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*J Immunol* published online 8 July 2009
http://www.jimmunol.org/content/early/2009/07/08/jimmunol.0803167
STAT1 Negatively Regulates Lung Basophil IL-4 Expression Induced by Respiratory Syncytial Virus Infection

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IL-4 contributes to immunopathology induced in mice by primary respiratory syncytial virus (RSV) infection. However, the cellular source of IL-4 in RSV infection is unknown. We identified CD3⁺ CD49b⁺ cells as the predominant source of IL-4 in the lungs of RSV-infected BALB/c mice. We ruled out T cells, NK cells, NKT cells, mast cells, and eosinophils as IL-4 expressors in RSV infection by flow cytometry. Using IL-4 GFP reporter mice (4get) mice, we identified the IL-4-expressing cells in RSV infection lungs of RSV-infected BALB/c mice. We also identified the IL-4-expressing cells in RSV infection as basophils (CD3⁻ CD49b⁺ FcεRI⁺ c-kit⁻). Because STAT1⁻/⁻ mice have an enhanced Th2-type response to RSV infection, we also sought to determine the cellular source and role of IL-4 in RSV-infected STAT1⁻/⁻ mice. RSV infection resulted in significantly more IL-4-expressing CD3⁻ CD49b⁺ cells in the lungs of STAT1⁻/⁻ mice than in BALB/c mice. CD49b⁺ IL-4⁺ cells sorted from the lungs of RSV-infected STAT1⁻/⁻ mice and stained with Wright-Giemsa had basophil characteristics. As in wild-type BALB/c mice, IL-4 contributed to lung histopathology in RSV-infected STAT1⁻/⁻ mice. Depletion of basophils in RSV-infected STAT1⁻/⁻ mice reduced lung IL-4 expression. Thus, we show for the first time that a respiratory virus (RSV) induced basophil accumulation in vivo. Basophils were the primary source of IL-4 in the lung in RSV infection, and STAT1 was a negative regulator of virus-induced basophil IL-4 expression. The Journal of Immunology, 2009, 183: 0000 – 0000.

Respiratory syncytial virus (RSV) is the leading cause of bronchiolitis and viral pneumonia in infants worldwide. In the United States, RSV infection results in the hospitalization of >100,000 infants per year (1). The immunologic features of primary RSV infection are not fully defined. In infants, expression of the Th2 cytokine IL-4 has been correlated with RSV disease severity, but not consistently (2–5). Mechanisms by which primary RSV infection leads to IL-4 expression are unexplained.

IL-4 is critical for the development of Th2-type inflammation (6). The role of IL-4 has been investigated in a mouse model of primary RSV infection. IL-4-deficient mice on a C57BL/6 (B6) background exhibit less lymphocytic inflammation in the lung than B6 control mice, suggesting that IL-4 contributes to recruitment of inflammatory cells (7). However, the cellular source of IL-4 in the mouse model of RSV infection has not been defined nor has the role of IL-4 in RSV-induced cytokine production been completely defined. BALB/c mice are more permissive to RSV replication than B6 mice and BALB/c is the most common mouse strain used for investigating primary RSV pathogenesis (8, 9). The role of IL-4 in the BALB/c mouse model of RSV infection is not known. Both B6 and BALB/c mice exhibit a dominant Th1-type response to primary RSV infection (8). In BALB/c mice, the immune response to primary A2 strain RSV infection is characterized by abundant IFN-γ-producing NK, CD4⁺, and CD8⁺ cells in bronchoalveolar lavage fluid and a robust CTL response (10, 11).

STAT1⁻/⁻ mice are deficient in transcriptional responses to type I (α/β) and type II (γ) IFNs, have increased susceptibility to viral disease, and have an enhanced Th2 phenotype with virus infection (12, 13). Compared with BALB/c controls, primary A2 strain RSV infection of STAT1⁻/⁻ mice results in increased viral titers, exacerbated disease, elevated Th2 cytokine levels in the lung, increased airway mucus, eosinophilia, and airway hyperresponsiveness (14, 15). The cellular source of IL-4 in RSV-infected STAT1⁻/⁻ mice has also not been elucidated. Thus, BALB/c and STAT1⁻/⁻ mice provide models of Th1 and enhanced Th2 inflammation in response to primary RSV infection, respectively.

In this study, we investigated the role of IL-4 in RSV infection in BALB/c (Th1 response) and STAT1⁻/⁻ (enhanced Th2-type response) mice. We also sought to identify the cell type producing IL-4 in RSV-infected mice. Basophils were the predominant IL-4-expressing cell type in the lung in RSV infection in both BALB/c and STAT1⁻/⁻ mice. In addition, we found that STAT1 is a regulator of lung basophil IL-4 expression in a model of primary respiratory virus infection.

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1 Abbreviations used in this paper: RSV, respiratory syncytial virus; i.n., intranasally; α-GalCer, α-galactosylceramide; p.i., post-infection; BMCC, bone marrow-derived mast cell; DC, dendritic cell.

The Journal of Immunology, 2009, 183: 0000 – 0000.

Received for publication September 23, 2008. Accepted for publication June 1, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

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DOI: 10.4049/jimmunol.0803167

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0803167
Materials and Methods

**Virus and mice**

The A2 strain of RSV was provided by R. Chanock (National Institutes of Health, Bethesda, MD). Viral stocks were propagated and titrated by plaque assay in HEp-2 cells as previously described (16). Female 6- to 8-week-old BALB/c mice were obtained from Charles River Laboratories. STAT1<sup>−/−</sup> mice and CD1<sup>d</sup>-<sup>−/−</sup> mice on a BALB/c background were described elsewhere (14, 17). CD1<sup>d</sup>-<sup>−/−</sup>STAT1<sup>−/−</sup> mice were generated by mating CD1<sup>d</sup>-<sup>−/−</sup> and STAT1<sup>−/−</sup> mice with subsequent genotyping. IL4<sup>−/−</sup> mice on a BALB/c background were obtained from The Jackson Laboratory. STAT1<sup>−/−</sup>/IL4<sup>−/−</sup> mice were generated by mating STAT1<sup>−/−</sup>/ and IL4<sup>−/−</sup> mice followed by genotyping. Mice expressing a knockin IL4/ires/EGFP transgene (4get mice, endogenous IL4 expression remains intact) are described elsewhere (18). 4get mice backcrossed to the BALB/c background from C129/H11002<sup>−/−</sup>/H11002<sup>−/−</sup> were obtained from The Jackson Laboratory and bred to STAT1<sup>−/−</sup> mice with subsequent genotyping, to produce STAT1<sup>−/−</sup>/4get mice. All mice were maintained under specific pathogen-free conditions. Six- to 11-week-old female mice were lightly anesthetized and infected intranasally with RSV or with mock-infected cell culture supernatant as previously described (16).

**Flow cytometric analysis of lung mononuclear cells**

Mice were euthanized with i.p. sodium pentobarbital (Fort Dodge Labs) and the lungs were removed. Lung mononuclear cells were isolated by Ficoll-Hypaque cushion centrifugation (1.09 specific gravity). Cells were counted with a hemacytometer, then treated and stained for cell surface molecules and intracellular cytokines as previously described (19). We used the following Abs from BD Pharmingen: anti-CD49b (DX5), anti-CD3, anti-CD8a, anti-CD4, anti-CD11b, anti-IL-4 (or isotype control rat IgG1), anti-IFN-γ, anti-Ly49 C/F/I/H and anti-Ly49 A/B 1:200, anti-CD16/32 Ab (BD Pharmingen) was obtained from eBioscience. We used anti-Ly49 C/F/I/H and anti-Ly49 A/B 1:200, and anti-B220. We used anti-NKG2D, anti-FcεRII (MAR-1), and anti-c-kit Abs obtained from eBioscience. We used anti-Ly49 C/F/I/H and anti-Ly49 A/B Abs obtained from BioLegend. Anti-CD16/32 Ab (BD Pharmingen) was used to prevent nonspecific staining. PE-labeled, α-galactosylceramide (α-GalCer)-loaded tetrameric CD1d molecules (α-GalCer-tetramer) were prepared as described previously (20, 21). For staining of intracellular cytokines, cells were stimulated in RPMI 1640/10% FBS/1 μM ionomycin (Sigma-Aldrich)/10 ng/ml PMA (Sigma-Aldrich)/1 μl/ml of GolgiStop (BD Pharmingen) for 6 h at 37°C and 5% CO<sub>2</sub>. Cells were analyzed using an LSR II flow cytometer (BD Biosciences). Three to 5 × 10<sup>6</sup> lung lymphocytes per animal were analyzed based on forward and side scatter properties. Data were analyzed using Win MDI 2.8 (The Scripps Research Institute) or FlowJo software (Tree Star).

**FACS and Wright-Giemsa staining**

Whole lung mononuclear cells were isolated from RSV-infected STAT1<sup>−/−</sup> mice 5 or 6 days post-infection (p.i.) as described above. Cells were pooled from 8 or 11 mice and stained with anti-CD49b and anti-IL-4 using the intracellular cytokine staining protocol described above. Lymphocytes were gated based on forward and side scatter properties. CD49b<sup>−/−</sup> IL-4<sup>−/−</sup> cells within the lymphocyte gate were sorted into 100% PBS using a FACSAria cell sorter (BD Biosciences). Post-sort flow cytometric analyses indicated that the purity of sorted CD49b<sup>−/−</sup>/IL-4<sup>−/−</sup> cells was >99%. The sorted cells were washed with PBS/3% FBS, applied to a glass slide by cytospin, and stained with Wright-Giemsa.

**Bone marrow-derived mast cells (BMMC)**

Bone marrow cells were obtained from BALB/c mouse femurs. BMMCs were derived as described elsewhere (22). Briefly, cells were cultured in MEM with 10% FBS, 2 mM l-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 50% WEHI-3 cell-conditioned medium. The cells were maintained for 3 wk and nonadherent cells were passed once per week to fresh medium. The cultures consisted of >80% differentiated mast cells as determined by flow cytometry (c-kit<sup>−/−</sup>/FceRI<sup>+</sup>) and toluidine blue staining characteristics.

**In vivo anti-FcεRI treatment**

RSV-infected STAT1<sup>−/−</sup> mice were treated i.p. daily on days 1–5 p.i. (seven total injections). Mice were kept 200 μl of 0.5 mg/ml anti-FcεRI Ab (MAR-1 clone) or affinity-purified Aramien hamster IgG as a control (both from eBioscience).

**Quantitation of viral load and cytokines in lung tissue**

Lungs were individually ground with precooled mortar and pestle and sterile glass. Glass and tissue debris were removed from lung homog

| Table I. Effect of IL-4 on viral load<sup>a</sup> |
|----------------|----------------|----------------|----------------|
| BALB/c (n = 7) | IL4<sup>−/−</sup> (n = 5) | STAT1<sup>−/−</sup> (n = 8) | STAT1<sup>−/−</sup>/IL4<sup>−/−</sup> (n = 8) |
| Day 6 | 4.1 ± 0.09 | 4.3 ± 0.11 | 4.9 ± 0.09 | 4.8 ± 0.12 |

<sup>a</sup> Mice were infected with 10<sup>5</sup> PFU of RSV. Lungs were harvested 6 days p.i. Infectious virus in the left lung homogenates was titrated by plaque assay. Data are log PFU/g ± SEM.

**Histopathology**

Heart-lung blocks were harvested 7 days p.i. and fixed in 4% paraformaldehyde overnight. Lungs were transferred to 70% ethanol and then embedded in paraffin blocks. Tissue sections (5 μm) were stained with H&E to assess histologic changes, periodic acid-Schiff to assess goblet cell hyperplasia, and Luna stain to assess eosinophilia. Slides were examined and scored by a single pathologist who was blinded to the experimental groups. Alveolar spaces, airways, interstitium, and vessels (both arteries and veins) were examined. Inflammatory infiltrates were assessed for location, severity, and composition (cell types: eosinophils, neutrophils, macrophages, small lymphocytes, transformed lymphocytes, plasma cells). We quantified mononuclear cell peribronchial cuffing as a measure of inflammation. H&E-stained slides were digitally scanned using a Zeiss Mirax MIDI microscope with a ×20 objective having a 0.85 numerical aperture (Zeiss). The maximum thickness of mononuclear cell cuffing around each airway was measured using Histoquant software (3D Histech). We considered 10 μm the limit of detection and assigned airways with no mononuclear cell cuffing a value of 5 μm All airways involved in the tissues were scored (39–87 airways/mouse).

**Statistical analyses**

Values of p were determined by a two-tailed t test, assuming equal variance, or by one-way ANOVA and Tukey multiple comparison tests. Data are representative of three experiments (except FACS sorting, two experiments) in which similar results were found in all replicate experiments.

**Results**

**IL-4 regulates lung cytokine levels and contributes to lung inflammation in RSV infection**

IL-4 contributes to lung inflammation in the context of enhanced Th2-type inflammation is not known. We hypothesized that IL-4 contributes to lung inflammation in RSV-infected BALB/c mice. The role of IL-4 in RSV infection in the context of enhanced Th2-type inflammation is not known. We hypothesized that IL-4 plays a role in RSV pathogenesis in the context of enhanced Th2-type inflammation. To test this hypothesis, we generated STAT1<sup>−/−</sup>/IL4<sup>−/−</sup> mice.

We determined the effect of IL-4 on viral load and disease severity in RSV infection in BALB/c and STAT1<sup>−/−</sup> mice. BALB/c, IL4<sup>−/−</sup>, STAT1<sup>−/−</sup>, and STAT1<sup>−/−</sup>/IL4<sup>−/−</sup> mice were infected with 10<sup>5</sup> PFU of A2 strain RSV. Lungs were harvested 6 and 8 days p.i. There was no difference in viral load at 6 days p.i. between BALB/c and IL4<sup>−/−</sup> mice, and there was no difference in viral load between STAT1<sup>−/−</sup> and STAT1<sup>−/−</sup>/IL4<sup>−/−</sup> mice (Table I). The viral loads were significantly higher (p < 0.05) in STAT1<sup>−/−</sup> and STAT1<sup>−/−</sup>/IL4<sup>−/−</sup> mice than in BALB/c and enates by centrifugation (15 min, 2000 rpm, 4°C). Infectious RSV was titrated by plaque assay in HEp-2 cells as previously described (16). Levels of IFN-γ and IL-13 were measured in lung homogenates using ELISA kits (R&D Systems). Dilutions of recombinant cytokines were included for the generation of standard curves.
Table II. IL-4 regulated IFN-γ and IL-13 levels in RSV infection

<table>
<thead>
<tr>
<th></th>
<th>BALB/c (n = 12)</th>
<th>IL4-/- (n = 9)</th>
<th>STAT1-/- (n = 15)</th>
<th>STAT1-/-IL4-/- (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6</td>
<td>IFN-γ</td>
<td>144 ± 13^a</td>
<td>260 ± 15^a</td>
<td>1552 ± 170</td>
</tr>
<tr>
<td></td>
<td>IL-13</td>
<td>ND</td>
<td>80 ± 5</td>
<td>92 ± 8</td>
</tr>
<tr>
<td>Day 8</td>
<td>IFN-γ</td>
<td>55 ± 8</td>
<td>71 ± 11</td>
<td>96 ± 14</td>
</tr>
<tr>
<td></td>
<td>IL-13</td>
<td>ND</td>
<td>73 ± 2^a</td>
<td>41 ± 4^a</td>
</tr>
</tbody>
</table>

* Mice were infected with 10^5 PFU of RSV and cytokines in the left lung homogenates were quantified. Data in pg/ml ± SEM are combined from two experiments.

^a These groups were significantly different (p < 0.05) comparing day 6 IFN-γ levels by ANOVA.

^b ND, None detected, below detection limit.

^c These groups were significantly different (p < 0.05) comparing day 6 and day 8 IL-13 levels in STAT1-/- and STAT1-/-IL4-/- by ANOVA.

IL4-/- mice, similar to published results (15). There were no detectable viral titers in RSV-infected BALB/c, IL4-/-, STAT1-/-, and STAT1-/-IL4-/- mice 8 days p.i. We measured weight loss as a surrogate for RSV illness severity (14, 16). BALB/c, IL4-/-, STAT1-/-, and STAT1-/-IL4-/- mice were infected with 10^5 PFU of A2 strain RSV, and the mice were weighed daily for 14 days. The BALB/c and IL4-/- mice did not lose weight in response to this dose of virus (data not shown). The STAT1-/- mice lost significantly more weight than BALB/c mice in response to RSV infection (data not shown), as previously published (14). There was no difference in RSV-induced weight loss between STAT1-/- and STAT1-/-IL4-/- mice (data not shown). Thus, IL-4 had no effect on viral load, viral clearance, and illness severity in RSV infection in BALB/c and STAT1-/- mice.

It was previously demonstrated that peak lung IFN-γ levels in RSV-infected BALB/c and STAT1-/- mice is 6 days p.i. and that infection induces higher IFN-γ levels in STAT1-/- mice than in BALB/c mice (14, 15). We found that RSV-infected IL4-/- mice had 1.8-fold higher IFN-γ levels than RSV-infected BALB/c mice, whereas there was no difference in lung IFN-γ levels between RSV-infected STAT1-/- and STAT1-/-IL4-/- mice (Table II).

 Compared with infected STAT1-/- mice, RSV-infected STAT1-/-IL4-/- mice had lower IL-13 levels 8 days p.i. (Table II). Thus, IL-4 regulated IFN-γ levels in RSV-infected BALB/c mice and IL-4 regulated IL-13 levels in RSV-infected STAT1-/- mice.

We determined the effect of IL-4 on histopathologic changes induced by RSV infection in BALB/c and STAT1-/- mice. We found that RSV-infected IL4-/- mice on a BALB/c background had less peribronchial lymphocytic inflammation than RSV-infected BALB/c mice (Fig. 1, A and C), results consistent with published data with IL4-deficient mice on a B6 background (7). RSV-infected STAT1-/- and STAT1-/-IL4-/- mice had equivalent bronchovascular (peribronchial and perivascular) cellular infiltrates consisting of eosinophils, neutrophils, macrophages, and small lymphocytes (Fig. 1, A and C). Strikingly, RSV-infected STAT1-/- mice had greater lung consolidation throughout alveolar spaces and more intrabronchial macrophages and eosinophils than RSV-infected STAT1-/-IL4-/- mice (Fig. 1, A and B). RSV-infected STAT1-/-IL4-/- mice did not exhibit significant intrabronchial inflammatory cells. Thus, in the context of Th1-type inflammation seen in BALB/c mice in primary RSV infection, IL-4 contributed to peribronchial lymphocytic inflammation. In the context of enhanced Th2-type inflammation seen in STAT1-/- mice in primary RSV infection, IL-4 contributed to lung consolidation and intrabronchial inflammation.

**RSV induced IL-4 expression by CD3-CD49b+ cells in BALB/c mice**

The immune response to primary A2 strain RSV infection in BALB/c mice is Th1 like and IL-4 was not detected in lungs by ELISA or the RNase protection assay (19). However, IL4-/- mice exhibited significantly decreased lymphocytic inflammation 7 days p.i. in primary RSV infection compared with control BALB/c mice (Fig. 1). We hypothesized that RSV infection will induce high levels of IFN-γ expression and low levels of IL-4 expression in CD4+ T cells in the lungs of BALB/c mice.

We measured intracellular IL-4 and IFN-γ by flow cytometry. As negative controls for flow cytometric quantification of IL-4, we used an isotype control Ab and IL4-/- mice. Lung mononuclear cells were isolated from mock-infected and RSV-infected BALB/c and IL4-/- mice 6 days p.i., the peak day of T cell numbers and lung IFN-γ levels in response to RSV infection in BALB/c mice.

**FIGURE 1.** IL-4 contributed to lung inflammation in RSV-infected BALB/c and STAT1-/- mice. BALB/c, IL4-/-, STAT1-/-, and STAT1-/-IL4-/- mice were mock infected (n = 2 per group) or infected with 10^8 PFU of RSV (BALB/c, n = 2; IL4-/-, STAT1-/-, and STAT1-/-IL4-/-, n = 3). Lungs were harvested 7 days p.i. and processed for H&E staining. A, Representative sections show peribronchial and perivascular inflammation (yellow arrowheads) and consolidation in alveolar spaces (RSV-infected STAT1-/- mice). Scale bars in upper left represent 100 μm. B, Two panels show intrabronchial inflammation (yellow arrowheads) in RSV-infected STAT1-/- mice. Scale bars in upper left represent 50 μm. C, Peribronchial inflammation was quantified as the maximal distance of mononuclear cell cuffing around bronchi, and all airways involved in the tissues were scored (see Materials and Methods). Gray lines show means. *, p < 0.05 (ANOVA). NS, Not significantly different.
Also shown is the percentage of gated CD4 IFN-γ cells, not CD4 T cells, in BALB/c mice. BALB/c mice (mock, n = 3; RSV, n = 4) and IL-4−/− mice (mock, n = 5; RSV, n = 5) were mock infected or infected with 10^3 PFU of RSV. Lung mononuclear cells were isolated 6 days p.i. and stained with anti-CD3-PE-Cy7, anti-CD4-PerCP-Cy5.5, anti-CD8-allophycocyanin-Cy7, anti-CD49b-biotin/streptavidin-allophycocyanin, anti-IFN-γ-PE-Cy7, and either anti-IL-4 or rat IgG1-PE as an isotype control for IL-4 staining. A–D, Representative dot plots show (A) the percentage of gated CD3^+CD49b^+ cells that were IFN-γ expressing or IL-4 expressing, (B) the IL-4 isotype control staining for CD3^+CD49b^+ cells, (C) the percentage of gated CD4^+ T cells that were IFN-γ expressing or IL-4 expressing, and (D) the IL-4 isotype control staining for CD4^+ T cells. E, The percentage of CD3^+CD49b^+ and CD4^+ T cells that were IL-4 expressing was determined for each mouse by subtracting the percentage of PE− events in the isotype control-stained series from the IL-4-expressing percentage. F, The total number of IL-4-expressing CD3^+CD49b^+ and CD4^+ T cells in the lungs. G, The total number of IFN-γ-expressing CD3^+CD49b^+ and CD4^+ T cells in the lungs. * p < 0.05 comparing RSV to mock.

Contrary to our hypothesis, we found that RSV induced IL-4 expression in CD3^+CD49b^+ cells, not CD4^+ T cells. The CD3^+CD49b^+ immunophenotype defined a cell pool containing both NK cells and basophils (24, 25). Representative dot plots show the percentage of gated CD3^+CD49b^+ cells that stained positively for IL-4 or IFN-γ (Fig. 2A) or isotype control Ab for IL-4 or IFN-γ (Fig. 2B). Also shown is the percentage of gated CD4^+ T cells that stained positively for IL-4 or IFN-γ (Fig. 2C) and the isotype control Ab for IL-4 or IFN-γ (Fig. 2D). Compared with mock infection, RSV infection increased the percentage of gated CD3^+CD49b^+ cells that express IL-4 from 0.3 ± 0.1% to 0.8 ± 0.1%, a 2.7-fold increase (Fig. 2E). RSV infection increased the total number of IL-4^+CD3^+CD49b^+ cells in the lung 6-fold, from 264 ± 21 in mock-infected mice to 1614 ± 253 in RSV-infected mice (Fig. 2F). RSV infection did not increase the percentage or total number of IL-4^+CD4^+ T cells (Fig. 2E and F). There were many IFN-γ-expressing CD3^+CD49b^+ cells and CD4^+ T cells (Fig. 2G), as well as IFN-γ-expressing CD8^+ T cells (data not shown), in the lungs of RSV-infected BALB/c mice. Taken together, the data are consistent with the phenotype of RSV-infected BALB/c mice being predominantly Th1 like. Nevertheless, primary RSV infection induced IL-4 expression in CD3^+CD49b^+ cells.

**CD3^+CD49b^+ cells were the primary source of IL-4 in RSV-infected STAT1−/− mice**

Primary i.n. RSV infection of STAT1−/− mice increases the levels of both Th2 cytokines and IFN-γ in the lung (14, 15). To identify the cellular source of IL-4 in RSV-infected STAT1−/− mice, we measured intracellular IL-4 by flow cytometry. For a negative control, we used an isotype control Ab. Whole lung mononuclear cells were isolated 6 days p.i. from STAT1−/− mice that were mock infected or infected with RSV, and lymphocytes were gated based on forward scatter and side scatter properties (data not shown). We chose 6 days p.i. because that is the day the number of IFN-γ-producing T cells in the lung (data not shown) and the IFN-γ levels in the lungs of RSV-infected STAT1−/− mice peak (15). Cells that

FIGURE 2. RSV infection induced IL-4 expression in CD3^+CD49b^+ cells, not CD4^+ T cells, in BALB/c mice. BALB/c mice (mock, n = 3; RSV, n = 4) and IL-4−/− mice (mock, n = 5; RSV, n = 5) were mock infected or infected with 10^3 PFU of RSV. Lung mononuclear cells were isolated 6 days p.i. and stained with anti-CD3-PE-Cy7, anti-CD4-PerCP-Cy5.5, anti-CD8-allophycocyanin-Cy7, anti-CD49b-biotin/streptavidin-allophycocyanin, anti-IFN-γ-PE-Cy7, and either anti-IL-4 or rat IgG1-PE as an isotype control for IL-4 staining. A–D, Representative dot plots show (A) the percentage of gated CD3^+CD49b^+ cells that were IFN-γ expressing or IL-4 expressing, (B) the IL-4 isotype control staining for CD3^+CD49b^+ cells, (C) the percentage of gated CD4^+ T cells that were IFN-γ expressing or IL-4 expressing, and (D) the IL-4 isotype control staining for CD4^+ T cells. E, The percentage of CD3^+CD49b^+ and CD4^+ T cells that were IL-4 expressing was determined for each mouse by subtracting the percentage of PE− events in the isotype control-stained series from the IL-4-expressing percentage. F, The total number of IL-4-expressing CD3^+CD49b^+ and CD4^+ T cells in the lungs. G, The total number of IFN-γ-expressing CD3^+CD49b^+ and CD4^+ T cells in the lungs. * p < 0.05 comparing RSV to mock.
stained positively for CD3, CD4, CD8, and CD49b; were defined by histogram gates around defined peaks (data not shown). The percentage of CD3⁺ CD49b⁺ cells that were IL-4⁺ was 13.8 ± 0.2 and the total number of IL-4⁺ CD3⁺ CD49b⁺ cells in the lungs of RSV-infected STAT1⁻/⁻ mice was 9499 ± 844 (Table III). Compared with mock infection, RSV dramatically increased the percentage (8-fold) and the number (13-fold) of IL-4-expressing CD3⁺ CD49b⁺ cells in the lungs of STAT1⁻/⁻ mice (Table III). The percentage of CD4⁺ T cells that were IL-4⁺ or IL-4⁻ was 0.1 in both mock-infected and RSV-infected mice (Table III). Thus, RSV infection increased CD3⁺ CD49b⁺ cell IL-4 expression in STAT1⁻/⁻ mice 6 days p.i. and did not increase CD4⁺ T cell IL-4 expression.

**STAT1 regulated CD3⁺ CD49b⁺ cell IL-4-expression in RSV infection**

Since RSV-infected STAT1⁻/⁻ mice have an enhanced Th2 phenotype with RSV infection (14, 15), we hypothesized that the percentage and total number of IL-4-expressing CD3⁺ CD49b⁺ cells would be greater in the lungs of RSV-infected STAT1⁻/⁻ mice than in the lungs of RSV-infected BALB/c mice. Lung mononuclear cells isolated 6 days p.i. from STAT1⁻/⁻ and BALB/c mice that were either mock infected or infected with RSV were analyzed by flow cytometry. The CD3⁺ CD49b⁺ gate is outlined in Fig. 3A. Representative dot plots show the percentage of gated CD3⁺ CD49b⁺ cells that were IL-4⁺ or IFN-γ⁺ (Fig. 3B). RSV infection increased the total number of IL-4-expressing CD3⁺ CD49b⁺ cells and IFN-γ⁺ CD3⁺ CD49b⁺ (NK) cells in both STAT1⁻/⁻ and BALB/c mice (Fig. 3C). The total number of IL-4-expressing CD3⁺ CD49b⁺ cells was 2.8-fold greater in RSV-infected STAT1⁻/⁻ mice than in RSV-infected BALB/c mice, whereas the number of IFN-γ-expressing NK cells was 2.6-fold greater in RSV-infected BALB/c mice than in RSV-infected STAT1⁻/⁻ mice (Fig. 3C). A similar pattern was observed when the data were analyzed as the percentage of gated CD3⁺ CD49b⁺ cells that were IL-4 expressing or IFN-γ expressing (Fig. 3D). The number of cells in the lung (Fig. 3C) was calculated using cell counts obtained with a hemacytometer (see Materials and Methods). The average total cell counts in each group were 9.27 × 10⁶ (BALB/c mock), 1.15 × 10⁹ (BALB/c RSV), 5.7 × 10⁸ (STAT1⁻/⁻ mock), and 1.6 × 10⁹ (STAT1⁻/⁻ RSV). There were significantly more cells in the BALB/c RSV group than in the BALB/c mock group and there were significantly more cells in the STAT1⁻/⁻ RSV group than in the STAT1⁻/⁻ mock group.

**Table III. Flow cytometric quantitation of CD3⁺ CD49b⁺ and T cell subsets in lung mononuclear cells isolated 6 days p.i. from STAT1⁻/⁻ mice that were mock infected or infected with 10⁵ PFU of RSV**

<table>
<thead>
<tr>
<th>Gate Hierarchy</th>
<th>Total No. of Cells in the Lunga</th>
<th>Percentage of Parent Gateb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte</td>
<td>Mock (n = 8) RSV (n = 7)</td>
<td>Mock (n = 8) RSV (n = 7)</td>
</tr>
<tr>
<td>CD3⁺ CD49b⁺</td>
<td>383,000 ± 10,800 1,080,000 ± 126,000</td>
<td>N/A</td>
</tr>
<tr>
<td>IFN-γ⁺ CD3⁺ CD49b⁺</td>
<td>43,073 ± 2,238</td>
<td>69,146 ± 6,225</td>
</tr>
<tr>
<td>IL-4⁺ CD3⁺ CD49b⁺</td>
<td>514 ± 204</td>
<td>1,599 ± 114</td>
</tr>
<tr>
<td>CD4⁺ T</td>
<td>724 ± 66</td>
<td>9499 ± 844</td>
</tr>
<tr>
<td>CD8⁺ T</td>
<td>120,357 ± 9,008</td>
<td>368,098 ± 75,963</td>
</tr>
<tr>
<td>CD49b⁺ T</td>
<td>314 ± 100</td>
<td>2,048 ± 223</td>
</tr>
<tr>
<td>IL-4⁺ CD4⁺ T</td>
<td>159 ± 28</td>
<td>494 ± 50</td>
</tr>
<tr>
<td>CD8⁺ CD4⁺ T</td>
<td>47,095 ± 4,969</td>
<td>128,203 ± 21,994</td>
</tr>
<tr>
<td>IL-4⁺ CD8⁺ T</td>
<td>3,438 ± 1,042</td>
<td>22,114 ± 4,776</td>
</tr>
</tbody>
</table>

* a Total number of cells ± SEM was obtained by calculating the percentage of gated events by the number of lung mononuclear cells isolated.
* b Lymphocytes were gated using forward and side scatter properties. Values represent the mean percentage of gated events within the parental gate ± SEM. For example, in mock-infected STAT1⁻/⁻ mice, CD3⁺ CD49b⁺ cells were 11.3% of lymphocytes and IFN-γ⁺ CD3⁺ CD49b⁺ cells were 1.2% of CD3⁺ CD49b⁺ cells, etc.
* c There is no gate parental to the lymphocyte gate.
* d There is no gate parental to the lymphocyte gate.
There was no significant difference in total cells between the BALB/c RSV and STAT1-/- RSV group. These data show that STAT1 negatively regulates IL-4 expression by CD3+CD49b+ cells and positively regulates IFN-γ expression by NK cells in RSV infection.

To confirm that CD3+CD49b+ cells and not CD4+ T cells are the predominant IL-4-expressing cells in RSV-infected STAT1-/- mice, we back-gated IL-4-expressing cells in RSV-infected STAT1-/- mice and found that ~85% of IL-4+ cells were CD3+CD49b+ (Fig. 4). We also performed time course studies to investigate the kinetics and cell type specificity of IL-4 expression in RSV-infected STAT1-/- mice. Whole lung mononuclear cells were isolated from STAT1-/- mice that were mock infected or infected with RSV 4, 5, 6, and 7 days p.i. RSV infection induced IL-4 expression by CD3+CD49b+ cells at all time points assayed, and there was no significant difference in the number of IL-4+CD3+CD49b+ cells at these time points (Fig. 5A). In contrast, RSV infection did not at any time induce IL-4 expression by CD4+ T cells (Fig. 5A). We also examined IFN-γ expression by CD3+CD49b+ cells. In BALB/c mice, CD3+CD49b+ is a typical NK cell immunophenotype (25). IFN-γ expressing CD3+CD49b+ cells in our system are likely NK cells. The total number of IFN-γ+ NK cells (Fig. 5B) was significantly higher in infected mice than mock-infected mice 4 days p.i., then decreased. The number of CD4+ T and CD8+ T cells (data not shown) and the number of IFN-γ+CD4+ T and IFN-γ+CD8+ T cells peaked 6 days p.i. (Fig. 5B). These data are similar to the observation that IFN-γ+ NK cells are detected before IFN-γ+ T cells in the bronchoalveolar lavage fluid of RSV-infected BALB/c mice (11).

Taken together, RSV infection of STAT1-/- mice resulted in IL-4+CD3+CD49b+ cells in the lung on days 4–7 and resulted in peak IFN-γ+ NK cells at day 4 followed by peak IFN-γ+ T cells at day 6.

![FIGURE 4. IL-4-expressing cells in RSV-infected STAT1-/- mice predominantly had a CD3+CD49b+ immunophenotype. Gating of IL-4-expressing lymphocytes (rectangular gate) in experiment described in Fig. 1. The percentages of gated IL-4-expressing lymphocytes from RSV-infected STAT1-/- mice that were CD3+CD49b+, CD3+CD49b-, CD3+CD49b, and CD3+CD49b were shown in the dot plot indicated by the arrow.](http://www.jimmunol.org/)

IL-4+ CD3+ CD49b+ cells in the lungs of RSV-infected STAT1-/- mice were not NKT cells

It has been shown that CD1-restricted NKT cells can produce abundant IL-4 and IFN-γ upon activation and regulate IL-4 and IFN-γ levels in vivo (26, 27). Also, activated NKT cells transiently down-regulate TCR expression upon activation in vitro (28, 29). Therefore, it was possible that IL-4-expressing CD3+CD49b+ cells in RSV-infected STAT1-/- mice are NKT cells. We tested the hypothesis that NKT cells are the IL-4-producing cells in RSV-infected mice in two ways. First, we quantified IL-4 expression by NKT cells in the lungs of RSV-infected STAT1-/- mice using tetramers loaded with α-GalCer. NKT cells recognize glycolipid Ags presented by MHC class I-like CD1d and NKT cells recognize the artificial ligand α-GalCer (28). Gated lymphocytes in lung mononuclear cells from RSV-infected mice 5 days p.i. contained large numbers of α-GC-tetramer+CD3+CD220+ NKT cells (Fig. 6A). Exclusion of B220+ cells is necessary for staining NKT cells because B cells can express CD1d (30, 31). The percentage of α-GC-tetramer+ B220+ NKT cells (CD3+ or CD3−) that expressed IL-4 was <0.1% compared with 4.21% of gated CD3+CD49b+ cells that expressed IL-4 (Fig. 6B). The second way we excluded NKT cells as IL-4-expressing cells in the lungs of RSV-infected STAT1-/- mice was by generating NKT cell-deficient STAT1-/-CD1d−/− mice and quantifying IL-4-expressing cells in these mice and STAT1-/- mice that were infected with RSV. There was no difference in the percent (Fig. 6C) or total number (Fig. 6D) of IL-4+ CD3+CD49b+ cells in the lungs of STAT1-/- and STAT1-/-CD1d−/− mice 5 days p.i. Taken together, these data demonstrate that IL-4-expressing CD3+CD49b+ cells in the lungs of STAT1-/- mice are not NKT cells with down-regulated CD3 expression. Furthermore, we identified NKT cells in RSV-infected STAT1-/- mice using tetramer staining and these cells did not express IL-4.
mock. Representative dot plots from infected mice show (IL-4+ CD3-CD49b+ cells, gated CD3-). We tested whether these cells are CD3+ CD49b+ gate NKT gate CD3+ cells in the lung in these mice than in BALB/c mice. Experiments showed above described that these cells are CD3-CD49b+ cells in the lungs of RSV-infected STAT1−/− mice were mock infected or infected with RSV, lung mononuclear cells were isolated on day 5 or day 6 p.i., and intracellular cytokine and cell surface markers were analyzed by flow cytometry.

Since CD3+ CD49b+ is a NK cell phenotype, we tested the hypothesis that the IL-4-expressing cells in RSV-infected STAT1−/− mice are NK cells. The IL-4+ CD3+ CD49b+ cells in RSV-infected STAT1−/− mice were NKG2D+ and CD122− (data not shown). We used a mixture of FITC-labeled anti-Ly49C/F/H/A Ab, FITC-labeled anti-Ly49A, and FITC-labeled anti-Ly49G2. The IL-4+ CD3+ CD49b+ cells in RSV-infected STAT1−/− mice were Ly49s− (data not shown). The IFN-γ+ CD3+ CD49b+ in the lungs of RSV-infected STAT1−/− mice were NKG2D+ CD122+ Ly49s+, confirming that these cells are NK cells (data not shown). Thus, IL-4+ CD3+ CD49b+ cells in RSV-infected STAT1−/− mice were not NK cells.

It has been shown that mast cells, eosinophils, and basophils can express IL-4, as measured by flow cytometric analyses of GFP expression and cell surface markers of peripheral blood and bone marrow cells of bicistronic IL-4 reporter 4GET mice (32). We tested whether the IL-4+ CD3+ CD49b+ cells in the lungs of RSV-infected STAT1−/− mice are either mast cells, basophils, or eosinophils by flow cytometry. We defined mast cells as SSCint FcRγI−CD11b−CD49b−, basophils as SSCint FcRγI+CD11b−CD49b−, and eosinophils as SSCint FcRγI−CD11b−CD49b− (32). We used BMNCs as positive controls for FcγRI and c-kit staining. Using intracellular cytokine staining, we found that IL-4+ CD3+ CD49b+ cells in the lungs of RSV-infected STAT1−/− mice were SSCint FcRγI−CD11b−CD49b− (data not shown). The IFN-γ+ CD3+ CD49b+ cells from the lungs of RSV-infected STAT1−/− mice CD11b+<sup>+</sup>Gr-1<sup>+</sup>B220<sup>+</sup> (data not shown). Thus, we ruled out mast cells and eosinophils as the identity of IL-4+ CD3+ CD49b+ cells in the lungs of RSV-infected STAT1−/− mice.

**FIGURE 6.** NKT cells were not the source of IL-4 in RSV-infected STAT1−/− mice. A–C, STAT1−/− mice were mock infected (n = 2) or infected with 10<sup>6</sup> PFU of RSV (n = 2). Lung mononuclear cells were isolated 5 days p.i. and stained with anti-CD3-PerCP-Cy5.5, anti-CD49b-biotin/streptavidin-allophycocyanin-Cy7, anti-CD4-PE-Cy7, anti-B220-FITC, α-GalCer-loaded CD1d tetramers-PE (αGC-tetramer), and anti-IL-4-allophycocyanin. B220<sup>+</sup> cells were excluded by gating in the plots in the right column. Representative dot plots from infected mice show (A) populations of CD3<sup>+</sup> CD49b<sup>+</sup> and NKT (αGC-tetramer<sup>+</sup>, CD3<sup>+</sup>, B220<sup>+</sup>) cells, (B) the percentage of gated CD3<sup>+</sup> CD49b<sup>+</sup> cells and αGC-tetramer<sup>+</sup> cells that were IL-4 expressing. C and D, STAT1−/− and STAT1+/+. CD11b+<sup>+</sup> mice were mock infected (n = 4 per group) or infected with 10<sup>6</sup> PFU of RSV (n = 6 per group). Lung mononuclear cells were isolated 6 days p.i. and stained with anti-CD3-FITC, anti-CD4-PerCP-Cy5.5, anti-CD8-allophycocyanin-Cy7, anti-CD49b-biotin/streptavidin-allophycocyanin, anti-IFN-γ-PE-Cy7, and either anti-IL-4 or rat IgG1-PE as an isotype control for IL-4 staining. For each mouse, the percentage of IL-4-expressing gated CD3<sup>+</sup> CD49b<sup>+</sup> cells (D) was obtained by subtracting the percentage of PE<sup>-</sup> gated CD3<sup>+</sup> CD49b<sup>+</sup> cells in the isotype control-gated series of cells. Percentage ± SEM is shown. E, The total number of IL-4-expressing CD3<sup>+</sup> CD49b<sup>+</sup> cells per lung ± SEM. *p < 0.05 comparing RSV to mock.

**IL-4+CD3+CD49b+ cells in the lungs of RSV-infected STAT1−/− mice are not NK cells, mast cells, or eosinophils**

We analyzed IL-4+ CD3+ CD49b+ cells in the lungs of RSV-infected mice by flow cytometry to identify their cell type. We used STAT1−/− mice because RSV induces more IL-4+ CD3+ CD49b+ cells in the lung in these mice than in BALB/c mice. Experiments described above showed that these cells are CD3<sup>−</sup>CD4<sup>−</sup>CD8<sup>−</sup> (Table III). In a series of experiments, STAT1−/− mice were mock infected or infected with RSV, lung mononuclear cells were isolated on day 5 or day 6 p.i., and intracellular cytokine and cell surface markers were analyzed by flow cytometry.

We investigated IL-4+ CD3+ CD49b+ cells in the lungs of RSV-infected mice histologically. We sorted these cells and stained them with Wright-Giemsa. Two experiments were performed. Whole lung mononuclear cells were isolated from RSV-infected STAT1−/− mice 5 or 6 days p.i. Cells were pooled from 8 or 11 mice and stained with anti-CD49b and anti-I-<sub>4</sub>. Lymphocytes were gated based on forward and side scatter properties. IL-4+ CD49b+ cells were 0.6% of cells within the FSC<sup>int</sup>SSC<sup>low</sup>“lymphocyte” gate (Fig. 7). Approximately 2500 CD49b+ IL-4<sup>+</sup> cells were obtained from the lungs of each RSV-infected STAT1−/− mouse. Sorted cells were cytopsin and stained with Wright-Giemsa. There is considerable debate about the histologic features of mouse basophils. Lee and McGarry (25) recently reviewed the literature on the flow cytometric immunophenotypes, morphology, and staining characteristics of human and mouse basophils. Basophils have “metachromatic-stained granules that are asymmetrically distributed throughout the cytoplasm and a lobulate nucleus that has comparatively weak staining chromatin” and mouse basophils “have relatively few, loosely packed granules of unequal size” (25). The hematologic appearance of the IL-4+ CD49b+ cells we sorted was consistent with basophil characteristics (Fig. 7).
IL-4-expressing cells in RSV-infected BALB/c and STAT1−/− mice were basophils

Using BMMCs, we found that the anti-FcεRI Ab yielded good positive staining when only cell surface markers were analyzed but did not yield definitive positive staining in our intracellular cytokine staining protocol, likely due to effects from cell permeabilization (data not shown). Therefore, we used 4get and STAT1−/− 4get mice to test the hypothesis that CD3−CD49b+ cells in the lungs of RSV-infected mice are basophils. 4get mice are useful for investigating IL-4 expression in BALB/c mice. Connective tissue mast cells as well as blood basophils and eosinophils from 4get mice constitutively transcribe IL-4 mRNA, but IL-4 protein expression requires cell stimulation (32). STAT1−/−, 4get, and STAT1−/− 4get mice were mock infected or infected with RSV. Lung mononuclear cells were isolated 6 days p.i. and stained with anti-CD49b-biotin/streptavidin-allophycocyanin, anti-CD3, anti-CD44, and anti-FcεRI Ab. Sorted cells were analyzed by flow cytometry. FSClowSSClow cells were gated (Fig. 8A). Gated CD3−CD49b+ cells from infected STAT1−/− mice exhibited no background autofluorescence in the GFP range (Fig. 8B). In both 4get and STAT1−/− 4get mice, RSV infection increased the percentage of CD3−CD49b+ cells that were GFP+ but did not increase the percentage of T cells that were GFP+ (Fig. 8C). Gated CD3−CD49b+ cells from RSV-infected STAT1−/− 4get mice (Fig. 8D) and RSV-infected 4get mice (data not shown) expressed high levels of FcεRI but did not express c-kit, whereas BMMC controls expressed high levels of FcεRI and c-kit (data not shown). RSV infection increased the total number of IL-4-expressing (GFP+) basophils in the lungs of 4get and STAT1−/− 4get mice, and there were significantly more IL-4-expressing basophils in the lungs of RSV-infected STAT1−/− 4get mice than RSV-infected 4get mice (Fig. 8E). These data are consistent with the total numbers of IL-4-expressing cells we obtained by intracellular cytokine staining in RSV-infected BALB/c and STAT1−/− mice (Fig. 3). Thus, we identified the IL-4-expressing CD3−CD49b+ cells in the lungs of RSV-infected mice as basophils.

Basophils contributed to lung IL-4 expression in RSV infection

The MAR-1 clone anti-FcεRI Ab has been shown to deplete basophils (33). We depleted basophils in RSV-infected STAT1−/− mice using this Ab or treated mice with an isotype control Ab. The treatment effectively depleted IL-4-expressing basophils from the lungs of RSV-infected STAT1−/− mice (Fig. 9). Gating on the FSClowSSC population typical for lymphocytes and basophils, we found that basophil depletion eliminated the majority of IL-4-expressing cells from the lungs of RSV-infected STAT1−/− mice (Fig. 9). We did not observe IL-4+ cells outside this FSClowSSC gate, similar to Fig. 4. Thus, basophils were the predominant IL-4-expressing cell type in the lungs of these RSV-infected mice. We assayed IL-4 protein levels by ELISA in RSV-infected STAT1−/− mice, but the levels were below the detection limit (data not shown). Thus, we were not able to determine the effect of
basophil depletion on whole lung IL-4 protein levels. We assessed the role of basophils in RSV-induced lung histopathologic changes in STAT1^{-/-} mice. STAT1^{-/-} mice were infected with 10^5 PFU of RSV, treated with anti-FceRI Ab or control Ab (see Materials and Methods), and lungs were harvested 7 days p.i. We observed no effect of basophil depletion on histopathology (data not shown).

Discussion

Our study shows for the first time that a respiratory virus (RSV) induces basophil accumulation and basophil IL-4 expression in vivo. We investigated the role and cellular source of IL-4 in RSV infection in BALB/c (Th1-type inflammation) and STAT1^{-/-} mice (enhanced Th2-type inflammation). Our data show that IL-4 is important in RSV pathogenesis in these models. RSV-infected IL4^{-/-} mice had higher IFN-γ levels in the lung than RSV-infected BALB/c mice. In contrast, RSV-infected STAT1^{-/-} IL4^{-/-} mice had lung IFN-γ levels similar to those of RSV-infected STAT1^{-/-} mice. We conclude from these data that IL-4 regulation of IFN-γ production is STAT1 dependent. RSV-infected STAT1^{-/-} IL4^{-/-} mice had lower IL-13 lung levels than RSV-infected STAT1^{-/-} mice 8 days p.i. but not 6 days p.i. Thus, there is IL-4-dependent and IL-4-independent IL-13 production in response to RSV infection in STAT1^{-/-} mice, results seen in mouse models of allergic inflammation (34, 35).

In addition to modulating cytokine levels in the lung, we found that IL-4 contributes to RSV-induced cellularity in the lung in BALB/c mice, in which Th1-type inflammation is characteristic, as well as in STAT1^{-/-} mice, in which enhanced Th2-type inflammation is characteristic. Interestingly, RSV-infected STAT1^{-/-} IL4^{-/-} mice had less lung consolidation and fewer intrabronchial inflammatory cells than RSV-infected STAT1^{-/-} mice. This is an important finding because RSV is the leading cause of mechanical ventilation for respiratory failure in infants (36) and it is thought that inflammatory cells contribute to airway blockage. IL4 and IL4Rα polymorphisms have been associated with severe RSV bronchiolitis in infants (37). IL-4 has been shown to mediate RSV disease severity in mice immunized with formalin-inactivated, alum-precipitated RSV and then challenged with RSV, a model of RSV vaccine-enhanced illness (38). Immune competent mice exhibit a Th1-dominant response to primary RSV infection. RSV pathogenesis has been studied in the setting of Th2 inflammation using mice immunized with formalin-inactivated virus, using mice primed with vaccinia virus that expresses RSV G, using RSV combined with mouse models of allergic inflammation, and using recombinant RSV that expresses IL-4 (8, 10). Our data with STAT1^{-/-} and STAT1^{-/-}IL4^{-/-} mice demonstrate that IL-4 contributed to lung inflammation in the setting of Th2-type inflammation induced by primary RSV infection.

We used two different doses of RSV in this study, 10^5 PFU and 10^6 PFU per mouse, for the following reasons. We performed experiments for Figs. 3–6 first using 10^5 PFU per mouse. Then we performed histopathology experiments using 10^5 PFU per mouse. At this high dose, STAT1^{-/-} and STAT1^{-/-} IL4^{-/-} mice were moribund as a result of RSV infection by day 7 postinfection (e.g., last time point Fig. 5). Because 10^6 PFU overwhelmed the STAT1^{-/-} mice by day 7, it was necessary to reduce the viral inoculum to 10^5 PFU per mouse to adequately assess histopathology (Fig. 1). We then performed viral load, cytokine measurements, and further flow cytometric analyses (Tables I–III and Figs. 2 and 7–9) using 10^5 PFU. Basophils were the predominant IL-4-expressing cell type in the lung in RSV infection in BALB/c and STAT1^{-/-} mice at both 10^5 and 10^6 PFU.

Activated NKT cells are known to produce IL-4 and to downregulate TCR expression upon activation (28, 29). We excluded NKT cells as the CD3^−CD49b^ IL-4-expressing cells in the lungs of RSV-infected STAT1^{-/-} mice. First, α-βC-loaded CD1 tetramer-staining, we showed that NKT cells were not the IL-4-expressing cells in the lungs of RSV-infected STAT1^{-/-} mice. Also, we generated NKT cell-deficient CD1d^{-/-}STAT1^{-/-} mice, and NKT cell deficiency had no effect on the percentage or number of IL-4-expressing CD3^-CD49b^ cells in the lungs of RSV-infected STAT1^{-/-} mice. Emerging cell immunophenotype data continuously redefines our classification of immune cells. For example, cells that express both NK and dendritic cell (DC) markers are described (39, 40). IFN-producing killer DCs can transition from cytotoxic effectors to DC-like Ag presentation upon stimulation in vitro (41). CD3^-CD49b^-IL-4^- cells in our studies are not IFN-producing killer DCs because they are B220^- (data not shown).

Several recent studies indicate that basophils play a key role in Th2 inflammation induction, immune memory, and Ag capture. IL-4-expressing basophils were found in the bronchoalveolar lavage of patients following segmental allergen challenge (42). In mice primed with goat anti-mouse IgD and then challenged with goat serum, a model of robust Th2 inflammation in vivo, basophils were found to initiate IL-4 production (43). Infection of mice with the Th2-inducing parasite Nippostrongylus brasiliensis results in basophil IL-4 expression and basophilia (44). Adoptive transfer of basophils in IgE-mediated allergic inflammation showed that these cells play a role in chronic inflammation (45). Coculture experiments demonstrated that basophil-supported CD4^+ T cell Th2 differentiation in vitro depends on IL-4 produced by basophils (46). Immunizing mice with the fluorophore allopseudocyanin showed that basophils can act as Ag-capturing cells (47, 48). Basophils migrated to the draining lymph node and expressed IL-4 and thymic stromal lymphopoeitin in mice immunized and challenged with papain (33). Basophils and thymic stromal lymphopoeitin were required for Th2 induction in this model (33). Thus, basophils are key innate regulators of Th2 inflammation. STAT1^{-/-} and STAT1^{-/-} IL4^{-/-} 4get mice may be useful for studying basophils because basophils are rare in wild-type mice (25).

We found that RSV infection induced pulmonary basophil accumulation and basophil IL-4 expression. Basophils were the vast
majority of IL-4-expressing cells in the lung in primary RSV infection. RSV-infected STAT1−/− mice had more IL-4-expressing basophils than RSV-infected BALB/c mice. Thus, STAT1 negatively regulated RSV infection-induced basophil accumulation. To our knowledge, this is the first report of basophil inflammation induced by a respiratory virus. As an important source of IL-4, basophils may provide a bridge between innate and adaptive immunity in RSV infection.

We investigated the role of basophils in RSV pathogenesis in STAT1−/− mice using anti-FcεRI Ab to deplete basophils in vivo as previously described (33). Basophils were indeed the primary source of IL-4 expression in the lungs of RSV-infected mice because basophil depletion ablated the majority of IL-4-expressing cells. We did not detect IL-4 protein by ELISA in lung homogenates in any RSV-infected mice in this study (data not shown). We did not observe a difference in mononuclear cell bronchovascular (perivascular and peribronchial) inflammation, a difference in lung consolidation in alveolar spaces, nor a difference in intrabronchial inflammatory cells between basophil-depleted and control Ab-treated RSV-infected STAT1−/− mice. This result was somewhat surprising because STAT1−/− IL-4−/− mice had less lung consolidation and intrabronchial inflammatory cells than STAT1−/−/− mice in the setting of RSV infection. It may be that systemic IgG Ab treatment (depleting and control Ab) has some immune effects that mask the effect of basophil depletion. Although the cells that have the flow cytometric characteristics of basophils are the most numerous cells producing IL-4, an alternative explanation for the failure of the basophil depletion to alter RSV-induced histopathology is that cells other than basophils are more potent producers of IL-4 on a per cell basis. Also, although the anti-FcεRI Ab clearly depleted IL-4-expressing basophils in this study and has been used by others for functional assessment of basophils in mice, the mechanism of depletion is not clear (33). It is possible that the anti-FcεRI Ab could have additional effects such as receptor cross-linking on basophils and/or mast cells which could result in immune activation and degranulation. The Ba103 mAb, which recognizes CD200R3, has been used to deplete basophils in mice, but this receptor is also expressed on mast cells (49). The field would benefit from a specific basophil-deficient mouse model.

In a collaborative study, we previously found that the line 19 strain of RSV induces greater IL-13, airway hyperreactivity, and airway mucus expression in BALB/c mice than the A2 laboratory strain of RSV induces greater IL-13, airway hyperreactivity, and airway mucus expression in BALB/c mice than the A2 laboratory strain of RSV infection in mice. RSV infection does not follow challenge with wild-type virus. Further studies will be required to clarify the role of CD1d in the hydrocarbon oil-induced model of lupus nephritis. Immunity 171: 2142–2153.


Acknowledgments

We thank John Schroeder (Johns Hopkins Medical School, Baltimore, MD) for helpful discussions.

Disclosures

The authors have no financial conflict of interest.

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