IL-13 Is Sufficient for Respiratory Syncytial Virus G Glycoprotein-Induced Eosinophilia After Respiratory Syncytial Virus Challenge

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IL-13 Is Sufficient for Respiratory Syncytial Virus G Glycoprotein-Induced Eosinophilia After Respiratory Syncytial Virus Challenge

Teresa R. Johnson,*† Robert A. Parker,§ Joyce E. Johnson,‡ and Barney S. Graham2✉†

Although well studied in settings of helmint infection and allergen sensitization, the combined contributions of IL-4 and IL-13 and their signaling pathways in models of viral pathogenesis have not been reported. Using a murine model of respiratory syncytial virus (RSV) infection, we evaluated the contribution of IL-4, alone and in conjunction with IL-4, during immunization with recombinant vaccinia virus expressing RSV G glycoprotein (vvGs) or with formalin-inactivated RSV (FI-RSV). We showed that both IL-4 and IL-13 activity must be inhibited to modulate G-specific responses resulting in severe RSV-induced disease. Inhibition of IL-4 or IL-13 activity alone had minimal impact on disease in vvGs-immunized mice. However, treatment of IL-4-deficient mice with IL-13Ra during vvGs immunization reduced IL-5, IL-13, and eotaxin production and pulmonary eosinophilia after RSV challenge. In contrast, FI-RSV-induced immune responses were diminished when either IL-4 or IL-13 activity was blocked. After RSV challenge, these type 2 T cell responses were also diminished in vvGs-primed IL-4Ra-deficient mice. Our data suggest that secreted vGs uses mechanisms requiring signaling through the IL-4Ra-chain by either IL-4 or IL-13 for induction of eosinophilia and is the first description of the relative contributions of IL-4, IL-13, and their receptors in viral pathogenesis. The Journal of Immunology, 2003, 170: 2037–2045.

Interleukin-13 is a cytokine produced by type 2 T cells with functions paralleling those of IL-4 (1–4). These shared functions of IL-4 and IL-13 are due in part to common usage of the IL-4Ra α-chain in both the IL-4 and IL-13 receptors (2, 5). Although IL-4 and IL-13 exhibit similar functions in B cell development and isotype switching, a preferential role for IL-13 in some inflammatory processes has been observed (6–9). Both IL-4 and IL-13 play important roles in resistance to parasitic infection in a complex regulatory network as reviewed (10). Yet, when the phenotypes of IL-4- and IL-13-deficient helmint-infected mice are compared, striking differences are observed, suggesting parallel yet distinct roles for IL-4 and IL-13 (8).

Type 2-associated cytokines have been implicated in the pathogenesis of asthma and atopy. IL-13 has been shown to be a very potent inducer of eotaxin production, suggesting a major role for this cytokine in asthma and pathogenic conditions associated with activation of type 2 T cells (6). Recent reports demonstrate that IL-13 rather than IL-4 may be the key immune mediator in asthma (7, 11). When IL-13 activity was blocked in animal models of asthma, airway hyperresponsiveness (AHR),3 eosinophil recruitment, and mucus production were inhibited, despite intact IL-4 production and function. In complementary studies, induction of IL-13 expression in transgenic mice resulted in increased AHR, infiltration of inflammatory cells (including eosinophils), mucus production, airway obstruction, and eotaxin production (9).

Respiratory syncytial virus (RSV) is the primary viral cause of respiratory infections in infants each year (12, 13) but also produces significant disease in elderly and immunocompromised patients (14–16). Although RSV infection normally results in mild to moderate disease, some infants experience more severe disease, caused by 51,000–82,000 and 63,000–100,000 hospitalizations in the U.S. each year in children younger than 1 year and 5 years of age, respectively (17).

There are two disease syndromes in humans associated with RSV infection that suggest a role for type 2 cytokines in RSV pathogenesis. Elevated levels of IgE in the serum (18) and airway secretions (19) and the presence of eosinophil cationic protein in nasal secretions (20) suggest the production of type 2 cytokines during primary RSV infection and may be a correlate of severe disease. The incidence of childhood asthma is increased in those infants who experience severe RSV disease (21, 22), and the overall rate of severe RSV-induced bronchiolitis is increasing coincident with the rising incidence of asthma (17).

The second syndrome involves immunization with a formalin-inactivated RSV vaccine (FI-RSV) in the early 1960s that resulted in no protection from natural infection and caused a vaccine-enhanced illness (23, 24). Studies in mice have shown that FI-RSV immunization primes for immune responses resulting in the production of IL-4, IL-5, and pulmonary eosinophilia and severe RSV disease (25, 26). Interestingly, immunization with the RSV attachment G glycoprotein alone can induce CD4+ T cells that produce type 2 cytokines and results in eosinophil recruitment and illness on RSV challenge (27, 28), with the secreted form of RSV G (Gs) predisposing for more severe disease than the membrane-anchored form (29, 30). An epitope has been identified in the G glycoprotein that is associated with this effect (31–33).

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RSV attachment protein allows induction of protective immunity without vaccine-enhanced lung eosinophilia (31), and in BALB/c mice the response to this epitope is dominated by Vβ14+CD4+ T cells (34). The G-specific response includes production of both the type 2 cytokines IL-4 and IL-13 and the type 1 cytokine IFN-γ (29, 31, 32, 34, 35).

An important question for vaccine development is whether the antigenic determinants of the RSV G were an essential factor in the FI-RSV vaccine-enhanced illness. The similar effects on induction of Th2 responses after subsequent RSV infection of G-immunized mice suggest that RSV G Ags may have played an important role in the FI-RSV vaccine-enhanced illness. However, RSV G is expressed on wild-type virus that, during a typical primary infection, leads to a Th1-type response, and primary infection with RSV causes severe disease in a minority of infants. In addition, the G-induced response in mice is dependent on the genetic background (36), is epitope dependent (31–33), and is restricted to selected TCRs (34). In contrast, FI-RSV immunization led to enhanced disease in virtually all individuals (23, 24), suggesting that it was not dependent on the genetic background or restricted to a single epitope or TCR. Finally, the pathology, illness, and immune responses elicited by FI-RSV can also be elicited by purified RSV F glycoprotein (25), especially when formulated in alum (37), indicating that this type of response is not necessarily dependent on G-specific epitopes.

Other differences have been observed in the pattern of type 2 cytokine expression in G-immunized mice compared with mice immunized with FI-RSV. For example, when IL-4 activity was inhibited during FI-RSV immunization, disease and IL-4 production following challenge was decreased, whereas production of IFN-γ was increased (38). However, when neutralizing anti-IL-4 Ab is administered during vaccinia virus (vv) Gs immunization or when IL-4-deficient mice were immunized, pulmonary eosinophilia and type 2 cytokine production were not reduced (35). With the dominant role for IL-13 in asthma (7, 11), we hypothesized IL-13 may be the primary mediator of RSV G-induced eosinophilia and enhanced lung pathology after subsequent RSV infection. In these studies, we sought to define the requirements for IL-4 and IL-13, alone or in combination, for the induction of those immune responses that predispose for severe disease following RSV challenge of vvGs-immunized mice. The contribution of each cytokine to immunization-induced immune responses was evaluated in IL-4Ra−/− mice and in IL-4−/− or IL-4−/− mice treated with IL-13 receptor antagonist (IL-13Ra).

Materials and Methods

Cells, viruses, and IL-13Ra

HEP-2 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in EMEM supplemented with 10% FCS, glutamine, and antibiotics (10% EMEM). A laboratory stock of RSV A2 strain was prepared as previously described (39). FI-RSV was prepared as previously described (25). Stocks of vv expressing either secreted RSV G (a gift from G. W. Wertz, University of Alabama, Birmingham, AL; designated vvGs) or β-galactosidase (a gift from B. Moss, National Institutes of Health, Bethesda, MD; designated vac-vvG) were generated as described (29). All virus stocks were determined to be free of Mycoplasma contamination by PCR analysis. Soluble IL-13Ra was constructed by in-frame fusion of the extracellular domains of the murine IL-13Rα1-chain with domains 2 and 3 from the constant region of the human IgG1 heavy chain (40). IL-13Ra was shown to specifically bind and neutralize IL-13 with no effect on IL-4 activity. The IL-13Ra reagent and the appropriate isotype control were provided by S. Goldman (Genetics Institute, Cambridge, MA).

Mouse immunization and challenge

BALB/c IL-4−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-4Ra−/− mice on a BALB/c genetic background (41) were a gift from N. Nohen-Troth (National Institutes of Health). Control (wild-type) BALB/c mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Mice were immunized with vac-lac, vvGs, or FI-RSV and challenged with RSV 6 weeks later as previously described (29). IL-13Ra was administered at priming or at challenge as specified in Table I. The mice were injected i.p. with 200 μg of control IgG or IL-13Ra daily at the indicated time points. Mice were anesthetized and challenged with 107 PFU RSV (in 0.1 ml) administered intranasally, then weighed, and scored for illness daily as described (29).

RSV titers

Mice were euthanized at the indicated days postchallenge, and lungs were removed and quick-frozen. PFU of virus were measured by plaque assay on confluent HEp-2 monolayers as previously described (29). Data are expressed as log10 (PFU per gram).

Cytokine levels

Cytokine protein levels in lung supernatants from day 4 plaque assays were quantitated by ELISA using cytokine-specific kits (R&D Systems, Minneapolis, MN).

Bronchoalveolar lavage (BAL) eosinophils

Seven days after challenge, BAL was performed as previously described (35). BAL cell pellets were differentially stained with Diff-Quick (Fisher Scientific, Pittsburgh, PA), and eosinophil numbers were counted as previously described (35).

Lung histopathology

Seven days after RSV challenge, mice were euthanized, and the left lung was removed and placed in phosphate-buffered formalin (10% formalin). Thin sections were cut from paraffin-embedded lungs and stained with H&E or with Wright’s Giemsa. The stained lung sections were analyzed and scored for degree and composition of lung inflammation as previously described (29). Briefly, inflammation was assessed according to the following scale: 0, no infiltrate; 1, mixed generalized increase in interstitial mononuclear cells without widening of alveolar septa; 2, dense septal mononuclear infiltrates with septal thickening and occasional foci of intraalveolar cells; 3, significant alveolar space consolidation (intra-alveolar edema, inflammation, or hemorrhage) in addition to interstitial inflammation.

Ab assays: Ag-specific ELISAs and RSV neutralization

Prechallenge sera were collected from immunized mice by retro-orbital bleeding the day before RSV challenge. Sera were stored at −20°C until Ab assays were performed. Ab ELISAs were performed as previously described (29), coating the plates with purified RSV Gs isolated from RSV subtype A virus and detecting bound serum Ab with HRP-conjugated Abs to mouse IgG1 or IgG2a (Southern Biotechnology, Birmingham, AL). Data are represented as log2 values of the serum dilution producing 0.100 OD550 unit and twice the Ag-negative well.

<table>
<thead>
<tr>
<th>Priming</th>
<th>Strain</th>
<th>Treatment</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>vac-lac</td>
<td>IL-4−/−</td>
<td>IgG control</td>
<td>IL-4−13+</td>
</tr>
<tr>
<td>IL-4−/−</td>
<td>IgG control</td>
<td>IL-4−13+</td>
<td></td>
</tr>
<tr>
<td>IL-4−/−</td>
<td>IL-13Ra</td>
<td>IL-4−13−</td>
<td></td>
</tr>
<tr>
<td>IL-4−/−</td>
<td>IL-13Ra</td>
<td>IL-4−13−</td>
<td></td>
</tr>
<tr>
<td>vvGs</td>
<td>IL-4−/−</td>
<td>IgG control</td>
<td>IL-4−13+</td>
</tr>
<tr>
<td>IL-4−/−</td>
<td>IgG control</td>
<td>IL-4−13+</td>
<td></td>
</tr>
<tr>
<td>IL-4−/−</td>
<td>IL-13Ra</td>
<td>IL-4−13−</td>
<td></td>
</tr>
<tr>
<td>IL-4−/−</td>
<td>IL-13Ra</td>
<td>IL-4−13−</td>
<td></td>
</tr>
<tr>
<td>FI-RSV</td>
<td>IL-4−/−</td>
<td>IgG control</td>
<td>IL-4−13+</td>
</tr>
<tr>
<td>IL-4−/−</td>
<td>IgG control</td>
<td>IL-4−13+</td>
<td></td>
</tr>
<tr>
<td>IL-4−/−</td>
<td>IL-13Ra</td>
<td>IL-4−13−</td>
<td></td>
</tr>
<tr>
<td>IL-4−/−</td>
<td>IL-13Ra</td>
<td>IL-4−13−</td>
<td></td>
</tr>
</tbody>
</table>

IL-4−/− or IL-4−/− BALB/c mice were immunized with vac-lac, vvGs, or FI-RSV. During immunization, mice were treated with IL-13Ra or IgG isotype control on days −1, 0, and +1. To denote cytokine status during priming, mice were designated IL-4−13−, IL-4−13+, IL-4−13−, or IL-4−13+ as shown. From 5 to 6 wk postimmunization, mice were challenged intranasally with 107 PFU live RSV.
Titers of RSV neutralizing Abs were determined by plaque reduction assay on HEp2 cell monolayers as described (42). The data are expressed as the log$_2$ of the serum dilution resulting in 40% of the PBS control titers (i.e., 60% neutralization).

**Statistical analysis**

Data from multiple experiments were combined and summarized as mean ± SD. Comparisons between groups used a generalized linear model approach and adjusted for experiments. Post hoc testing was done only when there was a significant overall group effect in the analysis (e.g., between the different cytokine treatment groups or between the different priming agents). Post hoc testing used Dunnett’s procedure that adjusts for multiple comparisons against a specific predefined group, either the IL-4$^{+/+}$ control group or the vac-lac immunization group as appropriate. These post hoc analyses also adjusted for experiment when results from more than one experiment were combined. All analyses were done in SAS version 6.12 (SAS Institute, Cary, NC). $p$ values < 0.05 were considered statistically significant.

**Results**

**Interference with IL-13 alone does not alter disease in vvGs-primed mice**

Eosinophil lung infiltration is induced in vvGs-primed mice (29) and is not inhibited by neutralization of IL-4 during vvGs immunization (35). We therefore examined the requirements of IL-13 for induction of eosinophilia in vvGs-immunized mice by blocking IL-13 function with the administration of IL-13Ra during vvGs priming or RSV challenge. Eosinophilia in IL-13Ra-treated vvGs-primed mice was examined 7 days after RSV challenge by differential staining of BAL cells. Significant recruitment of eosinophils occurred in mice immunized with vvGs, and eosinophilia was not inhibited by administration of IL-13Ra at the time of either vvGs priming or RSV challenge (Table II). Additionally, IL-13Ra administration at any time did not decrease the severity of illness or the production of type 2 associated cytokines in vvGs-immunized RSV-challenged mice (data not shown). Thus, those immune responses predisposing for severe illness and eosinophilia in vvGs-primed mice are induced in the absence of functional IL-13.

**Th2 cytokine production and eosinophilia are abolished in vvGs-primed IL-4Ra-deficient mice**

We have demonstrated that eosinophilia and type 2 cytokine production in vvGs-immunized RSV-challenged mice is not dependent on the individual activities of IL-4 (35) or IL-13 (Table II). Receptors for both IL-4 and IL-13 contain the IL-4Ra chain (2, 5). Therefore, to examine the effects of the constitutive loss of both IL-4 and IL-13 signaling on severe RSV disease, IL-4Ra-deficient mice were immunized with vvGs and then challenged with RSV. Illness and viral titers did not significantly differ between IL-4Ra$^{-/-}$ and IL-4Ra$^{+/+}$ mice within the vac-lac- or the vvGs-immunized groups (data not shown). After RSV challenge, eosinophilia was decreased in vvGs-primed IL-4Ra-deficient mice (Fig. 1, $p < 0.005$ relative to vvGs-primed IL-4Ra$^{+/+}$ mice). These data demonstrate that IL-4Ra-mediated signals contribute to the induction of immune responses during vvGs immunization that predispose for eosinophilia on RSV challenge.

IL-5 and IL-13 levels were significantly reduced in vvGs-immunized IL-4Ra-deficient mice (Fig. 2; $p < 0.05$ relative to vvGs-primed IL-4Ra$^{+/+}$ mice). In addition, significant decreases were also seen in eotaxin production ($p < 0.05$), suggesting regulation of IL-5 and eotaxin by IL-4Ra-mediated signals. IFN-γ levels, however, were not altered in IL-4Ra-deficient mice (relative to wild-type controls). These data suggest that signals mediated by IL-4Ra engagement, presumably by the ligands IL-4 or IL-13, are required for the induction of type 2 cytokines in vvGs-immunized mice after RSV challenge.

**Induction of eosinophilia in vvGs-immunized mice requires either IL-4 or IL-13, whereas both IL-4 and IL-13 are required for eosinophilia in FI-RSV-immunized mice**

Inhibition of IL-4 (35) or IL-13 (above) alone does not reduce eosinophil recruitment in vvGs-primed mice in contrast to the effects observed in FI-RSV-immunized mice (35, 38), whereas the loss of both IL-4 and IL-13 signaling in vvGs-primed IL-4Ra-deficient mice results in reduced pulmonary eosinophilia and type 2 cytokine production (above). To evaluate the relative contributions of IL-4 and IL-13 in the generation of RSV-induced pulmonary eosinophilia and type 2 cytokine production, the functions of either IL-4 or IL-13 or both were inhibited during vvGs or FI-RSV immunization (Table I), and eosinophilia after subsequent RSV challenge was measured. In IL-4$^{+/-}$ mice immunized with vvGs or with FI-RSV, significant eosinophilia was observed in BAL 7 days after RSV challenge (Fig. 3; $p < 0.0001$ relative to vac-lac-primed mice). In FI-RSV-primed mice, eosinophilia was significantly reduced when the function of IL-4 or IL-13—either alone or in combination—was interrupted (Fig. 3; $p < 0.001$ comparing FI-RSV-primed IL-4$^{+/-}$ mice with each of the other FI-RSV-immunized groups). Conversely, in IL-4$^{-/-}$ and IL-4$^{+/-}$ mice primed with vvGs, eosinophil numbers were similar to that of IL-4$^{+/-}$ mice ($p > 0.05$). However, in IL-4$^{+/-}$ vvGs-immunized RSV-challenged mice, eosinophilia was significantly reduced (compared with IL-4$^{+/-}$, IL-4$^{-/-}$, and IL-4$^{+/-}$ vvGs-primed mice, $p < 0.001$). Importantly, inhibition of eosinophil-recruiting immune responses during vvGs immunization required blockade of both IL-4 and IL-13 activity, whereas inhibition of IL-4 or IL-13 alone reduced eosinophilia in FI-RSV-primed mice. Thus, the cytokine requirements for eosinophil recruitment are different in vvGs-and FI-RSV-immunized mice.

**Table II. BAL eosinophilia in IL-13Ra-treated vvGs-immunized mice**

<table>
<thead>
<tr>
<th>Priming</th>
<th>Treatment at Priming</th>
<th>Treatment at Challenge</th>
<th>% Eosinophils</th>
<th>No. of Eosinophils ($\times 10^5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vac-lac</td>
<td>IgG control</td>
<td>IgG control</td>
<td>2.8 ± 0.7</td>
<td>8.1 ± 8.0</td>
</tr>
<tr>
<td></td>
<td>IL-13Ra</td>
<td></td>
<td>2.7 ± 0.5</td>
<td>7.5 ± 7.6</td>
</tr>
<tr>
<td></td>
<td>IL-13Ra</td>
<td></td>
<td>3.4 ± 0.7</td>
<td>5.4 ± 1.8</td>
</tr>
<tr>
<td>vvGs</td>
<td>IgG control</td>
<td>IgG control</td>
<td>18.1 ± 4.0$^b$</td>
<td>69.4 ± 29.8$^b$</td>
</tr>
<tr>
<td></td>
<td>IL-13Ra</td>
<td></td>
<td>12.7 ± 5.9$^b$</td>
<td>90.8 ± 25.2$^b$</td>
</tr>
<tr>
<td></td>
<td>IL-13Ra</td>
<td></td>
<td>14.6 ± 3.9$^b$</td>
<td>31.4 ± 8.6$^b$</td>
</tr>
</tbody>
</table>

$^a$ BAL was performed 7 days after RSV challenge, and the BAL cells were differentially stained and counted. Data represent the mean ± SD for $n = 5$–6 mice from a single experiment.

$^b$ Eosinophilia is significantly greater than in vac-lac-primed mice of any treatment group ($p < 0.005$).
Inhibition of IL-4 and IL-13 alters the composition and the degree of lung inflammation

Immunization of IL-4+13+ mice with vvGs resulted in significant eosinophil recruitment to the bronchovascular bundles after RSV challenge. However, as with eosinophil infiltration to the BAL compartment, eosinophilia in the lung tissue of vvGs-immunized mice was not reduced in IL-4−13− or IL-4+13− vvGs-immunized mice but was significantly reduced in IL-4−13− mice primed with vvGs and challenged with RSV compared with IL-4+13− mice (Table III). In FI-RSV-immunized IL-4+13− mice significant eosinophilia was observed in the tissue with eosinophil numbers reduced in IL-4−13−, IL-4+13−, and IL-4−13− FI-RSV-primed mice (p < 0.05 relative to FI-RSV-immunized IL-4+13− mice).

The severity of inflammation in the distinct histologic compartments of the lung (i.e., bronchovascular, perivenous, and interstitium) was graded using an established scoring system that has been described (29). As has been previously described (29, 30, 43, 44), vvGs-immunized RSV-challenged IL-4+13+ mice had significantly more severe lung inflammation than did vac-lac-primed IL-4+13− mice (Table III). Generally, the severity of inflammation in any histologic region of the lung was not significantly reduced in IL-4+13−, IL-4−13−, or IL-4−13− vvGs-primed RSV-challenged mice (Table III). The only significant decrease in inflammation (compared with IL-4+13− vvGs-immunized mice) was observed in the interstitial region of the IL-4−13− mice (p < 0.05). In contrast, interference with IL-4 function decreased the degree of inflammation in FI-RSV-immunized mice. In the bronchovascular region, significantly less leukocyte infiltration was observed in the IL-4− deficient mice regardless of the IL-13 status. Furthermore, the FI-RSV-primed IL-4−13− mice had significantly less inflammation in the interstitial region. These data suggest that in the setting of FI-RSV immunization IL-4 has a more dominant role than IL-13 in predisposing for severe pathology after RSV challenge. Thus, the requirement for each cytokine is dependent on the nature of vaccine Ag with the disease-enhancing properties of FI-RSV immunization being more sensitive to the influences of either cytokine, particularly IL-4.

**FIGURE 1.** Gs-induced BAL eosinophilia is reduced in IL-4Rα-deficient mice. IL-4Rα+/+ or IL-4Rα−/− mice were immunized with either vac-lac or vvGs and then challenged with RSV. BAL was performed 7 days after challenge, and cell pellets were differentially stained. Data represent the mean ± SD of five mice from a single experiment.

**FIGURE 2.** Production of type 2-associated cytokines is reduced in vvGs-primed IL-4Rα−/− mice. Mice were primed and challenged as described in Fig. 1. Lung supernatants at 4 days postchallenge were examined in cytokine-specific ELISAs. Data represent the mean ± SD of five mice from a single experiment.

**FIGURE 3.** Eosinophilia is reduced by inhibition of both IL-4 and IL-13 during vvGs immunization. IL-4+/+ or IL-4−/− mice were treated with IL-13Ra or control IgG on days −1, 0, and 1 around immunization with vac-lac, vvGs, or FI-RSV and then challenged with RSV as described in Table I. BAL was performed 7 days after challenge, and cell pellets were differentially stained. Data represent the mean ± SD for n = 10 from 2 combined experiments for most groups. For vac-lac- and FI-RSV-primed IL-4−13− groups, n = 9, and for the vvGs-primed IL-4+13+ group, n = 6.
IL-13 levels were significantly increased by priming with IL-4 or IL-13, and eosinophils in lung supernatants at day 4 postchallenge were measured by ELISA (Table IV). Cytokine production in vac-lac-immunized mice was not significantly altered by any intervention. Similarly, IL-10 and IFN-γ levels were not significantly altered in vvGs-immunized mice by any intervention when compared with IL-4+/13+ mice, and only in the FI-RSV-vaccinated IL-4/13+ mice were IL-10 levels significantly reduced (p < 0.01) with no significant changes in IFN-γ production. IL-4+/13+ mice immunized with vvGs or FI-RSV produced significantly more IL-4, IL-5, IL-10, IL-13, IFN-γ, and eosinophils than did vac-lac-immunized mice (p < 0.05). In vvGs-immunized mice, IL-4 and IL-13 levels were significantly reduced in IL-4+/13+ and IL-4+/13+ mice (p < 0.05 relative to IL-4+/13+ mice), but not in IL-4+/13+ mice, whereas IL-5 production in vvGs-immunized mice was significantly altered only in vvGs-immunized IL-4+/13+ mice (p < 0.05). The fact that IL-13Ra treatment of IL-4+/13+ vvGs-immunized mice did not reduce IL-13 levels is not unexpected because the IL-13Ra reagent blocks the function of secreted IL-13, not production of the cytokine. It is also possible that IL-13Ra administration increased IL-13 levels by altering its clearance. Furthermore, the levels of IL-4, IL-13, and eotaxin showed a statistically significant interaction between the IL-4 groups (IL-4+/13+ vs IL-4+/13−) and the IL-13 groups (ILG- vs IL-13Ra-treated) with cytokine levels for the IL-4+/13+ group significantly different from the levels expected from the combination of the IL-4 group effect and the IL-13 group effect. These data suggest that the immune responses induced by vvGs immunization require either IL-4 or IL-13 and that modulation of these responses at the time of immunization requires interference with the activity of both cytokines.

Table IV. Cytokine production in immunized mice after RSV challenge$^a$

<table>
<thead>
<tr>
<th>Priming</th>
<th>Group</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-10</th>
<th>IL-13</th>
<th>IFN-γ</th>
<th>Eotaxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>vac-lac</td>
<td>IL-4+/13+</td>
<td>13.0 ± 11.9</td>
<td>2.1 ± 3.1</td>
<td>149.1 ± 138.8</td>
<td>9.2 ± 12.5</td>
<td>269 ± 227</td>
<td>48.3 ± 47.8</td>
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<tr>
<td></td>
<td>IL-4+/13−</td>
<td>2.0 ± 1.6</td>
<td>52.9 ± 91.0</td>
<td>264.1 ± 270.8</td>
<td>17.7 ± 28.2</td>
<td>246 ± 294</td>
<td>61.4 ± 26.7</td>
</tr>
<tr>
<td></td>
<td>IL-4+/13+</td>
<td>6.9 ± 7.0</td>
<td>1.8 ± 1.5</td>
<td>236.8 ± 173.9</td>
<td>4.5 ± 5.2</td>
<td>247 ± 263</td>
<td>42.5 ± 32.5</td>
</tr>
<tr>
<td></td>
<td>IL-4+/13−</td>
<td>5.0 ± 1.6</td>
<td>7.9 ± 16.8</td>
<td>204.2 ± 155.8</td>
<td>1.4 ± 1.0</td>
<td>77 ± 82</td>
<td>57.5 ± 40.4</td>
</tr>
<tr>
<td>vvGs</td>
<td>IL-4+/13+</td>
<td>119.7 ± 39.7</td>
<td>109.6 ± 108.7</td>
<td>274.9 ± 264.4</td>
<td>352.4 ± 159.9</td>
<td>1323 ± 827</td>
<td>296.5 ± 127.6</td>
</tr>
<tr>
<td></td>
<td>IL-4+/13−</td>
<td>7.5 ± 3.7$^b$</td>
<td>46.6 ± 59.1</td>
<td>260.6 ± 314.3</td>
<td>134.8 ± 135.0$^b$</td>
<td>2339 ± 1088</td>
<td>210.9 ± 141.9</td>
</tr>
<tr>
<td></td>
<td>IL-4+/13+</td>
<td>226.4 ± 108.1$^b$</td>
<td>168.8 ± 93.0</td>
<td>299.3 ± 294.9</td>
<td>514.9 ± 206.7</td>
<td>2139 ± 1967</td>
<td>426.2 ± 183.8</td>
</tr>
<tr>
<td></td>
<td>IL-4+/13−</td>
<td>6.3 ± 5.6$^b$</td>
<td>21.9 ± 33.7$^b$</td>
<td>250.5 ± 239.1</td>
<td>88.1 ± 78.5$^b$</td>
<td>1715 ± 1080</td>
<td>151.8 ± 86.7</td>
</tr>
<tr>
<td>FI-RSV</td>
<td>IL-4+/13+</td>
<td>166.3 ± 93.2</td>
<td>157.0 ± 106.2</td>
<td>456.2 ± 363.0</td>
<td>545.2 ± 196.9</td>
<td>1377 ± 656</td>
<td>772.8 ± 264.1</td>
</tr>
<tr>
<td></td>
<td>IL-4+/13−</td>
<td>3.8 ± 2.3$^b$</td>
<td>13.0 ± 16.2$^b$</td>
<td>334.8 ± 314.3</td>
<td>112.2 ± 85.6$^b$</td>
<td>954 ± 720</td>
<td>373.0 ± 220.4$^b$</td>
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<tr>
<td></td>
<td>IL-4+/13+</td>
<td>114.2 ± 67.4</td>
<td>74.2 ± 41.7</td>
<td>398.6 ± 331.9</td>
<td>367.7 ± 150.8$^b$</td>
<td>2415 ± 1785</td>
<td>377.1 ± 233.2$^b$</td>
</tr>
<tr>
<td></td>
<td>IL-4+/13−</td>
<td>3.7 ± 4.8$^b$</td>
<td>55.2 ± 82.1$^b$</td>
<td>214.3 ± 161.8$^b$</td>
<td>51.4 ± 27.7$^b$</td>
<td>2183 ± 2225</td>
<td>192.3 ± 82.0$^b$</td>
</tr>
</tbody>
</table>

$^a$ Cytokine levels in day 4 lung supernatants were measured by specific ELISA. Data are represented as mean ± SD for 8–10 mice from 2 combined experiments. Limit of detection is 2 pg/ml for IL-4, IL-5, IL-10, and IL-13 kits and 10 pg/ml for IFN-γ and eotaxin kits.

$^b$ Statistically significantly difference relative to IL-4+/13+ mice of same priming group.
IL-4 and IL-13 inhibit viral clearance in FI-RSV-immunized mice but not in vvGs-primed mice

Peak RSV replication occurs 3–5 days after infection of mice, and virus is cleared around 8 days postinfection (39). Lack of IL-4 or IL-13 did not alter peak viral titers in vac-lac- or vvGs-primed mice (Table V). In FI-RSV-immunized mice, neutralization of IL-4 during priming has been shown to increase cytotoxic T lymphocyte killing and reduce peak viral titers (38). Therefore, the decrease in day 4 viral titers of FI-RSV-primed IL-4+13+ and IL-413− would be predicted. However, the decreased titers in IL-4+13+ FI-RSV-immunized mice are unexpected and may suggest a role for IL-13 in CTL activity. A recent report demonstrates the ability of IL-13 to synergize with GM-CSF and CD40 ligand to increase CTL activity (45). These data suggest that, whereas both IL-4 and IL-13 affect the induction of those immune responses that result in viral clearance in FI-RSV-primed mice, neither cytokine is critical to the induction of antiviral responses during vvGs immunization.

Table VI. Ab responses in immunized mice

<table>
<thead>
<tr>
<th>Priming</th>
<th>Group</th>
<th>G-Specific ELISA Ab Production</th>
<th>Neutralizing Ab Titors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgG1 titers</td>
<td>IgG2a titers</td>
</tr>
<tr>
<td>vac-lac</td>
<td>IL-4+13+</td>
<td>NDT</td>
<td>NDT</td>
</tr>
<tr>
<td></td>
<td>IL-4+13+</td>
<td>NDT</td>
<td>NDT</td>
</tr>
<tr>
<td></td>
<td>IL-4+13+</td>
<td>NDT</td>
<td>NDT</td>
</tr>
<tr>
<td></td>
<td>IL-4+13+</td>
<td>NDT</td>
<td>NDT</td>
</tr>
<tr>
<td>vvGs</td>
<td>IL-4+13+</td>
<td>10.69 ± 2.07</td>
<td>10.64 ± 2.62</td>
</tr>
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<td></td>
<td>IL-4+13+</td>
<td>9.82 ± 2.14</td>
<td>13.62 ± 1.02b</td>
</tr>
<tr>
<td></td>
<td>IL-4+13+</td>
<td>12.15 ± 0.88b</td>
<td>11.71 ± 2.53b</td>
</tr>
<tr>
<td></td>
<td>IL-4+13+</td>
<td>10.84 ± 1.14</td>
<td>13.01 ± 0.81b</td>
</tr>
<tr>
<td>FI-RSV</td>
<td>IL-4+13+</td>
<td>12.27 ± 1.50</td>
<td>9.72 ± 2.60</td>
</tr>
<tr>
<td></td>
<td>IL-4+13+</td>
<td>8.86 ± 1.37b</td>
<td>10.77 ± 2.47b</td>
</tr>
<tr>
<td></td>
<td>IL-4+13+</td>
<td>11.44 ± 2.27</td>
<td>8.98 ± 2.07</td>
</tr>
<tr>
<td></td>
<td>IL-4+13+</td>
<td>9.41 ± 1.31b</td>
<td>12.45 ± 0.36b</td>
</tr>
</tbody>
</table>

* Sera were collected from immunized mice before RSV infection. G-specific Ab titers were determined by ELISA, and data are expressed as the log_{10} of the serum dilution producing 0.100 OD_{450}. Neutralizing Ab titors were measured in an RSV plaque reduction assay. Data are expressed as the log_{10} of the serum dilution resulting in 40% plaque formation of the PBS control wells. Data represent the mean ± SD of 10 serum samples from 2 separate experiments. The limits of detection are 6.0 and 3.25 for the ELISA and neutralization assays, respectively. NDT, Not detected.

IL-4 and IL-13 alter Ag-specific Ab levels but not neutralizing Ab activity

The reduction in viral titers reported in Table V above may be due to increased titers of RSV-specific Abs. Therefore, RSV G-specific Ab and RSV-neutralizing Ab responses were measured (Table VI). As reported previously (29), vvGs immunization induced very low levels of neutralizing Ab. FI-RSV immunization resulted in higher levels of neutralizing activity that were significantly higher than vac-lac- or vvGs-primed mice (p < 0.001). Additionally, interference with IL-4 or IL-13 function did not alter neutralizing activity in most treatment groups. Only in FI-RSV-immunized IL-4+13− mice was a significant increase in neutralizing Ab titers observed (p < 0.05 relative to IL-4+13+ FI-RSV-primed mice). Therefore, the increased antiviral activity that reduced viral titers in FI-RSV-immunized IL-4+13+ and IL-4+13− mice was not due to increased neutralizing Ab activity.

Abs to RSV Gs were not detected in any vac-lac-primed mouse, whereas significant Ab responses were present in both vvGs- and FI-RSV-immunized mice (Table VI). Although unable to make IgE, the capacity of IL-4-deficient mice to produce IgG is intact (46). However, it has been reported that responses are biased to Abs with an IgG2a isotype with some decrease in IgG1 isotype. This pattern is reflected in our data in which titers of G-specific IgG2a Abs are significantly greater in IL-4+13+ and IL-4+13− mice relative to IL-4+13− mice primed with either vvGs or FI-RSV. However, only in FI-RSV-primed IL-4+13+ and IL-4+13− mice is a concomitant decrease in the IgG1 isotype observed. Furthermore, in vvGs-primed IL-4+13− mice, inhibition of IL-13 activity alone significantly increases both IgG1 and IgG2a Ab titers, underscoring a more prominent role for IL-13 in G-specific responses. Although significant differences were observed in G-specific Ab responses, these alterations did not reflect changes observed in viral titers (Table V) or lung histopathology (Table III; particularly in vvGs-immunized mice), suggesting that these parameters are correlates of altered T cell responses.

Discussion

Immunization with vvGs does not require IL-4 to induce pulmonary eosinophilia following RSV challenge (35). In contrast, FI-RSV-induced eosinophilia is IL-4 dependent (35, 38). However,
interference with IL-4 activity during FI-RSV immunization results in only a partial reversal of the type 2-associated disease profiles (35, 38). Additionally, in FI-RSV-immunized RSV-challenged mice, IL-4 is the dominant cytokine found in the lung, whereas IL-13 is expressed at higher levels in vvGs-primed mice (35). Therefore, we examined the role of IL-13 in RSV disease, alone and in combination with IL-4, in vvGs- and FI-RSV-immunized mice. The studies presented here describe the first detailed analysis of the combined contribution of these cytokines to the induction of virus-specific type 2 T cell responses and demonstrate different requirements for IL-4 and IL-13 in the induction of RSV-specific immune responses during immunization with vvGs and with FI-RSV.

Inhibition of either IL-4 or IL-13 alone reduces eosinophilia, the severity of pulmonary inflammation, and type 2 cytokine production in FI-RSV-immunized RSV-challenged mice. In contrast, both IL-4 and IL-13 activity must be blocked to diminish vvGs-induced eosinophilia and type 2 cytokine production. These data suggest that different immune mechanisms are used by vvGs and FI-RSV to initiate CD4+ T cell responses that predispose for eosinophilia and type 2 cytokine production after RSV challenge. Several factors may contribute to these different cytokine requirements by vvGs and FI-RSV. First, the effects of adjuvant during immunization must be considered, because RSV-specific immune responses are influenced quantitatively and qualitatively by adjuvant (47). Thus, the alum-precipitated FI-RSV and the live vaccinia virus vector of vvGs may activate distinct signaling pathways. Yet, it has been demonstrated that it is not merely the adjuvant that establishes eosinophil-recruiting immune responses, but rather the viral Ag also contributes to the phenotype of these responses (43). Secondly, the site of immunization may contribute to the induction of these distinct responses. Bembridge et al. (30) demonstrated that vvGs given intradermally led to pulmonary eosinophilia, but vvGs given i.p. did not. These data may suggest that the context of Ag presentation is important and that APC subpopulations influence differentiation of naive cells into Th1 or Th2 cells (48). Thirdly, antigenic load impacts the T cell differentiation process (49–52). This may play a role in the differential cytokine requirements for vvGs- and FI-RSV-induced immune response because FI-RSV, a formalin-inactivated virus, is administered at a single time point whereas immunization with vvGs, the replication of which may be detected for >2 wk postinfection (53), results in prolonged production of Ag.

The precise antigenic composition of an immunogen may influence the cytokine requirements for T cell differentiation. For example, requirements for IL-4 have been shown to vary depending on the strain of parasite used for infection, given that IL-4-deficient mice are resistant to only some strains of Leishmania major (54). It has also been shown that the stage of parasite life cycle influences cytokine requirements with IL-13 required for expulsion of Nipponstrongylus brasiliensis adult worms, but not eggs (55). Hancock et al. (43) demonstrated that responses to purified RSV fusion (F) glycoprotein might be modulated by the Th1-inducing adjuvant QS-21, whereas immune responses induced by purified RSV G were not significantly altered.

Our data underscore the complexity of the responses that result in immunopathology following viral challenge. Although interference with IL-4 or IL-13 activity resulted in concomitant decreases in IL-5 production and pulmonary eosinophilia in FI-RSV-immunized mice after RSV challenge, only inhibition of both IL-4 and IL-13 resulted in significant decreases in IL-5 production and in pulmonary eosinophilia in vvGs-immunized RSV-challenged mice. There are conflicting data describing the requirements of IL-5 for eosinophil recruitment in paramyxovirus-infected mice. Whereas Schwarze et al. (56) report a lack of eosinophilia and airway hyperresponsiveness in IL-5-deficient mice during primary infection with RSV, other groups have been unable to reproduce the findings that primary RSV infection elicits IL-5 and eosinophilia in wild-type mice. In addition, using pneumonia virus of mice (PVM) or Sendai virus (natural paramyxovirus pathogens in mice), Domachowske et al. (57) observed significant pulmonary eosinophilia in IL-5-deficient mice. Macrophage-inflammatory protein-1α (MIP-1α) is produced after RSV and PVM infection of mice (58–60). PVM infection of mice deficient in either MIP-1α or its receptor CCR1 resulted in reduced eosinophilia (59, 60). Similarly, RSV-infected MIP-1α−/− mice had significantly less pulmonary inflammation, although no eosinophils were present in either MIP-1α−/− or MIP-1α+/− mice (58). Thus, the fact that interference with either IL-4 or IL-13 function alone during vvGs immunization did not significantly alter IL-5 production or eosinophil recruitment may suggest that other inflammatory mediators such as MIP-1α may be more important in RSV-specific eosinophilic responses.

It has recently been reported that the enhanced respiratory disease in FI-RSV-immunized mice, as measured by AHR, is the result of immune complex deposition in the lung after RSV challenge, with AHR being reduced in C3- and IgG-deficient mice (61). Thus, the formation of immune complexes could contribute to the enhanced illness and pathology described in our studies. However, pulmonary histopathology was not reduced in FI-RSV-immunized RSV-challenged C3- or IgG-deficient mice (61). Additionally, interference with IL-4 or IL-13 function resulted in changes in pulmonary eosinophilia or illness without concomitant changes in Ab production. Therefore, although a role for Ab cannot be ruled out, these data suggest that T cells and their products are the factors that mediate the illness and more severe pathology observed in vvGs-immunized mice.

These studies have not defined which components of the FI-RSV vaccine-induced immune response were responsible for modifying the RSV titers after challenge. Although both IL-4−/− and IL-4−/− IL-13−/− mice immunized with FI-RSV had lower virus titers postchallenge, there is no indication that neutralizing activity or Ab levels were different between groups. Previous work from our laboratory has demonstrated that anti-IL-4 treatment of FI-RSV-immunized mice (38) or FI-RSV immunization of IL-4−/− mice resulted in decreased RSV titers and increased CTL activity (38). We and other groups have also shown that excessive amounts of IL-4 inhibit CTL function and can alter the mechanism of CTL activity (62–65) to a less efficient Fas-Fas ligand-dominated pathway (66).

Antiviral activity of eosinophils has been described for paramyxoviruses (67, 68) and for HIV-1 (69). Although direct antiviral activity of isolated eosinophils and of recombinant eosinophil RNases have been shown in vitro against RSV (67, 68), our data demonstrate high viral titers after RSV challenge of vvGs- and FI-RSV-immunized mice that develop abundant pulmonary eosinophilia. However, this is not necessarily a contradiction. In in vivo studies reported by Rosenberg and Domachowske (68), eosinophil infiltration peaks on day 3 postinfection when virus is still present in the lung and, therefore, when the antiviral activity of eosinophils may be detected. However, the kinetics of eosinophil recruitment after RSV challenge is different with peak eosinophilia occurring days 6 and 7 postchallenge (36). Peak viral titers occur at day 4 postchallenge, a time at which little pulmonary inflammation has yet occurred, and virus is cleared on days 7–9 postinfection (39). In immunized mice, viral clearance occurs 1–2 days sooner. Thus, in vvGs- or FI-RSV-immunized mice, viral clearance is
IL-13 REQUIREMENTS FOR RSV Gs-INDUCED EOSINOPHILIA

Acknowledgments

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References


