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Bronchiolitis caused by respiratory syncytial virus (RSV) infection is a major cause of hospitalization in children under 1 year of age. RSV causes common colds in older children and adults, but can cause serious disease in immunodeficient patients and the elderly. Development of effective vaccines and treatments for RSV infection is therefore a priority. Because bronchiolitis and vaccine-augmented disease are thought to be caused by exuberant T cell activation, attention has focused on the use of immunomodulators that affect T cell responses. In mice, IL-12 treatment down-regulates type 2 cytokine responses to the attachment protein G of RSV, reducing lung eosinophilia but further enhancing illness. We now show that CD8\(^+\) T cells are responsible for enhanced weight loss, whereas IL-12-activated NK cells express high levels of IFN-\(\gamma\) and inhibit lung eosinophilia without causing illness. Moreover, unlike immunocompetent mice, virus is detected in the mediastinal lymph nodes after elimination of both CD8\(^+\) T cells and NK cells. These studies show that innate immune responses to viral infections direct the pattern of subsequent specific immunity and are critical to the development of nonpathogenic antiviral effects. We speculate that IL-12 treatment might be beneficial and safe in T cell-deficient patients with RSV pneumonia.


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2 Address correspondence and reprint requests to Dr. Tracy Hussell, Room 366, Respiratory Medicine, Imperial College School of Medicine, Norfolk Place, Paddington, London W2 1PG, U.K. E-mail address: t.hussell@ic.ac.uk

3 Abbreviations used in this paper: RSV, respiratory syncytial virus; G, the attachment protein of RSV; rVV, recombinant vaccinia virus; \(\beta_2\)-microglobulin; \(\beta\)-gal, \(\beta\)-galactosidase; BAL, bronchoalveolar lavage; ELISPOT, enzyme-linked immunospot.

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IL-12-Activated NK Cells Do Not Cause Enhanced Illness

Clearance of RSV was assessed in lung homogenates at days 2 and 4 after virus challenge. Lungs were removed from four mice per group and homogenized. After centrifugation at 4000 rpm for 4 min, supernatant was titrated in doubling dilutions on HEP-2 cell monolayers in 96-well flat-bottom plates. Twenty-four hours later, monolayers were washed and incubated with peroxidase-conjugated anti-mouse IgG. Between each addition, the plates were washed six times with sterile PBS. Spots were enumerated per well after air drying on an inverted microscope. The results are expressed as spots per million.

Results

Role of NK, CD8+ T cells, and IFN-γ on the anti-eosinophilic effects of IL-12

To study the mechanisms responsible for reduced pulmonary eosinophilia after IL-12 treatment, rVV-G primed BALB/c mice were depleted of CD8+ T cells or IFN-γ at the time of RSV challenge. Lung lavage was performed 7 days after RSV challenge. As in previous studies, IL-12 treatment reduced lung eosinophilia in normal, rVV-G-primed BALB/c mice. This effect was also observed in BALB/c mice depleted of CD8+ T cells, showing that the reduced eosinophilia can be mediated by other cell types.

We have previously shown that immunocompetent C57BL/6 mice do not develop G-induced eosinophilia, but do so when CD8+ T cells are impaired (such as in CD8, β2m, and TAP-1 knockout mice) (8). To confirm that the reduction of eosinophils by IL-12 does not require CD8+ T cells, C57BL/6 mice with CD8 and TAP-1 gene deletions were examined. Similar to anti-CD8-treated BALB/c mice, IL-12 treatment resulted in a reduction of lung eosinophilia (TAP−/− and CD8−/− control in Fig. 1). Therefore, CD8+ T cells are not necessary for the reduction of lung eosinophilia by IL-12, and this effect does not depend on the background of the mouse strain.

IL-12 treatment enhances Th1 and Tc1 but inhibits Th2 cell development. We have previously shown that IFN-γ depletion induces eosinophilia to the G protein of RSV in immunocompetent C57BL/6 mice that are otherwise resistant. CD8+ T cells are the

110 IL-12-Activated NK Cells Do Not Cause Enhanced Illness

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most potent subset producing IFN-\(\gamma\) in this model. To determine whether the IL-12 reduced eosinophilia in the absence of CD8 T cells depends on IFN-\(\gamma\), BALB/c, and C57BL/6 CD8 knockout mice were additionally depleted of IFN-\(\gamma\). With IFN-\(\gamma\) depletion, IL-12 did not reduce G-induced lung eosinophilia (Fig. 1). Identical results were observed in TAP-1 knockout mice and C57BL/6 mice treated with anti-CD8 Abs (data not shown), showing that, although CD8 T cells are not necessary for inhibition of eosinophilia by IL-12, IFN-\(\gamma\) production is essential.

Because IFN-\(\gamma\) production was necessary for reduction of eosinophilia by IL-12, we sought to determine the role of a second major source of IFN-\(\gamma\), the NK cell. Unlike the role played by CD8\(^+\) T cells in C57BL/6 mice, depletion of NK cells from immunocompetent C57BL/6 mice does not result in lung eosinophilia and is consequently not affected by treatment with IL-12 (C57BL/6 + anti-NK in Fig. 1). When NK and CD8\(^+\) T cells were absent (anti-NK1.1-treated CD8 knockout mice), IL-12 treatment had no effect on eosinophilia. We were unable to perform similar studies in BALB/c mice because the DX5 Ab that recognizes NK cells in this strain does not deplete NK cells in vivo, and anti-asialo-GM\(_1\) treatment caused complications in that (as previously reported (21)) it also removed some CD45RB low activated CD8\(^+\) T cells (data not shown).

These results show that IL-12-activated NK cells inhibit lung eosinophilia and that the mechanism of inhibition requires IFN-\(\gamma\). They also show that IL-12 has no effect on eosinophilia in the absence of NK and CD8 T cells, and therefore does not operate via any other cell population (such as CD4\(^+\) T or B cells).

Role of in NK, CD8\(^+\) T cells, and IFN-\(\gamma\) in IL-12-enhanced illness

We have previously shown that IL-12 treatment enhances illness in G-primed, RSV-challenged BALB/c mice. This effect is seen regardless of the timing of IL-12 administration, but is most pronounced if IL-12 is given at the time of initial priming. Doses higher than 300 ng do not cause significantly greater weight loss, whereas lower doses, though causing less weight loss, do not significantly reduce lung eosinophilia (20). Therefore, we chose this fixed dose for the current studies. IL-12 treatment alone, in the absence of virus infection, has no effect on weight loss, showing that the IL-12 treatment itself is not causing a septic condition (data not shown).

To confirm the mechanism of IL-12-enhanced weight loss, BALB/c mice were depleted of CD8\(^+\) T cells using specific Ab. Removal of CD8\(^+\) T cells prevented IL-12-enhanced weight loss (Fig. 2A). Depletion of IFN-\(\gamma\) using specific Ab produced a similar effect (Fig. 2A). These data implicate CD8\(^+\) T cells and IFN-\(\gamma\) in causing enhanced weight loss in IL-12-treated mice. Depletion of CD8\(^+\) T cells or IFN-\(\gamma\) also prevented the more mild G-induced weight loss that occurred in the absence of IL-12 (data not shown).

The effect of IL-12 has not been described in other mouse strains. Therefore, we tested the effect of IL-12 treatment on illness in G-primed C57BL/6 mice, with or without CD8 or TAP-1 gene deletions. As in BALB/c mice, C57BL/6 mice show enhanced weight loss when treated with IL-12 (Fig. 2B, □); this was further enhanced when IL-12 was administered at both vaccination and challenge.

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Reduction of eosinophilia by IL-12 treatment in CD8 or TAP-1 knockout C57BL/6 mice is caused by IFN-\(\gamma\)-producing NK cells. Normal or knockout mice were scarified with rVV expressing RSV G-protein and treated with PBS (•, ○) or IL-12 (+, □) daily from day −2 to day +2 of sensitization. Two weeks later, all mice were challenged with RSV intranasally. Some mice were depleted of IFN-\(\gamma\) or NK cells before and during RSV challenge. Percent lung eosinophilia (\(y\)-axis) in BAL was assessed on cytrentrifuge preparations 7 days after challenge. Results from individual mice are shown. * Data that is significantly different from control treated mice in the same group (\(p < 0.01\)).

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** IL-12-augmented weight loss does not occur in the absence of CD8\(^+\) T cells or IFN-\(\gamma\). Mice were primed and challenged as described in Fig. 1, and treated with IL-12 or placebo. Day zero represents a pool of the weights recorded up to 2 days before the experiment. The percent weight loss (\(y\)-axis) is then determined from this original basal weight of the mice. The mean and SDs of at least four mice per group are shown. Similar results were obtained in two to three replicate experiments. A, Results from IL-12-treated BALB/c mice with or without anti-CD8 or anti-IFN-\(\gamma\) treatment. B, Weight loss in IL-12-treated C57BL/6 mice with or without gene deletions for TAP-1 or CD8 or anti NK1.1 treatment. C, Weight loss in immunocompetent C57BL/6 mice treated with anti-NK1.1 or anti-IFN-\(\gamma\) but not IL-12.
challenge (data not shown). However, IL-12-enhanced weight loss was not observed in CD8 or TAP-1 knockout animals (Fig. 2B, ○ and ●, respectively), showing that CD8 T cells are required for enhanced weight loss to occur.

Because NK cells also produce IFN-γ, we examined whether they contributed to enhanced weight loss. Depletion of NK cells from IL-12-treated immunocompetent C57BL/6 mice (i.e., with CD8 cells) did not prevent the enhanced weight loss (Fig. 2B, ■). CD8 or TAP-1 knockout mice also depleted of NK cells showed a similar weight loss profile to nondepleted knockout animals (data not shown). Immunocompetent G-primed C57BL/6 mice also display mild weight loss after RSV challenge even without IL-12 treatment (○ in Fig. 2C). Depletion of IFN-γ (●) or CD8+ T cells but not NK cells (■ in 2C) also abrogated this illness (Fig. 2C).

Collectively, these data indicate that CD8+ T cells and IFN-γ are responsible for IL-12-induced weight loss, whereas IL-12-enhanced NK cell responses are nonpathogenic. Regardless of immune status or IL-12 treatment, all mice had recovered by day 9 or 10 after RSV challenge.

**Antiviral effects of NK, CD8+ T cells, and IFN-γ**

As in previous studies (20), IL-12 treatment did not affect vaccinia virus replication during G protein priming. Mice were infected with recombinant vaccinia expressing β-gal, and a colorimetric assay was used to show that IL-12 did not affect expression of galactosidase in lesions from C57BL/6 or knockout mice (data not shown). In G-primed mice, RSV is cleared from the lung by day 4 after intranasal challenge. Spread outside the lung has not been observed. Fig. 3 shows that the presence of NK cells (in C57BL/6, TAP-1−/−, and CD8−/− mice) or CD8+ T cells (C57BL/6 and C57BL/6 + anti-NK1.1) results in elimination of RSV from the lung with similar kinetics to that seen in immunocompetent mice. If both subsets are absent (TAP-1−/− or CD8−/− mice treated with anti-NK1.1), virus persists in the lungs and can also be recovered from mediastinal lymph nodes. This phenomenon occurs irrespective of IL-12 treatment. Untreated animals still failed to clear the virus in the absence of both CD8 and NK cells (data not shown). Either CD8 or NK cells are therefore sufficient to eliminate virus infection.

**Role of NK, CD8+ T cells, and IFN-γ in cell recruitment in IL-12-treated mice**

Previous studies show that IL-12 treatment enhances cell recruitment in G-primed immunocompetent BALB/c mice challenged with RSV (20). This enhancement also occurred in C57BL/6 mice, and those depleted of NK cells (Table I). However, cell recruitment was unaffected or even reduced by IL-12 treatment in CD8 or TAP-1 knockout mice (Table II) or immunocompetent mice depleted of IFN-γ (see Table I). Depletion of IFN-γ or NK cells reduced total cell recruitment to the lung still further in untreated knockout mice, an effect even more evident after IL-12 treatment (Table II). These results suggest that the enhanced cell recruitment induced by IL-12 depends on the presence of CD8+ T cells.

**The effect of IL-12 treatment on T cell recruitment and cytokine production**

We have previously shown that IL-12 treatment of G-primed BALB/c mice enhances CD4+ T cell recruitment and intracellular IFN-γ expression (20). We observed similar enhancement of IFN-γ production in the present studies using ELISPOT and intracellular cytokine staining. In addition, we found that untreated C57BL/6 mice generally produced more IFN-γ-secreting cells than BALB/c mice (p = 0.043), particularly after IL-12 treatment (p = 0.023). This enhancement of IFN-γ expression by CD4+ T cells was not affected if C57BL/6 mice were depleted of NK cells (Table I).

We wished to determine whether the enhanced disease caused by IL-12-activated CD8+ T cells was accompanied by enhanced cell recruitment to the lung. In the absence of CD8+ T cells (CD8 and TAP knockout mice), IL-12 did not cause an increase in IFN-γ production by CD4 T cells as shown by intracellular staining (not significant) (Table II). The marginal increase in IFN-γ observed by ELISPOT probably represents IL-12-boosted production by NK cells, although there were only 5–10% NK cells in the BAL at the time when samples were harvested. However, by intracellular cytokine staining, we observed that more NK cells from CD8 knockout animals expressed intracellular IFN-γ after IL-12 treatment (12% ± 1.9 and 32% ± 4.3 in untreated or IL-12-treated CD8 knockout mice, respectively). As expected, depletion of IFN-γ from either immunocompetent (Table I) or CD8 knockout animals (Table II) almost abolished the capacity of the remaining cells to produce IFN-γ (p = 0.008 comparing IFN-γ production in CD8 knockouts to those additionally depleted of IFN-γ). Depletion of NK cells in CD8 knockout mice further reduced, but did not abolish, the number of cells producing IFN-γ (p = 0.027 comparing CD8−/− to CD8−/− + anti-NK1.1). IL-12 treatment reduced the frequency of IL-4- and IL-5-producing cells in all mice, except CD8 knockout mice depleted of IFN-γ or NK cells (Table II). Collectively, these results show that IL-12 treatment works through IFN-γ in reducing Th2-associated cytokines, and that NK cells are a significant source of IFN-γ in CD8 knockout animals.

**Discussion**

Our results show that IL-12-induced IFN-γ production by either NK or CD8+ cells inhibits lung eosinophilia, and that either cell type alone mediates viral clearance. The role of NK and CD8 cells is, however, quite different with respect to disease augmentation:
although IL-12-stimulated CD8\(^+\) T cells enhance the severity of weight loss during RSV challenge, IL-12-stimulated NK cells are purely protective. CD4\(^+\) T cells also produce IFN-\(\gamma\) but do not appear to contribute to enhanced illness. This is supported by the observation that IL-12-treated mice do not experience weight loss when CD8\(^+\) T cells and/or NK cells are absent.

Our current understanding of the RSV murine model is that lung eosinophilia depends on effective CD4\(^+\) T cell priming. Eosinophilia is abolished if CD4\(^+\) T cells are depleted (5), a strong CD4 T cell epitope is removed (22), or T cells are skewed toward a Th1 phenotype (25, 26). It is also possible that they act by enhancing Ag presentation, cognate interactions, and the expression of costimulatory molecules (B7-1, B7-2, MHC class I and II) by dendritic cells (26). It is also possible that they act by enhancing Ag presentation, cognate interactions, and the expression of costimulatory molecules (B7-1, B7-2, MHC class I and II) by dendritic cells and macrophages. Alternatively, macrophages and dendritic cells are susceptible to lysis by NK cells (27), which could therefore affect eosinophilia indirectly by killing APCs. This second possibility is supported by the observation that total cell recovery from the lung was reduced in CD8 knockout animals, and that IFN-\(\gamma\) levels in CD4 cells were not affected.

A recent study by Ohteki et al. (28) shows potent IFN-\(\gamma\) production by dendritic cells after IL-12 treatment in mice lacking T, B, and NK cells. This pathway does not appear to play a significant role in our studies, because IL-12 had no effect on eosinophilia in mice depleted of both CD8 and NK cells. In the OVA challenge model of airway eosinophilia, NK cells (but not NK T cells) and, to a lesser extent, \(\gamma\)6 T cells, are essential for the production of eosinophilia (29). By contrast, our present results showed that NK cells inhibit lung eosinophilia. The critical timing of NK cell depletion also differs in our models. In the case of OVA-induced eosinophilia, depletion of NK cells is only effective if performed at the time of G protein-priming activates CD8\(^+\) T cells in general so that when they are recruited to the lung during RSV challenge they respond more quickly. The same may be true for C57BL/6 mice, but further studies are needed to determine whether a MHC H-2\(^b\)-restricted CD8 epitope is present in the G protein.

Kos and Engleman (24) have shown that NK cells are required for CD8\(^+\) T cells to mature into effector cells. Therefore, NK cells may not directly influence CD4\(^+\) T cells in our model, but may do so via the induction of activated CD8\(^+\) T cells. This may also be true of other studies where NK cells are thought to affect the differentiation of CD4\(^+\) T cells into those with a Th1 phenotype (25, 26). It is also possible that they act by enhancing Ag presentation, cognate interactions, and the expression of costimulatory molecules (B7-1, B7-2, MHC class I and II) by dendritic cells and macrophages. Alternatively, macrophages and dendritic cells are susceptible to lysis by NK cells (27), which could therefore affect eosinophilia indirectly by killing APCs. This second possibility is supported by the observation that total cell recovery from the lung was reduced in CD8 knockout animals, and that IFN-\(\gamma\) levels in CD4 cells were not affected.

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cells isolated ex vivo express abundant IFN-γ but never IL-4 or IL-5 (demonstrable by intracellular staining), even in mice with intense lung eosinophilia. Similarly, NK cells expressing TCR have been described in the mouse and man (32, 33) but were not evident in our studies (NK T cells) have been described in mouse and man (32, 33).

Enhanced IL-5 (demonstrable by intracellular staining), even in mice with enhancement in RSV-infected mice (37). A recent study by Liu et al. showed that CD8 clones and lines cause severe disease in immunodeficient mice and those additionally depleted of IFN-γ or NK cells were sensitised to the G protein and treated with IL-12 (+) or placebo (-). Total cell recruitment, expression of IFN-γ in CD4+ T cells and the frequency of cytokine-producing cells were determined. The results represent the mean and SD of four to six individual mice.

IL-12-activated NK cells do not cause enhanced illness

Table II. Cellular recruitment and cytokine production in immunodeficient mice treated with IL-12*

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-12</th>
<th>Total Cells (x10^6)</th>
<th>% CD4/IFN-γ</th>
<th>No. of Cells Secreting Cytokine by ELISPOT (cells/10^6)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>IL-12</td>
<td>IFN-γ</td>
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<tr>
<td>CD8−/−</td>
<td>–</td>
<td>1.19 (0.2)</td>
<td>71.4 (6.1)</td>
<td>1000 (87)</td>
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<tr>
<td></td>
<td>+</td>
<td>0.79 (0.4)</td>
<td>62.1 (5.2)</td>
<td>1521 (121)</td>
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<tr>
<td>TAP−/−</td>
<td>–</td>
<td>1.43 (0.2)</td>
<td>59.1 (2.1)</td>
<td>1221 (16)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.57 (0.4)</td>
<td>60.2 (3.1)</td>
<td>1721 (118)</td>
</tr>
<tr>
<td>CD8−/− and anti-IFN-γ</td>
<td>–</td>
<td>0.82 (0.1)</td>
<td>43.1 (2.1)</td>
<td>52 (29)</td>
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<tr>
<td></td>
<td>+</td>
<td>0.57 (0.2)</td>
<td>40.9 (6.9)</td>
<td>96 (11)</td>
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<tr>
<td>CD8−/− and anti-NK1.1</td>
<td>–</td>
<td>0.91 (0.1)</td>
<td>22.1 (0.8)</td>
<td>632 (12)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.54 (0.1)</td>
<td>29.7 (1.4)</td>
<td>700 (69)</td>
</tr>
</tbody>
</table>

* Immunodeficient mice and those additionally depleted of IFN-γ or NK cells were sensitised to the G protein and treated with IL-12 (+) or placebo (-). Total cell recruitment, expression of IFN-γ in CD4+ T cells and the frequency of cytokine-producing cells were determined. The results represent the mean and SD of four to six individual mice.

**T** cells producing TNF-α

**References**


