Ragweed-induced expression of GATA-3, IL-4, and IL-5 by eosinophils in the lungs of allergic C57BL/6J mice

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ASTHMA IS CHARACTERIZED by pulmonary inflammation, reversible airflow obstruction, and airway hyperresponsiveness (1, 2, 7, 10). The asthma phenotype evolves from a complex cascade of immunological responses to aeroallergens, which leads to leukocyte recruitment to the airways and subsequent remodeling of the inflamed tissue (11, 14, 18). The recruitment of eosinophils is associated with increased production of the Th2 cytokines [interleukin (IL)-4 and IL-5], and ectopic expression within the airways of these two factors has been shown to induce eosinophil recruitment (21, 31). The biological role of IL-5 is limited to control of eosinophil proliferation, differentiation, activation, and survival (6, 9, 23, 34, 37, 43). In contrast, the biological significance of IL-4 is broader and includes stimulation of CD4+ T-cell differentiation and proliferation (40), B-cell class switching (25), and the induction of eosinophil-specific chemokine secretion (38). Thus IL-4 and IL-5 are key factors in asthma pathogenesis.

Studies suggest that CD4+ T cells are the primary source of IL-4 and IL-5 in allergic inflammation (1, 15, 17, 32). However, there is evidence that eosinophils are capable of providing positive feedback to the inflammatory response via production and secretion of these cytokines (16). In murine lungs infected with Schistosoma mansoni, eosinophils are reported to be the dominant source of IL-4 and IL-5 and to cause prolonged eosinophil recruitment and continued granuloma formation (33). To date, a similar role for eosinophils in the development of allergic asthma has not been established.

Transcription of both the IL-4 and IL-5 genes is regulated in part by the helix-loop-helix transcription factor GATA-3 (3, 30, 46). In murine T lymphocytes, GATA-3 is a major positive regulatory factor for IL-4 production (46, 47), and GATA-3 is required for IL-5 expression in T cells (45, 46). Ouyang and colleagues (27) recently established that GATA-3 is part of a master switch for Th2 commitment in T cells. This response to GATA-3 is independent of STAT-6, which indicates that the effect is not mediated by the IL-4/STAT-6 signaling pathway.

Evidence supporting a role for GATA-3 in the development of asthma is accumulating. Biopsies from human asthmatic patients have demonstrated a significant increase in the number of GATA-3 mRNA-positive cells in the airways compared with tissue from nonasthmatic individuals (26). Using a murine model of asthma, Zhang and colleagues (45) demonstrated that T-cell-specific expression of a GATA-3 dominant-negative mutant during the sensitization and challenge processes attenuated allergen-induced airway eosino-
philia. These observations together with studies correlating eosinophilic inflammation with Th2 cytokine production (24) suggest that GATA-3 plays a key role in eosinophilic inflammation of the airways.

In the current study, ragweed (RW)-sensitized C57BL/6J mice were used to define the pattern of GATA-3 expression in the lungs over a 72-h period after allergen challenge. Maximum expression of GATA-3 was observed 12–24 h after RW challenge and corresponded with the appearance of IL-4 and IL-5 in bronchoalveolar lavage (BAL) fluid. Greater than 95% of the GATA-3 mRNA expressing cells in the lungs were eosinophils. Furthermore, in vitro stimulation of RW-primed lung eosinophils demonstrated transcriptional dependence for Th2 cytokine production.

MATERIALS AND METHODS

Animals. Male 6–10-wk-old C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). NJ.1638 mice were obtained from J. Lee and N. Lee (Mayo Clinic, Scottsdale, AZ). All mice were maintained by the Department of Comparative Medicine at the Brody School of Medicine at East Carolina University. Animals were fed Purina laboratory chow and sterilized tap water ad libitum. All studies were approved by the Animal Welfare and Use Committee at the Brody School of Medicine.

Sensitization and challenge of animals. A 14-day protocol was used. Mice were sensitized by intraperitoneal injection of RW on days 0 and 4 (80 μg/injection, lot 56-29; endotoxin content <2.3 ng/mg RW; Greer Laboratories, Lenoir, NC). Sensitization solution consisted of 1 mg of RW in 1 ml of 0.9% NaCl (Baxter, Deerfield, IL) plus 333 μl of Imject alum (Pierce, Rockford, IL). On day 11, animals were anesthetized with ketamine (90 mg/kg body wt) and xylazine (10 mg/kg body wt) and challenged by intratracheal administration of RW (10 μg of RW in 0.1 ml of 0.9% NaCl).

Lung lavage, tissue fixation, and sectioning. Lungs were lavaged with a single 1.0-ml aliquot of PBS (GIBCO-BRL, Grand Island, NY). Samples were centrifuged at 2,000 rpm for 5 min, and the supernatants were removed and stored at −80°C. Lungs were removed, infused with 4% paraformaldehyde (in PBS) for 30 min, rinsed with PBS, and immersed in 0.5 M sucrose (in PBS) overnight at 4°C. Lungs were inflated with and embedded in optimal cutting temperature compound (Sakura FineTek, Torrance, CA) and stored at −80°C.

Cytokine determination. Concentrations of IL-4 and IL-5 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 both IL-4 and IL-5.

In situ hybridization. In situ hybridization was performed as described by Helton and colleagues (13). Lung sections (10–20 μm) were placed on Superfrost Plus microscope slides (VWR Scientific, West Chester, PA), acetylated using 0.25% acetic acid anhydride, and dehydrated in ethanol. Sections were hybridized using synthetic DNA oligonucleotides with the following sequences: GATA-3 antisense (5′-CTCGGCT-GTGGCTGCGCCCT-3′) and GATA-3 sense (5′-AGGGCAGG-GACGACAGCGGAG-3′). Oligonucleotides were synthesized by GIBCO-BRL and labeled on the 3′ end using α-35S-labeled dATP (1,075 Ci/mmol, New England Nuclear, Boston, MA) and terminal deoxynucleotidyl transferase (Boehringer Mannheim, Indianapolis, IN) to a specific activity of ~0.9–1.8 μCi/μmol. Sections were incubated with 1 × 106 counts/min of α-35S-labeled dATP per 25 μl of hybridization buffer for 12 h at 37°C. Slides were dipped in Kodak NTB-3 emulsion (Sigma Chemical, St. Louis, MO), exposed for 72 h, and processed using Dektol and Kodak fixer (Sigma). Quantification of the mRNA hybridization signal was performed using NIH Image 1.60 software (W. Rasband, National Institute of Mental Health) and a Macintosh G3 computer. Integrated density for GATA-3 mRNA expression in the lung sections was determined using the NIH imager program. The area of the cell being scanned and the mean density of the scanned region were multiplied to yield the integrated density. Numbers of GATA-3 mRNA-expressing cells were normalized per millimeter of basement membrane for airways and blood vessels. Enumeration of GATA-3 mRNA values for the alveolar region were normalized per square millimeter of distal lung area.

Fig. 1. In situ hybridization for GATA-3 mRNA in murine lung tissue. GATA-3 antisense RNA probe was hybridized to lung-tissue sections from C57BL/6J mice that were sensitized to ragweed (RW) and challenged with either PBS (A) or RW (B). Intense black deposits represent GATA-3-positive cells visualized by nuclear tract bulk staining of 35S-labeled antisense probes. Arrows in the PBS-challenged group indicate GATA-3 mRNA-positive cells in the alveolar region. Sections from 8–12 mice per group were analyzed; representative photographs from each group are shown.
Table 1. Time course of GATA-3 mRNA positive cell infiltration of the lungs

<table>
<thead>
<tr>
<th>Time After Challenge, h</th>
<th>Blood Vessel, cells/mm basement membrane</th>
<th>Alveoli, cells/mm²</th>
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<tbody>
<tr>
<td>0</td>
<td>PBS ND ND</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>PBS 27 ± 6‡</td>
<td>ND</td>
</tr>
<tr>
<td>24</td>
<td>PBS 39 ± 4‡</td>
<td>61 ± 4‡</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 3 mice for PBS groups and n = 5 mice for ragweed-challenged group. Lungs were removed and fixed in paraformaldehyde at indicated times after ragweed challenge. Lung sections were hybridized with 35S-labelled GATA-3 antisense oligonucleotide probe. Hybridization of probe was visualized by autoradiography and numbers of GATA-3 positive cells were quantified by microscopy. PBS, phosphate-buffered saline; ND, not detectable. 

In vitro stimulation of eosinophils. Purified peripheral blood and lung eosinophils were resuspended in RPMI-1640 (GIBCO-BRL) and 5% fetal calf serum (GIBCO-BRL) at a cell density of 1 x 10⁶ cells/ml for both peripheral blood and lung eosinophils. The cells were stimulated with 10⁻⁷ M phorbol 12-myristate 13-acetate (PMA) and 10⁻⁷ M A-23187 (Sigma). Actinomycin D was added at a concentration of 10⁻⁶ M when indicated. Samples were stimulated in 96-well plates at 37°C for 30 min, 1 h, and 16 h.

Western blot analysis of GATA-3 and nuclear factor-κB. Purified peripheral blood eosinophils from NJ.1638 mice were collected by centrifugation and resuspended in extraction buffer that contained 10 mM Tris-HCl, pH 7.4, 0.32 M sucrose, 2 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 μg/ml pepstatin A. The cells were disrupted by sonication on ice twice for 20 s. Lysates were centrifuged for 1 h at 100,000 g. The supernatants were collected and protein concentrations were determined (Bio-Rad). Proteins were separated by 12% SDS-PAGE and transferred to 0.45-μm polyvinylidene difluoride membranes (Immobilon). GATA-3 and the p50 subunit of nuclear factor (NF)-κB were identified by immunoblotting with goat anti-GATA-3 Ab and goat anti-NF-κB p50 Ab (both from Santa Cruz Biotechnology), respectively, followed by horseradish peroxidase-conjugated donkey anti-goat IgG Ab. Immunoreactive protein complexes were detected by using enhanced chemiluminescence detection reagents.

Statistical analysis. All experimental values within the individual data sets were compared by ANOVA and Tukey’s HSD post hoc test (P < 0.05). Values presented in this report represent means ± SE.
RESULTS

GATA-3 mRNA-positive cells are recruited to the airways after RW allergen challenge. In situ hybridization revealed that GATA-3 mRNA expression increased over the 24 h after RW challenge. The GATA-3-positive cells were located along the basement membrane of the airways and blood vessels and were broadly dispersed in the alveolar region (Fig. 1). A significant increase in the number of GATA-3-positive cells was observed in the airways and blood vessels 12 h after RW challenge (Table 1). In contrast, an increase in expression in the alveolar region was delayed. In sensitized mice, a significant increase in the number of GATA-3 mRNA-positive cells was not observed until 24 h after RW challenge. Interestingly, recruitment to the airways and blood vessels was only observed after the airway challenge, whereas sensitization alone elicited expression of GATA-3 in the alveolar region. Kinetics of the GATA-3 mRNA-positive cell distribution reflected the pattern of eosinophil recruitment beginning with extravasation through the blood vessels at 12 h, localization around vessels and large airways by 24 h, and a minor increase in alveolar eosinophilia at these time points.

Expression of GATA-3 mRNA and GATA-3 protein colocalizes with MBP-positive cells. Combined in situ hybridization for GATA-3 mRNA and immunofluorescence for MBP protein demonstrated that GATA-3 mRNA colocalized with MBP in the airways (Fig. 2). Of the GATA-3 mRNA-positive cells, >95% costained for MBP. Colocalization was also observed at the protein level (Fig. 3). These results indicate that the majority of cells expressing GATA-3 in the airways after allergen challenge are eosinophils. To confirm this finding, peripheral blood eosinophils from nonsensitized and nonchallenged NJ.1638 mice were isolated, and Western blot analysis was performed (Fig. 4). Expression of GATA-3 and the p50 subunit of NF-κB, the upstream modulator of GATA-3, were detected.

Changes in GATA-3 mRNA expression at the cellular level were assessed by measuring the integrated density of silver grains over individual cells in autoradiographs of lung sections after in situ hybridization. The integrated density was significantly elevated 24 h

![Image of autoradiograph with GATA-3 expression](image_url)

![Image of Western blot analysis](image_url)

Fig. 3. Colocalization of GATA-3 protein and MBP in lung tissue after RW challenge. Expression of GATA-3 and MBP was analyzed by dual immunocytochemistry. GATA-3 protein was visualized by diaminobenzidine staining, and MBP expression was visualized using a Cy5-labeled secondary antibody (inset). Figure is representative of 4 animals.

Fig. 4. Expression of GATA-3 and nuclear factor-κB (NF-κB) proteins by peripheral blood eosinophils. Peripheral blood eosinophils from NJ.1638 mice were isolated and the cytosol fraction was assessed for GATA-3 (A) and NF-κB (B) expression by Western blot analysis. Procedure was repeated on three separate occasions, and a representative Western blot is shown.
Fig. 5. Increased cellular expression of GATA-3 mRNA 24 h after RW challenge. Amount of GATA-3 mRNA expression per cell was analyzed by determining the integrated density for individual cells within the submucosa of the airways. At least 100 cells per section were assessed. Only sections containing ≥3 airways were analyzed; 3–6 mice were evaluated at each time point. Values represent means ± SE; *P < 0.05.

after RW challenge (Fig. 5). By 72 h postchallenge, GATA-3 expression returned to baseline levels. The increased integrated density was indicative of either increased synthesis or decreased degradation of GATA-3 mRNA after the allergen challenge.

IL-4 and IL-5 levels in BAL fluid increase after RW challenge. IL-4 and IL-5 concentrations in the BAL fluid of RW-challenged mice were increased significantly 24 h postchallenge (Table 2). Comparison of the IL-4 and IL-5 levels with the expression of GATA-3 (shown in Fig. 5) indicates that the three parameters changed in parallel, and that eosinophils were a probable source of IL-4 and IL-5 in the lungs after allergen challenge.

Nonspecific activation of airway and peripheral blood eosinophils in vitro leads to de novo production of IL-4 and IL-5. The ability of eosinophils to produce the Th2 cytokines IL-4 and IL-5 was assessed in vitro using cells isolated from peripheral blood of NJ.1638 mice and the lungs of RW-challenged wild-type mice.

Table 2. Effect of ragweed challenge on Th2 cytokine concentration in bronchoalveolar lavage fluid

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intraperitoneal ragweed, intratracheal PBS</th>
<th>Intraperitoneal ragweed, intratracheal ragweed</th>
</tr>
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<tbody>
<tr>
<td>IL-4 (pg/ml)</td>
<td>2.0 ± 0.6</td>
<td>69.0 ± 7.0</td>
</tr>
<tr>
<td>IL-5 (pg/ml)</td>
<td>1.8 ± 0.4</td>
<td>49.0 ± 10.0</td>
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Values are means ± SE. Lungs were lavaged with 1.5 ml of ice-cold PBS 24 h after intratracheal ragweed challenge and were stored at ~80°C. Lavage fluid was not concentrated. Concentrations of interleukin-4 and -5 ([IL-4] and [IL-5], respectively) were analyzed by sandwich ELISA. *P < 0.05, significant difference from PBS-challenged mice.

Incubation with PMA and A-23187 for 18 h induced the release of both IL-4 (Fig. 6A) and IL-5 (Fig. 6B). Release of preformed IL-4 and IL-5 was unlikely, because exposure of the cells to PMA or A-23187 for 30–120 min did not lead to the release of these cytokines (data not shown). In addition, incubation of the cells with the RNA polymerase II inhibitor actinomycin D abolished IL-4 and IL-5 release, which suggests that cytokine production occurred de novo.
production is a function of transcriptional activation of these Th2 cytokine genes.

**DISCUSSION**

The results of this study provide evidence that allergen challenge induces expression of GATA-3 and GATA-3-responsive genes in pulmonary eosinophils. A role for GATA-3 in the development of asthma was initially demonstrated by Nakamura and colleagues (26), who reported a significant elevation of GATA-3 mRNA in lung tissue from asthma patients compared with lung tissue from nonasthmatic subjects. Further evidence of a role for GATA-3 in allergen-induced lung inflammation was provided by Zhang and colleagues (45), who found a dominant negative mutant expressed for GATA-3 in mice and demonstrated that the pulmonary response to allergen challenge was attenuated. Expression of the GATA-3 mutant was restricted to T cells and was induced during both the sensitization and airway-challenge periods. The results of these studies were therefore consistent with the known importance of Th2 lymphocytes in asthma and the role of GATA-3 in the growth of this cell type. The results of the present study indicate that GATA-3 plays a broader role in allergic inflammation. Expression by pulmonary eosinophils and the capacity of that cell type to produce IL-5 provide a mechanism for positive feedback in the inflammatory response at the site of the allergen challenge.

There is considerable evidence that GATA-3 regulates IL-5 gene expression in human and murine T cells. For example, expression of IL-5 in EL-4 and myeloid cells requires the presence of GATA-3 (3, 36, 44), mutation of the GATA-3 binding site in EL-4 cells abolishes antigen or cAMP-induced IL-5 production (44), GATA-3 binds to the IL-5C promoter site in EL-4 cells (20), and retroviral infection with GATA-3 cDNA enhances IL-5 production in developing Th1 T-cell clones (8). The role of GATA-3 in the regulation of IL-4 expression is more controversial. In contrast to IL-5, early studies of IL-4 expression demonstrated that ectopic expression of GATA-3 did not induce IL-4 production (46), and GATA-3 antisense oligonucleotides did not inhibit IL-4 promoter activity (30). However, recent studies suggest that GATA-3 plays a more direct role in IL-4 transcription. Retroviral expression of GATA-3 in committed Th1 cells enhances IL-4 production (8), and expression of a dominant negative mutant for GATA-3 inhibits allergen-induced IL-4 production (45). In addition, introduction of GATA-3 into STAT6-deficient T cells induces DNase I hypersensitive sites in the IL-4 locus and triggers STAT6-independent IL-4 production, which is a novel concept in the paradigm of IL-4 gene regulation (27). Taken together, the evidence indicates that GATA-3 is an important factor in the regulation of both IL-4 and IL-5.

The molecular mechanism by which GATA-3 enables the polarization of T-cell subsets into the Th2 category has also been described by Ouyang and colleagues (28), who observed that developing Th1 cells could inhibit GATA-3 expression through an IL-12-dependent mechanism. Conversely, GATA-3 expression was found to inhibit IL-12 signaling in T cells. These findings have been further substantiated by the demonstration that ectopic expression of GATA-3 in developing Th1 cells inhibits interferon-γ production and commitment to the Th1 phenotype (8). Collectively, these findings indicate that the role of GATA-3 in both the upregulation and downregulation of gene expression is critical to the development of a Th2 immune response.

The present study demonstrates that murine tissue eosinophils express GATA-3 after allergic sensitization and challenge. A previous report by Zon and colleagues (48) demonstrated GATA-3 expression in the HL-60-C15 and 3+C−5 sublines of the human eosinophil-like cell line, HL-60. GATA-3 has also been shown to downregulate the expression of genes in HL-60 cells (35). The finding that eosinophils express GATA-3 in vivo is therefore a logical outcome of the present study.

The observation that GATA-3 is expressed and is functionally active in eosinophils has important implications in the progression and self-perpetuation of allergic reactions that lead to asthma. Production of IL-5 by tissue eosinophils should contribute to a microenvironment that supports the localization, longevity, activation, and proliferation of these cells, perhaps independent of Th2 cells. However, its importance in human asthma is unknown. The acute sensitization and challenge protocol used in this study results in a high degree of eosinophil accumulation in the airways with minimal recruitment of lymphocytes. Therefore, the role that eosinophil GATA-3 expression plays in allergic airway inflammation is likely to be amplified in this model. In contrast, chronic allergic airway inflammation such as is observed in human asthma is characterized by significant accumulation of lymphocytes in the airways. Nakamura and colleagues (26) observed that 60–90% of the GATA-3-positive cells in human lung tissue were CD3-positive T cells; however, <15% of the GATA-3-positive cells were identified as eosinophils. Thus, in human asthma, eosinophil expression of GATA-3-responsive genes is not likely to be the primary source of proinflammatory cytokines leading to airway inflammation but rather may provide a redundant source of Th2 cytokine to support the chronic inflammatory process.

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