology was not increased in IL-12−/− and IL-18−/− mice during RSV infection. The reason for the difference in lung histopathology between the above study and the present study is unknown. IL-23 is another important cytokine in regulating IFN-γ production (59, 60). However, because IL-12p40 is an important component of IL-23, a heterodimeric cytokine (61), IL-23 regulation of IFN-γ production is likely lost in IL-12p40−/− mice. Similarly, IL-12p40 homodimer, IL-12p80, is likely absent in the IL-12p40−/− mice. This cytokine acts as an IL-12 and IL-23 antagonist by competing at their receptors (17) and is associated with enhanced airway macrophage accumulation in Sendai virus infection (62). The present study does not determine the cellular sources of IFN-γ production. Lymphocytes are an important source of IFN-γ, and IFN-γ production is induced by IL-12 (13) and/or IL-18 (19). However, NK cells and macrophages are other important sources of IFN-γ production (18), and IFN-γ production from these cells may be induced directly by viral infection, independent of IL-12 or IL-18 regulation. Since NK cell recruitment is impaired in the IL-12p40−/− mice (30), we would speculate that lung macrophages are the likely source of IFN-γ in these studies. Thus, other cellular sources may be responsible for IFN-γ production and viral clearance in the absence of IL-12 and/or IL-18 or when the levels of these cytokines are low.

Abrogation of IL-18-mediated signaling in IL-12p40−/− mice increased IL-13 production, lung inflammation, myofibroblast hyperplasia, and mucus expression, suggesting an interaction or synergy between IL-18 and IL-12 in mediating T cell commitment during RSV infection. This finding is consistent with that found in a mouse model of asthma (20). Particularly noteworthy in the present study is the increased myofibroblast hyperplasia induced by IL-18 neutralization in IL-12p40−/− mice after RSV infection, and this may significantly contribute to the pathophysiology of reactive or chronic airway disease (63). As IL-12 protein levels (14, 15) and IL-18 mRNA levels (23) are decreased in RSV-induced bronchiolitis, administration of exogenous IL-12 (35) and/or IL-18 may, in part, limit the Th2 predominant immune response and lung inflammation during RSV infection. The findings in this study suggest a compensatory role for IL-18 in Th0 cell commitment following RSV infection.

The Th1/Th2 concept is evolving and becoming more complicated. The Th1 cytokine IFN-γ is usually believed to be protective in allergic and inflammatory responses. However, a recent study indicates that influenza virus-induced Th2 immune response and lung inflammation in OVA-sensitized mice is mediated by IFN-γ (64). Similarly, the function of IL-18, an IFN-γ-inducer, has also been controversial. It has been shown that IL-18 can increase allergic sensitization, serum IgE, Th2 cytokines, and airway eosinophilia in a mouse model of allergic asthma (65). IL-18 and Ag together can also stimulate memory Th1 cells to induce severe airway inflammation and AHR in naive hosts (66). The effect of IL-18 or IFN-γ on lung inflammation and AHR may be related to the previous sensitization or the functional status of memory T cells. Interestingly, the number of IL-18-positive cells is increased in the nasal brush samples of infants with RSV bronchiolitis, but not from infants with RSV upper respiratory tract infection (67), indicating that the IL-18 response may be related to disease severity or site of infection.

In summary, IL-12p40 deficiency increases lung inflammation, Th2 immune response, and mucus production in acute RSV infection but does not affect IFN-γ production and viral clearance. In the absence of IL-12p40, IL-18 neutralization further increases lung inflammation, mucus expression, and IL-13 production but does not alter IFN-γ production and viral clearance. These findings indicate that IL-12 and IL-18 are important mediators in limiting lung inflammation and Th2 immune responses to RSV infection, and deficiency in IL-12 and IL-18 production may lead to persistent Th2 immune responses to RSV infection in children, causing atopy and asthma later in their lives.
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