IL-10 gene knockout attenuates allergen-induced airway hyperresponsiveness in C57BL/6 mice

J. PAUL JUSTICE, 1 Y. SHIBATA, 1 S. SUR, 2 J. MUSTAFA, 1 M. FAN, 1 AND M. R. VAN SCOTT 1

1Departments of Physiology and Pharmacology, Brody School of Medicine at East Carolina University, Greenville, North Carolina 27858; and 2Department of Internal Medicine, University of Texas Medical Branch, Galveston, Texas 77555

Received 10 February 2000; accepted in final form 19 September 2000

In this study, the development of airway hyperresponsiveness (AHR) following ragweed (RW) allergen challenge was compared in IL-10-deficient (IL-10-KO) and wild-type (WT) C57BL/6 mice. Adoptive transfer of mononuclear splenocytes to IL-10-KO mice at the time of allergen challenge reduced the magnitude of inflammation but reinstated AHR development in IL-10-KO mice. Treatment with recombinant murine IL-10 (rmIL-10) into the airways of BALB/c mice at the time of allergen challenge reduces IL-4 and IL-5 concentrations in BALF and subsequent recruitment of eosinophils to the airways (24, 27). Despite the reduction of Th2 inflammatory responses, instillation of IL-10 into the airways does not reduce AHR but rather increases methacholine (MCh) responsiveness (24).

In this study, the development of AHR following ragweed (RW) allergen challenge was compared in IL-10-deficient (IL-10-KO) and wild-type (WT) C57BL/6 mice to test the hypothesis that IL-10 reduces airway inflammation concurrent with an augmentation of AHR. The results demonstrate that, in the absence of IL-10, AHR to MCh exposure does not occur despite upregulation of Th2 inflammation. In addition, instillation of rmIL-10 at the time of RW challenge induces AHR development while reducing multiple inflammatory components by ~50% in IL-10-KO mice. These data support a dual role for IL-10 as an anti-inflammatory signal that independently augments AHR.

MATERIALS AND METHODS

Animals. Male C57BL/6J WT, IL-10-KO C57BL/6J, and B6.CB.17 scid [severe combined immunodeficient (SCID)] mice 6–10 wk old were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained by the Department of

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
L364

IL-10 PROMOTES AIRWAY HYPERRESPONSIVENESS

Comparative Medicine at the Brody School of Medicine at East Carolina University (Greenville, NC). All experimental procedures were approved by the Brody School of Medicine Animal Care and Use Committee.

Allergic sensitization of C57BL/6J WT and IL-10-KO mice. Mice were sensitized by two intraperitoneal injections of RW (83 μg/injection, Lot 56–129; endotoxin content <2.3 mg/ml; Greer Laboratories, Lenoir, NC) in 25 μl of Imject Alum (Pierce, Rockford, IL) and 75 μl of PBS. Injections were given 4 days apart. Seven days after the second intraperitoneal injection, mice were anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine and challenged by intratracheal administration of 8 μg of RW in PBS.

Inflammation and airway reactivity were evaluated in separate groups of animals on day 14 (i.e., 72 h after allergen challenge). The mice were anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine and challenged by intratracheal instillation of 8 μg of RW in 100 μl of PBS. For protocols calling for reconstitution of IL-10 levels in IL-10 KO mice, rmIL-10 (R&D Systems, Minneapolis, MN) was administered intratracheally on day 11 concomitant with allergen challenge. IL-10 was mixed with the RW challenge solution to achieve a dose of 1 ng per animal.

BALF cell counts and cytokine determinations. Animals were anesthetized with ketamine (90 mg/kg body wt) and xylazine (10 mg/kg body wt), and the lungs were lavaged with two 0.75-ml aliquots of PBS to evaluate inflammation. The BALF cells and supernatants were collected by centrifugation at 800 g for 5 min. The recovered cells were resuspended in a constant volume, and total cell counts were determined with a hemacytometer. Differential cell counts were performed on cytocentrifuged preparations stained with Diff-Quik (Baxter Healthcare, Miami, FL). BALF supernatants were flash-frozen in liquid nitrogen at the time of collection.

Concentrations of IL-4, IL-5, IL-13, and interferon-γ (IFN-γ) in BALF were determined using two-site immunoenzymometric assay kits (Endogen, Cambridge, MA) according to the manufacturer’s instructions. The lower limits of detection were 1 pg/ml for IL-4, 1 U/ml for IL-5, 40 pg/ml for IFN-γ, and 5 pg/ml for IL-13.

Determination of serum immunoglobulins. Serum samples were obtained by heart puncture. Serum was diluted 40-fold with buffer (PBS + 5% BSA). Total IgE was measured by sandwich ELISA using anti-IgE and biotinylated anti-IgE antibodies (PharMingen, San Diego, CA). RW-specific IgA and IgG1 were determined by applying RW (50 μg/ml) in buffer (0.1 M sodium acetate and 1.0 M NaCl, pH 6.5) to 96-well plates overnight, followed by addition of serum samples. Detection of specific immunoglobulin isotypes was performed using biotinylated detection antibodies (PharMingen).

Adaptive transfer of WT and IL-10-KO splenocytes into SCID mice. Prospective SCID recipient mice were primed with 8 μg of RW it 24 h before cell transfer. Splenocytes were isolated from RW-sensitized WT and IL-10-KO donor mice on day 12 of the immunization protocol described above. More nucleated cells were further enriched by discontinuous density gradient centrifugation (30%/60% Percoll, 2,000 g for 20 min). The SCID recipients received 5 × 10⁷ cells ip and 10⁷ splenocytes iv. Twenty-four hours following the transfer, SCID recipients were challenged with 8 μg of RW it. Control SCID mice were primed and challenged with RW, but no cells were transferred.

Evaluation of airway reactivity. Airway reactivity was assayed using three different methodologies. First, enhanced pause (Pₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑἐ떤가요. First, enhanced pause (Pₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉееےے  was measured in conscious animals using a whole body plethysmograph system (Buxco Electronics, Sharon, CT). At the time of study, the animals were placed in the plethysmograph chamber, and activity was allowed to stabilize for 10 min. Following equilibration, animals were exposed to nebulized saline for 2 min, and Pₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑᵉₑᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉᵉےے was recorded for 5 min to establish a baseline. MCh was dissolved in saline to concentrations of 0.75–48 mg/ml. Animals were exposed to increasing doses of nebulized MCh for 2 min. Pₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑᵉᡝےے was monitored continuously for 5 min following each dose of MCh.

Second, airway resistance in anesthetized animals was measured using a Mused pulmonary function monitoring system (PMS, Mumford, London, UK). Animals were anesthetized with 95 mg/kg ketamine and 5 mg/kg xylazine delivered by intraperitoneal injection. A 20-gauge cannula was inserted into the trachea and connected to a Fleisch 00000 pneumotachograph to monitor air flow. PE-60 tubing was inserted into the esophagus to the level of the thorax to measure transpulmonary pressure. Airway resistance (Raw) was calculated as the quotient of the changes in flow and pressure between isovolumetric points on inspiration and expiration. A 30-gauge cannula was inserted into the tail vein for injection of MCh (Sigma, St. Louis, MO). For doses <300 μg/kg, Raw was recorded for 1 min. For higher doses, Raw was recorded for 2 min after each injection of MCh to allow ample time for breathing to stabilize.

Third, tracheas were excised 72 h after allergen challenge to assess smooth muscle reactivity to MCh. Excessive tissue was removed, and the tracheas were cut into rings 2 mm wide. Rings were fixed to a strain gauge suspended in Krebs-Ringer solution containing (in mM) 115 NaCl, 0.4 KH₂PO₄, 2.4 K₂HPO₄, 25 NaHCO₃, 1.2 CaCl₂, and 1.2 MgCl₂ in 5% CO₂-95% O₂. After a 1-h equilibration period, resting tension was set to 1 g. KCl was added to the bathing solution to achieve a final concentration of 50 mM. This was repeated three times, and the maximal tension to 50 mM KCl was recorded. Responses to 10⁻¹⁰ to 10⁻⁴ M MCh were then measured. The protocol was performed twice for each tissue and the values were averaged. Values are reported as a percentage of the maximum tension developed on stimulation of the preparations by KCl.

Statistical analysis. Differences between groups for BALF eosinophils, lymphocytes, IL-4, IL-5, IL-13, and IFN-γ content and serum levels of IgA, IgG1, and IgE were determined by ANOVA and Tukey’s honestly significant difference post hoc test was performed to assess significant differences between treatment groups. Differences in dose-response relationships were analyzed using a general linear model for full factorial analysis of repeated measures optimized for polynomial contrast of doses (SPSS, Chicago, IL). F value was corrected for the correlation structure using the Greenhouse-Geisser epsilon factor.

RESULTS

Absence of AHR development in RW-sensitized and challenged IL-10-KO mice. AHR was first evaluated by measuring changes in Raw to intravenous MCh in anesthetized, spontaneously breathing mice. PBS-challenged WT and IL-10-KO mice exhibited identical dose-response relationships (Fig. 1A). However, following RW challenge, WT mice exhibited a 2.5-fold increase in Raw to MCh compared with PBS-challenged animals (WT and IL-10-KO) as well as RW-challenged IL-10-KO mice (Fig. 1A, P = 0.001). These observations indicated that IL-10 was involved in development of allergen-induced bronchial hyperresponsiveness to MCh.

Differences in reactivity to MCh were also observed in isolated tracheal ring preparations. In the absence of
RW sensitization and challenge (naive groups), minimal reactivity to MCh was detected in either WT or IL-10-KO mice (Fig. 1B). In addition, hyperresponsiveness to MCh was not observed in either PBS-challenged WT or IL-10-KO mice. However, tracheal rings from RW-challenged WT mice exhibited greater responsiveness to MCh than the naive and PBS-challenged groups (Fig. 1B). In contrast, responsiveness of rings from RW-sensitized and -challenged IL-10-KO mice was not different from the naive or PBS-challenged groups (Fig. 1B). These results demonstrated lack of hyperresponsiveness in IL-10 KO mice at the level of the airway smooth muscle.

$P_{enh}$ measurements in conscious unrestrained mice were performed as a noninvasive method of evaluating AHR development (Fig. 1C). Consistent with Raw, RW-challenged WT mice exhibited AHR, whereas RW-challenged IL-10-KO mice exhibited no AHR to nebulized MCh in the range of 0.75–48 mg/ml (Fig. 1C). Hamelmann et al. (9) previously demonstrated that $P_{enh}$ is a valid indicator of lower airway function, and since all three of the methods used to evaluate AHR yielded consistent results, further studies of lung function were performed by assessments of $P_{enh}$ alone.

Reconstitution of AHR by rmIL-10 in IL-10-deficient mice. One nanogram of rmIL-10 was administered concomitant with RW challenge to reconstitute IL-10 levels in the airways. In contrast to IL-10-deficient ani-
mals, AHR was observed in IL-10-KO animals treated with rmIL-10 (Fig. 2).

Development of AHR in SCID mice following adoptive transfer of WT and IL-10-KO splenocytes. Airway reactivity was evaluated 72 h after intratracheal challenge in SCID mice adaptively transferred with mononuclear splenocytes from either WT or IL-10-KO mice. SCID mice receiving WT splenocytes exhibited AHR compared with control SCID mice receiving allergen challenge alone. SCID recipients receiving splenocytes from IL-10-KO mice exhibited no AHR (Fig. 3). These results indicated that the effect of IL-10 on AHR development could be adoptively transferred.

Effect of IL-10 deficiency on Th2 inflammatory parameters. Both RW-sensitized RW-challenged WT and IL-10-KO mice exhibited significant inflammation compared with the corresponding PBS-challenged controls. However, eosinophil and lymphocyte infiltration into the airways 72 h following allergen challenge was greater in IL-10-KO mice than in WT animals (Table 1). Administration of 1 ng rmIL-10 at the time of allergen challenge reduced inflammation in IL-10-KO mice by ~50% but had minimal effects in WT mice.

Twenty-four hours following RW challenge, both IL-10-KO and WT mice exhibited marked elevations in the levels of IL-4 and IL-5 in the BALF compared with PBS-challenged mice (Table 2). The concentrations of IL-4 and IL-5 in BALF following RW challenge were 40-fold and 3-fold higher, respectively, in IL-10-KO mice compared with similarly treated WT mice. Administration of rmIL-10 (1 ng) in IL-10-KO mice decreased both IL-4 and IL-5 levels in knockout mice, whereas no effect was observed in WT animals. Measurements of IL-13 levels in BALF yielded similar results. Twenty-four hours after RW challenge, BALF from IL-10-KO con-

Table 1. BALF cellularity 72 h after challenge

<table>
<thead>
<tr>
<th>Condition</th>
<th>Eosinophils, 10^4/ml</th>
<th>Lymphocytes, 10^4/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT/it PBS</td>
<td>0.27 ± 0.13</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>WT/it RW</td>
<td>30.0 ± 11.8</td>
<td>1.3 ± 0.40</td>
</tr>
<tr>
<td>WT/it RW + 1 ng rmIL-10</td>
<td>20.0 ± 4.0</td>
<td>0.60 ± 0.10</td>
</tr>
<tr>
<td>IL-10-KO/it PBS</td>
<td>0.38 ± 0.17</td>
<td>0.49 ± 0.13</td>
</tr>
<tr>
<td>IL-10-KO/it RW</td>
<td>97.0 ± 15.0</td>
<td>6.8 ± 0.09</td>
</tr>
<tr>
<td>IL-10-KO/it RW + 1 ng rmIL-10</td>
<td>43.3 ± 10.0</td>
<td>2.5 ± 0.74</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6. Total numbers of cells recovered were analyzed by visual counts with a hemacytometer. Differential counts were obtained by Wright-Giemsa staining of cytosin samples and visual identification and counting. Animals were sensitized with ragweed (RW) and challenged with either PBS (WT/it PBS; IL-10-KO/it PBS), RW (WT/it RW; IL-10-KO/it RW), or RW + 1 ng of recombinant murine interleukin-10 (rmIL-10) (WT/it RW + 1 ng rmIL-10; IL-10-KO/it RW + 1 ng rmIL-10). BALF, bronchoalveolar lavage fluid; WT, wild type; IL, interleukin; IL-10-KO, IL-10-deficient; it, intratracheal. †Significant difference from PBS-challenged animals of the same strain. ‡Significant difference from similarly treated WT. §Significant difference from RW-challenged animals of the same strain. P < 0.05.

Table 2. Cytokine levels in BALF 24 h after challenge

<table>
<thead>
<tr>
<th>Condition</th>
<th>IL-4, pg/ml</th>
<th>IL-5, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT/it PBS</td>
<td>0.98 ± 0.24</td>
<td>12 ± 7</td>
</tr>
<tr>
<td>WT/it RW</td>
<td>22.9 ± 7.6*</td>
<td>25 ± 4*</td>
</tr>
<tr>
<td>WT/it RW + 1 ng rmIL-10</td>
<td>26.9 ± 9.8</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>IL-10-KO/it PBS</td>
<td>0.0 ± 0.2</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>IL-10-KO/it RW</td>
<td>932.5 ± 208.0*</td>
<td>76 ± 14*</td>
</tr>
<tr>
<td>IL-10-KO/it RW + 1 ng rmIL-10</td>
<td>177.7 ± 80.7*</td>
<td>41 ± 4*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6. Twenty-four hours after challenge, mice were anesthetized, the tracheas were cannulated, and lungs were lavaged with two 0.75-ml aliquots of saline. Lung lavages were centrifuged to separate cells from supernatant. Supernatants were assayed for Th2 cytokine concentrations. *Significant difference from PBS-challenged animals of the same strain. †Significant difference from similarly treated WT. §Significant difference from RW-challenged animals of the same strain. P < 0.05.
The inflammatory parameters measured in this study (i.e., BALF levels of IL-4, IL-5, IL-13, eosinophils, lymphocytes and serum levels of IgG1, IgA, and IgE) have been associated with AHR development. Specifically, Garlisi et al. (5) and Hogan et al. (10) independently demonstrated a correlation between airway eosinophilia and AHR in mice. Wills-Karp et al. (25) and Grunig et al. (8) have both demonstrated a role for IL-13 in allergen-induced AHR development. It has been reported that IL-5-deficient mice do not develop AHR (4) and overexpression of IL-5 in the airways of nonsensitized nonchallenged mice is sufficient to induce AHR (12). Shi and colleagues (20) have shown that inhalation of IL-4 by asthmatic individuals induces AHR. Consistent with the effect of IL-4 on B cells, the production of several immunoglobulins (IgE, IgA, and IgG1) has been shown to be elevated in the airways and serum of asthmatic individuals (17). Due to the significant elevation in all of these factors in IL-10-deficient mice, the data presented in this study suggest that, while these factors may be associated with AHR development, alone they cannot precipitate AHR development. Although the activation state of eosinophils was not addressed in these studies (as well as in this report), an earlier study demonstrated that the levels of eosinophil peroxidase and leukotriene C4 were significantly elevated within the airways of IL-10-deficient mice compared with those in WT control mice (14). Therefore, the absence of AHR development in IL-10-deficient mice also does not appear to be due to the lack of activated eosinophils within the airways of these mice.

The mechanism by which IL-10 facilitates development of AHR is not known, but the evidence indicates that it acts in concert with one or more inflammatory factors to increase smooth muscle reactivity. The following observations support this theory. First, allergic sensitization and challenge are required to observe the effect of IL-10. In WT animals, IL-10 alone has no effect on airway responses to MCh (24). Second, reactivity to MCh is similar in naive WT and IL-10-deficient mice, indicating that IL-10 does not directly stimulate airway constriction. Third, a strong correlation exists between Th2 inflammation and AHR in IL-10-deficient animals, but that correlation breaks down in the absence of IL-10. Fourth, a similar degree of hyperresponsiveness is observed in WT mice regardless of whether the readout is Raw, Pmv, or tracheal ring contraction. AHR in this model is therefore a reflection of smooth muscle activation. Fifth, as discussed above, IL-10 production by Th2 cells is critical to AHR development in this model, which indicates the need for

Table 3. Serum levels of Th2 immunoglobulins 72 h after challenge

<table>
<thead>
<tr>
<th></th>
<th>Ragweed-Specific IgA, A550</th>
<th>Ragweed-Specific IgG1, A550</th>
<th>Total IgE, μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT/tt PBS</td>
<td>0.4 ± 0.7</td>
<td>0.58 ± 0.16</td>
<td>0.17 ± 0.06</td>
</tr>
<tr>
<td>WT/tt RW</td>
<td>0.45 ± 0.07</td>
<td>0.41 ± 0.09</td>
<td>0.25 ± 0.08</td>
</tr>
<tr>
<td>IL-10-KO/tt PBS</td>
<td>0.47 ± 0.1</td>
<td>0.54 ± 0.12</td>
<td>0.91 ± 0.15†</td>
</tr>
<tr>
<td>IL-10-KO/tt RW</td>
<td>0.91 ± 0.13*</td>
<td>1.17 ± 0.21*</td>
<td>1.68 ± 0.23*</td>
</tr>
</tbody>
</table>

Values are means ± SE; *n ≥ 6. Mice were anesthetized and blood samples were taken by heart puncture. A550, absorbance at 550 nm. †Significant difference from PBS-challenged animals of the same strain. *Significant difference from similarly treated WT. P < 0.05.
other coincident lymphocyte-derived signals in the process. Thus AHR in this model involves the induction of smooth muscle hyperresponsiveness.

Shibata and colleagues (21) previously demonstrated that splenocytes from RW-sensitized WT C57BL/6 mice produce IL-4, IL-5, and IL-10 but not IFN-γ when stimulated with RW in vitro. Depletion of the CD4+ cells by complement-dependent cell lysis reduces IL-4 and IL-5 production by >80% and IL-10 production by 94%, indicating that RW-specific Th2 cells are the major source of IL-10 in isolated splenocytes. These observations, in combination with the results of the adoptive transfer experiments in the present study, indicate that IL-10 production by Th2 lymphocytes is required for development of AHR in this model.

In summary, current evidence indicates that IL-10 downregulates allergen-induced Th2 inflammation within murine airways, yet is critical for the development of AHR in this model. While the mechanism is unclear, it is likely that IL-10 functions in concert with one or more inflammatory factors to precipitate AHR via an indirect effect on airway smooth muscle.

We thank Dr. Don Holbert for assistance with statistical analysis. This project was supported in part by National Heart, Lung, and Blood Institute Grant 5RO1-HL-50049 and an East Carolina University Brody Institute of School of Medicine Faculty Research Grant.

REFERENCES


5. Garlisi CG, Falcone A, Hey JA, Paster TM, Fernandez X, Mottonen M, Isomaki P, Saario R, Toivanen P, Punnonen K, Lee JJ, McGarry MP, Farmer SC, Denzler KL, Larson KA, Ullrich SE. Interleukin-5-producing CD41 T cells are the major source of IL-10 in isolated splenocytes. These cells by complement-dependent cell lysis reduces IL-4 and IL-5 production by >80% and IL-10 production by 94%, indicating that RW-specific Th2 cells are the major source of IL-10 in isolated splenocytes. These observations, in combination with the results of the adoptive transfer experiments in the present study, indicate that IL-10 production by Th2 lymphocytes is required for development of AHR in this model.


