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Inhibition of Allergic Inflammation in a Murine Model of Asthma by Expression of a Dominant-Negative Mutant of GATA-3

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Summary

The cytokines IL-4, IL-5, and IL-13, secreted by Th2 cells, have distinct functions in the pathogenesis of asthma. We have previously shown that the transcription factor GATA-3 is expressed in Th2 but not Th1 cells. However, it was unclear whether GATA-3 controls the expression of all Th2 cytokines. Expression of a dominant-negative mutant of GATA-3 in mice in a T cell–specific fashion led to a reduction in the levels of all the Th2 cytokines IL-4, IL-5, and IL-13. Airway eosinophilia, mucus production, and IgE synthesis, all key features of asthma, were severely attenuated in the transgenic mice. Thus, targeting GATA-3 activity alone is sufficient to blunt Th2 responses in vivo, thereby establishing GATA-3 as a potential therapeutic target in the treatment of asthma and allergic diseases.

Introduction

Asthma is a debilitating disease of the lower airways that affects millions of people worldwide. Both the prevalence and severity of asthma is increasing at an alarming rate in developed countries, including the United States. Bronchoalveolar lavage (BAL) and biopsy of patients with mild to moderate asthma has provided impressive evidence for complex airway inflammation in asthma (Holgate, 1997; Busse, 1998; Gleich et al., 1988). The hallmarks of allergic asthma are infiltration of eosinophils into the bronchial wall and lumen, elevated serum IgE levels, mucus production in the airways, and airway hyperresponsiveness (AHR) to specific and nonspecific stimuli (Holgate, 1997; Busse, 1998; Gleich et al., 1988; Wills-Karp, 1999). The most striking and consistent pathophysiology in asthma is damage to the bronchial epithelium caused by the cytotoxic proteins released by the infiltrated eosinophils (de Monchy et al., 1985; Gleich et al., 1988; Frick et al., 1989). Studies of human asthma as well as of animal models of allergic inflammation/asthma highlight a critical role for $CD4^+$ T helper 2 (Th2) cells as orchestrators of this disease (Robinson et al., 1992; Maggi and Romagnani, 1994; Gleich and Kita, 1997; Umetsu and DeKruyff, 1997).

The effector functions of Th2 cells are mediated by the cytokines they secrete—IL-4, IL-5, and IL-13. Recent work utilizing mice with specific genetic deficiencies show nonredundant roles for each of these cytokines in the pathophysiology of asthma. Although IL-4 and IL-13 share the IL-4Rα subunit and transduce signals through Stat6, the two cytokines appear to have distinct roles in the disease process. IL-4 is the key Th2-differentiating cytokine (Kopf et al., 1993; Seder and Paul, 1994; Morel and Oriss, 1998). The Th2 cells, in turn, produce more IL-4 together with IL-5 and IL-13. Using IL-4Rα$^{-/-}$ mice, we have recently shown that airway mucus production is critically dependent on the IL-4Rα chain but not on IL-4 or IL-5 (Cohn et al., 1999a). A specific inhibitor of IL-13 was recently shown to cause severe inhibition of mucus production and AHR in a murine model of asthma, substantiating our studies in IL-4Rα$^{-/-}$ mice (Grunig et al., 1998; Wills-Karp et al., 1998). Use of the IL-13 inhibitor, however, did not reduce airway eosinophilia in antigen-challenged animals (Wills-Karp et al., 1998). The cytokine that is most intimately associated with eosinophil biology is IL-5 (Coffman et al., 1989; Dent et al., 1990; Foster et al., 1996; Kopf et al., 1996; Cohn et al., 1999a). It not only regulates the proliferation, differentiation, and activation of eosinophils, it also provides an essential signal for the rapid mobilization of eosinophils from the bone marrow and cooperates with chemokines such as eotaxin in the homing of eosinophils to sites of allergic inflammation (Lopez et al., 1988; Yamaguchi et al., 1990; Collins et al., 1995). Thus, IL-4, IL-5, and IL-13 all have distinct roles in the pathogenesis of asthma.

We and others have previously shown that the transcription factor GATA-3 is expressed in a Th2-specific fashion (Zhang et al., 1997; Zheng and Flavell, 1997). We showed that *GATA-3* mRNA expression is substantially increased at 48 hr during Th2 development and plateaus thereafter (Zhang et al., 1997). In contrast, no such increase is seen in developing Th1 cells, and there is actually a progressive decline in *GATA-3* mRNA expression in developing Th1 cells (Zhang et al., 1997). In a recent study, forced expression of GATA-3 in developing Th1 cells caused abrogation of IL-12Rβ$_2$ subunit expression and IFNγ production (Ouyang et al., 1998). We and others have shown that GATA-3 directly activates the IL-5 promoter (Siegel et al., 1995; Zhang et al., 1997; Lee et al., 1998) and is critical and sufficient for IL-5 gene expression in a non-Th2 environment (Zhang et al., 1997; Zhang et al., 1998). In recent studies, we have demonstrated a significant increase in *GATA-3* mRNA expression in asthmatic airways compared to those of control subjects (Nakamura et al., 1999). The increase in GATA-3 expression correlated significantly with IL-5 expression and AHR in the asthmatics (Nakamura et al., 1999).

A previous report suggested that GATA-3 is sufficient for *IL-4* gene expression but has a minimal effect on *IL-5* or *IL-13* gene expression (Zheng and Flavell, 1997). In

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contrast, we (Zhang et al., 1998) and others (Ouyang et al., 1998; Ranganath et al., 1998) have recently shown that GATA-3 is not sufficient for optimal *IL-4* gene expression in a non-Th2 environment. This suggests that T cell factors such as c-Maf (Ho et al., 1996, 1998) (also Th2-specific) and NF-ATc (Ranger et al., 1998; Yoshida et al., 1998) together with GATA-3 might control optimal *IL-4* gene expression in Th2 cells (reviewed in Glimcher and Singh, 1999). In addition, although GATA-3 is not sufficient for *IL-4* gene expression in either B cells (Zhang et al., 1998) or in Th1 cells (Ouyang et al., 1998; Ranganath et al., 1998), it is sufficient for *IL-5* gene expression in the same cells. There is as yet no evidence that either c-Maf or NF-ATc is important for *IL-5* or *IL-13* gene expression. Intracellular cAMP-increasing agents such as prostaglandins, which have been recognized as important stimuli of Th2 responses (Phipps et al., 1991), are potent inducers of *IL-5* but not *IL-4* gene expression in effector T cells (Snijdewint et al., 1993). We have recently shown that *IL-5* but not *IL-4* gene expression is ablated in mice deficient in the p50 subunit of NF-κB (Yang et al., 1998). Thus, it has been unclear whether targeting GATA-3 activity alone in vivo would be sufficient to inhibit the expression of the key Th2 cytokine genes *IL-4*, *IL-5* and *IL-13* and, thus, the characteristic airway pathology in asthma. Since deficiency of the *GATA-3* gene causes embryonic lethality with severe impairment of fetal liver hematopoiesis and brain development (Pandolfi et al., 1995), we used an alternate approach to address our question. We generated transgenic mice expressing a dominant-negative mutant of GATA-3, in which amino acid residues KRR located between 304 and 306 were altered to AAA (Smith et al 1995.; will be referred to as KRR hereafter). This mutant was previously shown to inhibit GATA-3-dependent reporter gene activation (Smith et al., 1995). Our studies show that inhibition of GATA-3 activity attenuates asthma pathogenesis in vivo.

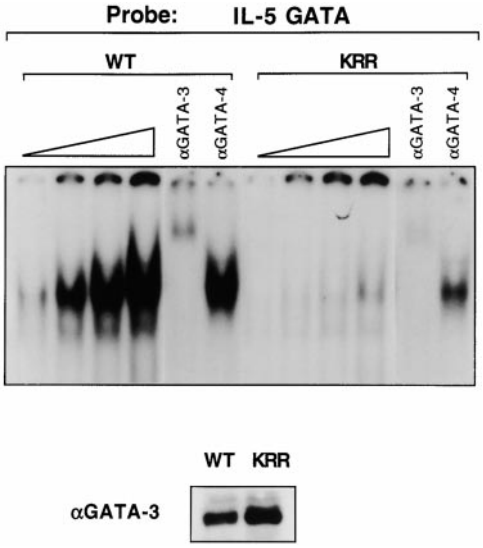

Figure 1. The Dominant-Negative Mutant of GATA-3, KRR, Binds Weakly to the GATA-3 Doublet Present in the IL-5 Promoter

(Upper panel) In vitro translated wild-type (WT) GATA-3 or mutant (KRR) was obtained using the TNT wheat germ lysate system of Promega. Equal volumes (0.5, 1, 2, and 6 μl) of the programmed TNT lysates were allowed to interact with a double-stranded oligonucleotide containing sequences between −73 and −54 containing the double GATA site present in the *IL-5* gene ($^{-73}$CCTCTATCTGATT GTTAGCA^{-54}) as described previously (Siegel et al., 1995; Zhang et al., 1997). The anti-GATA-3 antibody was a mouse monoclonal IgG1 that does not cross-react with GATA-1,-2, or −4 (Santa Cruz Biotechnology) but recognizes both the WT and mutant GATA-3 proteins. The anti-GATA-4 antibody was kindly provided by Dr. D. Wilson. The binding reactions were analyzed by electrophoresis on 6% native poly acrylamide gels (acrylamide:bisacrylamide = 30:1). Gels were dried and subjected to autoradiography. Shown is a representative experiment of two.

(Lower panel) Western blot analysis of the TNT lysates. The same volumes of the TNT lysates (2 μl) were resolved by SDS-PAGE and immunoblotting was performed with anti-GATA-3 antibody.

Results

KRR Binds Weakly to a GATA Doublet

Among all the Th2 cytokine genes, a functional GATA element has been identified only in the *IL-5* gene (Siegel et al., 1995). We previously described the presence of a double GATA site in the IL-5 promoter located between −70 and −60 that bound GATA-3 and was critical for activation of the promoter by multiple stimuli including antigen and cAMP (Siegel et al., 1995; Zhang et al., 1997). We have further shown that both GATA elements in the GATA doublet are important for IL-5 promoter activation (Zhang et al., 1998). Therefore, we first examined the DNA-binding ability of KRR to the IL-5 double-GATA site. As shown in Figure 1, compared to wild-type GATA-3, an equivalent amount of KRR bound weakly to the IL-5 GATA doublet. Thus, although KRR binds with an affinity similar to the wild-type protein to single GATA elements (Smith et al., 1995), its binding to GATA doublets, which are preferred binding sites for GATA proteins (Ko and Engel, 1993), is impaired.

Generation of KRR Transgenic Mice

We used the tetracycline (tet)-inducible expression system (Gossen and Bujard, 1992; Gossen et al., 1995) for the expression of KRR, similar to a system we previously developed for transgene expression in an inducible and lung epithelial cell–specific fashion in mice (Ray et al., 1997). For inducible expression of the *KRR* gene in a T cell–specific fashion, the reverse tetracycline transactivator (rtTA) was expressed under the control of the T cell–specific *lck* proximal promoter/*CD2* locus control region (LCR) (Allen et al., 1992), which permits expression of a linked transgene in both thymic and peripheral T cells (Figure 2A) (Perez et al., 1995; Youn et al., 1998). The *KRR* cDNA was inserted in a second plasmid downstream of a tet operator/promoter (O/P) and a cytomegalovirus (CMV) minimal promoter. Human growth hormone intronic and polyadenylation sequences were ligated to the 3′ end of both cDNAs for stable expression of the linked genes. We first tested the tet system and KRR function in vitro in Th2 cells. Th2 (D10) cells were transfected with an IL-5 promoter-reporter construct containing 1.7 kb of the IL-5 promoter together with the tet O/P-KRR plasmid with or without the lck-rtTA plasmid, and the cells were treated with dox. As shown in Figure 2B, antigen-induced IL-5 promoter activity was inhibited by 50%–60% when KRR expression was induced by rtTA. The partial inhibition of IL-5 promoter

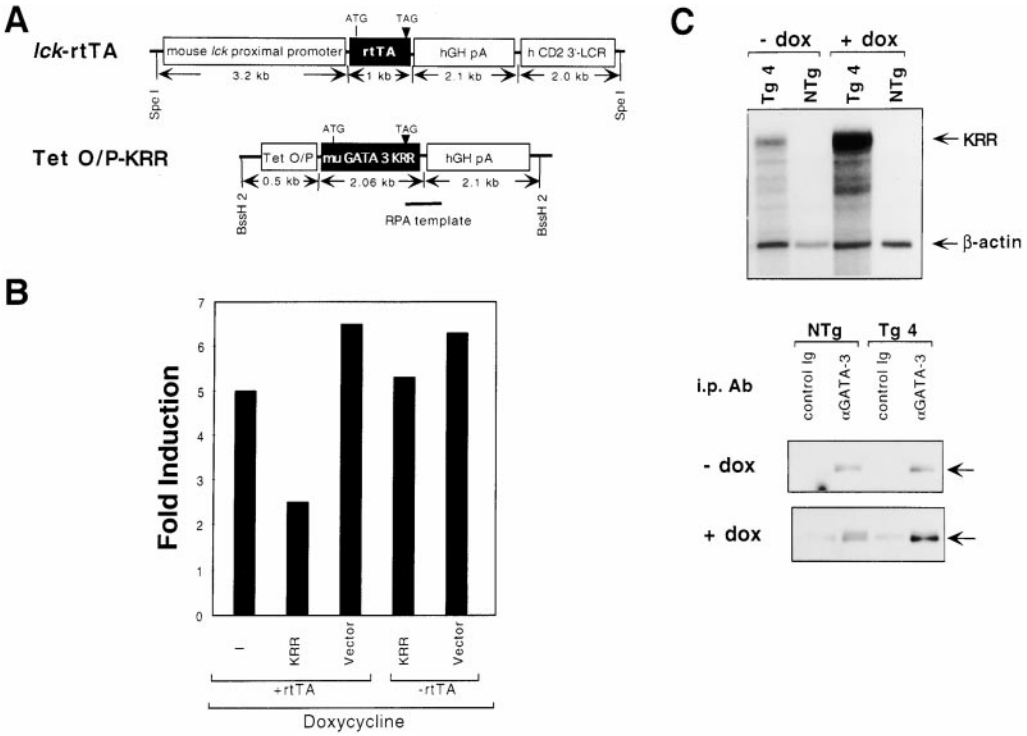

Figure 2. Generation of KRR Mice

(A) Schemtaic diagram of constructs used for T cell–specific, inducible expression of KRR. The 309 bp template that was used to determine KRR mRNA expression by RPA is shown.

(B) Inhibition of antigen-induced IL-5 promoter activation by KRR. Rested D10 cells were first stimulated with the antigen conalbumin and mitomycin C–treated and T cell–depleted APCs in complete medium containing 5 U/ml IL-2 for 72 hr and then subjected to electroporation as described previously. After 18–20 hr, cells were harvested and luciferase and β-galactosidase assays were performed. The fold activation of the promoter by antigen is shown with results representing the average of three experiments and normalized for β-galactosidase activity. The deviations were <15% between experiments. The average absolute luciferase activity in samples without KRR expression was 3000 relative light units.

(C) Expression of *KRR* RNA and protein in nontransgenic (NTg) and transgenic (Tg) mice in the presence or absence of dox (1 mg/ml in drinking water). Four days after dox induction, different organs of the animals including thymus (shown), spleen, and liver were harvested. RNA was extracted from the organs for determination of transgene RNA expression by RNase protection analysis (RPA). To determine protein expression (arrow), whole cell extracts were prepared from thymus (shown) or spleen and subjected to a sequential immunoprecipitation (i.p.)-Western blot analysis using the murine anti-GATA-3 antibody (Santa Cruz Biotechnology). Note that in the absence of dox, the protein expressed was predominantly the WT protein, which was equivalent in the NTg and Tg mice. In the presence of dox, KRR expression was induced only in the Tg animals, which accounts for the net increase in protein expression in these animals. The faint band migrating above the GATA-3 band and also seen in some of the control lanes is the immunoglobulin heavy chain partly released from the beads during boiling in SDS buffer.

activation by KRR was likely due to the high basal expression of GATA-3 in D10 cells.

We next used the plasmid constructs encoding rtTA and KRR to generate transgenic mice. We obtained five transgenic lines, out of which three lines displayed relatively high levels of transgene expression upon dox induction in both the thymus (Figure 2C) and the spleen. No expression was detected in the liver or other organs that are not enriched in T lymphocytes (data not shown). We also confirmed expression of the transgene in purified $CD4^+$ T cells isolated from the spleen. The ratio of $CD4^+$ and $CD8^+$ T cells and expression of the differentiation marker CD3 were normal in the transgenic mice (data not shown). Thus, although there was a low level leaky expression of KRR in the absence of dox, this did not impair T cell development in mice expressing the GATA-3 mutant. This was consistent with previous studies in which inhibition of GATA-3 activity in the fetal liver but not in the fetal thymus affected T cell development (Hattori et al., 1996). All studies were carried out with animals that were treated with dox to induce high level expression of KRR in the T cells of the mice.

Inhibition of Th2 Cytokine Gene Expression and Eosinophil Infiltration in the Lungs of KRR Mice

To test the effect of inhibition of GATA-3 activity on Th2 cytokine gene expression in vivo, transgenic mice and nontransgenic littermate controls were treated with dox and sensitized and challenged with Ova by inhalation (hereafter referred to as Ova/Ova). Twenty-four hours after challenge with Ova, mice were anesthetized, bronchoalveolar lavage fluid (BALF) was recovered from the animals, and cytokine levels were measured in BALF. As shown in Figure 3A, the predominant cytokines present in the BALF obtained from the control mice were of the Th2-type, as was expected in this model. The levels

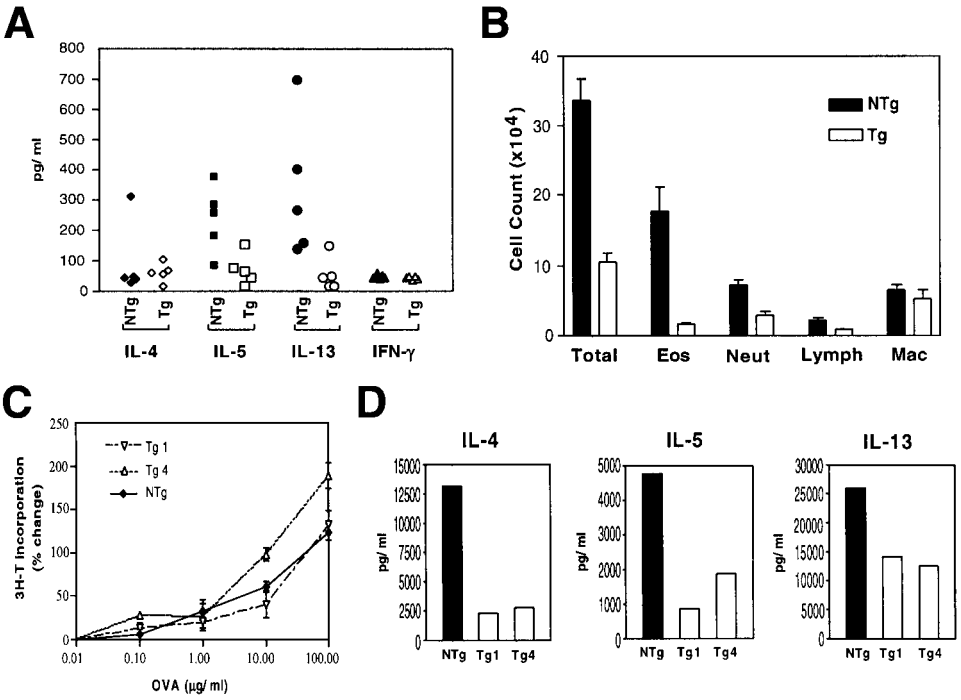

Figure 3. Decreased Cytokine Production and Leukocyte Infiltration in the Airways of KRR Mice

(A) Estimation of cytokines in BALF by ELISA. Mice were supplied with drinking water containing dox (1 mg/ml) for 4 days and then sensitized and challenged with Ova. BAL was performed at 24 hr after aerosol challenge. Mice were anesthetized and the lungs and heart were surgically exposed. The trachea were cannulated and the lungs were lavaged twice with 1 ml aliquots of PBS. The live cells (excluded trypan blue) recovered were counted in a hemocytometer. Cytospin preparations of BAL cells were stained with Diff-Quik (Baxter HealthCare) and cell differentials were enumerated based on morphology and staining profile. IL-4 levels, in general, were low in most mice, as has been previously observed by us and other investigators (Alexander et al., 1998; Cohn et al., 1999).

(B) The total and differential cell counts in the BAL of wild-type and KRR mice are shown. Shown are mean ± SEM (n = 5 per group). Results shown are representative of three experiments with three to five animals per group in each experiment.

(C) Similar proliferative responses of $CD4^+$ T cells isolated from WT and KRR mice. WT (closed symbols) and KRR (open symbols) mice were treated with dox and sensitized with Ova plus alum on days 0 and 5, and on day 12 splenic T cells isolated from these mice were stimulated in vitro with different concentrations of Ova (0.1–100 μg/ml) and mitomycin C–treated and T cell–depleted antigen-presenting cells (APC). Aliquots of cells were pulsed with [^{3}H]thymidine and incorporated counts were determined. Results shown are mean ± SEM. Shown is a representative experiment of two.

(D) WT and KRR mice (transgenic lines 1 and 4) were sensitized with Ova plus alum on days 0 and 5. On day 12, splenic $CD4^+$ T cells were isolated, pooled from two to three mice in each group, and incubated with Ova, APC, IL-4, and anti-IFNγ (Th2-skewing conditions) for 5 days. Next, the cells were restimulated with Ova, APC, and IL-2 (10 U/ml) for 72 hr and culture supernatants were used for cytokine estimations by ELISA.

of the Th2 cytokines IL-4, IL-5, and IL-13 present in the BALF of transgenic mice were lower than those recovered from their nontransgenic littermates (Figure 3A). The inhibition of IL-5 and IL-13 production by KRR is consistent with our previous results demonstrating the importance of GATA-3 in IL-5 gene expression (Zhang et al., 1997, 1998) and also with those of Ouyang et al. (1998), which show increased IL-5 and IL-13 expression in GATA-3-expressing Th1 cells. The total cell count in the BALF recovered from the transgenic mice upon antigen challenge was 4- to 5-fold lower than that in the BALF derived from the nontransgenic mice (Figure 3B). After antigen provocation, while 60%–70% of the cells present in the airways of the wild-type mice were eosinophils, only 5%–10% of the cells were eosinophils in the airways of the KRR mice (Figure 3B). Although the airway infiltration of other cells was also reduced in the transgenic mice, the most striking difference between the transgenic and nontransgenic littermates was the difference in eosinophil numbers in the airways and lung parenchyma (Figures 3B and 4).

Similar Proliferative Responses but Differential Cytokine Production by $CD4^+$ T Cells from Wild-Type and KRR Mice

We investigated whether the decrease in airway cytokine production in the KRR mice in response to antigen challenge was due to a defect in the ability of $CD4^+$ T cells to respond to antigen. Wild-type and KRR mice were immunized with Ova intraperitoneally and recall proliferative responses of spleen $CD4^+$ T cells were analyzed by [^{3}H]thymidine incorporation. The $CD4^+$ T cells from the KRR mice proliferated as efficiently as the wild-type cells in response to antigen, indicating that antigen priming was intact in the transgenic animals (Figure 3C). Cytokine production was measured in culture supernatants of the Ova-restimulated cells. As illustrated in Figure 3D, even when cytokine production was boosted by prolonged incubation of the cultures in vitro under Th2-differentiating conditions, the production of all three cytokines, IL-4, IL-5, and IL-13, was reduced to between 10% and 40% of the levels produced by the wild-type cells (Figure 3D). The residual cytokine expression in

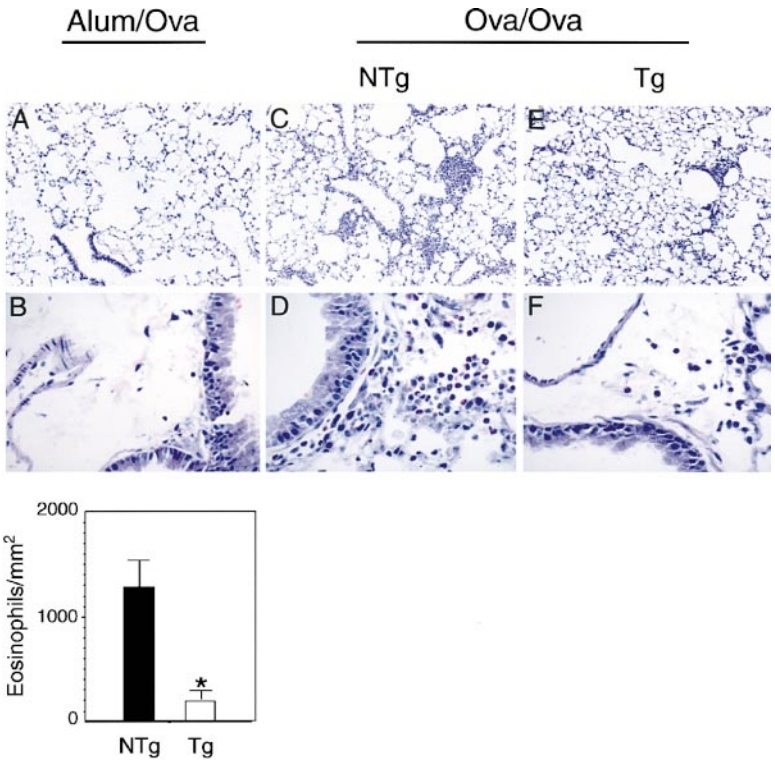

Figure 4. Inhibition of Airway Inflammation in the Lungs of KRR Mice

(Upper panel) Lungs were prepared for histology by perfusing the animal through the right ventricle with PBS to remove all blood. Lungs were then inflated to constant pressure (20 cm water) with Streck Tissue fixative (Streck Labs) instilled through a tracheostomy tube. Lung sections (5 μM) of WT or KRR mice either Ova/Ova-treated ([C–F], respectively) or Alum/Ova-treated (A and B) were mounted on slides and stained with Giemsa or hematoxylin-eosin (H and E) according to established procedures. WT Ova/Ova-treated mice had impressive peribronchiolar and perivascular eosinophilic inflammation (C and D). The cells with a pink cytoplasmic stain are eosinophils. KRR mice displayed a significant reduction in eosinophilic inflammation (E and F). Magnifications: (A), (C), and (E), 10×; (B), (D), and (F), 40×.

(Lower panel) Quantitation of eosinophils in the airways of WT and KRR mice. Eosinophils were quantitated in tissue sections using an eosinophil tissue index, which is the number of eosinophils per square millimeter of bronchovascular area. The significance of difference between the two groups was analyzed using Student's unpaired t test. Differences in means were considered significant if $P < 0.05$. *$P < 0.02$ compared with eosinophil numbers present in the airways of WT mice. Results shown are representative of three independent experiments.

the Th2 cells was likely due to incomplete inhibition of endogenous wild-type GATA-3 activity by KRR. Since the affinity of KRR for GATA doublets is considerably lower than that of the wild-type protein, complete inhibition of wild-type protein activity can be expected to require sufficiently high levels of mutant protein expression. For example, a 10-fold excess of dominant-negative NF-ATc is reportedly required to achieve ~90% inhibition of wild-type NF-ATc activity (Northrop et al., 1994; Ranger et al., 1998). However, the precise mechanism by which KRR interferes with wild-type activity, whether as monomers, homodimers, or heterodimers with the wild-type or other interacting proteins, is presently unknown. Collectively, these results demonstrate that the decrease in cytokine production by the T cells isolated from the KRR mice was not due to a defect in T cell priming.

Reduced Eosinophil Accumulation in the Lungs of KRR Mice

We next examined the lungs of the antigen-challenged mice histologically to determine the consequence of low Th2 cytokine gene expression in the airways. As shown in Figures 4C and 4D, nontrangenic mice displayed a prominent eosinophilic inflammatory response in the bronchovascular bundles. The KRR mice, on the other hand, had sparse eosinophilic inflammation in their airways upon antigen challenge (Figures 5E and 5F). In some KRR mice, a few patchy areas of a low degree of inflammation were noted in which eosinophils were barely detectable (Figure 4E). There were 30- to 50-fold fewer eosinophils/mm^2 in the lung parenchyma of the KRR mice compared to that in the controls (Figure 4, lower panel). The lungs of animals sham sensitized with alum only and challenged with Ova (Figures 4A and 4B) were totally devoid of any inflammation.

Inhibition of Mucus Production in the Lungs of KRR Mice

A typical feature of asthma pathophysiology is mucus overproduction in the airway epithelium. This feature of human asthma is also mimicked in murine models of asthma/allergic inflammation (Grunig et al., 1998; Wills-Karp et al., 1998; Cohn et al., 1999a). As shown in Figure 5, compared to sham-immunized animals (Figure 5A), there was an impressive increase in mucus production in the airway epithelium of nontransgenic mice (Figure 5B). In contrast, mucus production was only marginally increased in the lungs of the KRR mice (Figure 5C).

Reduction of Ova-Specific IgE Levels in the Sera of KRR Mice

A key effector function of IL-4 is class switching of Ig molecules to IgE. Since IL-4 was not consistently detectable at high levels in the BALF of the antigen-challenged wild-type or KRR animals, we assessed the effect of inhibition of GATA-3 activity on Ova-specific IgE production. Measurement of Ova-specific IgE in the sera derived from the animals showed ~10-fold more IgE levels in the control mice compared to that in the KRR mice (Figure 6).

KRR Has Mutant Activity on Th2 Cytokine Production but Wild-Type Effects on IFNγ Production

We examined the effect of KRR on cytokine production in unskewed activated CD4$^+$ T cell cultures. As shown

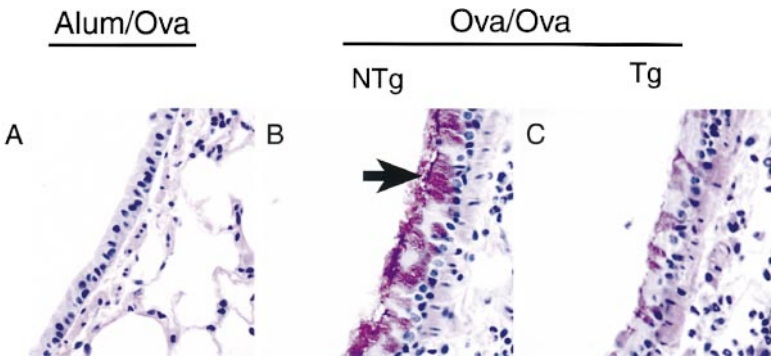

Figure 5. Inhibition of Mucus Production in the Bronchial Epithelium of Antigen-Challenged KRR Mice

Lung sections (5 μM) were mounted on slides and stained with periodic acid-Schiff base (PAS), which stains mucus. Compared to the sham-sensitized controls (A), the WT mice challenged with Ova (B) show increased mucus production in the goblet cells. Note the reduced mucus staining in the bronchial epithelium of the Ova-challenged KRR mice (C).

in Figure 7, KRR inhibited production of all the Th2 cytokines similar to its effects in Th2-skewed populations. Although KRR behaved as a dominant-negative mutant in regards to Th2 cytokine production, its effect on IFNγ production was similar to that of the wild-type protein in that it inhibited IFNγ production. This was also evident in Th1-skewed cultures as was observed by Ouyang et al. (1998). This suggests that the mechanism by which GATA-3 promotes Th2 gene expression is different from that involved in inhibition of IFNγ gene expression requiring distinct regions of the protein.

Discussion

Airway inflammation in asthma and allergic diseases is a complex phenomenon driven predominantly by Th2-type cells. Studies in both humans and mice show that Th2 cell–derived cytokines are critical regulators of asthma pathogenesis. An understanding of the molecular mechanisms that regulate Th2 cytokine production in vivo is of critical importance for the development of novel therapies for asthma and allergic diseases. We previously showed that the transcription factor GATA-3 is expressed in Th2 but not Th1 cells and plays a critical role in *IL-5* gene expression (Zhang et al., 1997). In this study we asked whether inhibition of GATA-3 activity

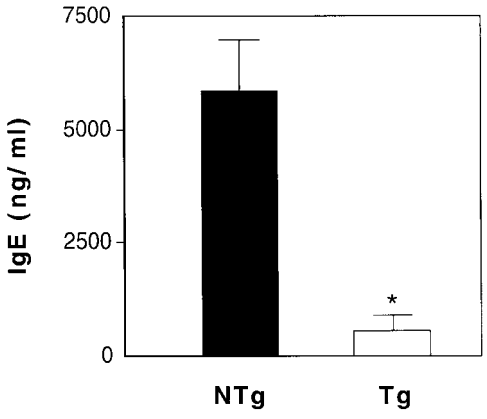

Figure 6. Reduction of Ova-Specific IgE in the Sera of OVA-Challenged KRR Mice

Before performing BAL, sera were obtained from anesthetized mice. Serum IgE concentrations were measured by ELISA. *$P < 0.01$ compared to IgE in controls. Shown is a representative experiment of two, with three to five animals per group in each experiment.

alone in vivo would be sufficient to inhibit the pathophysiology of asthma. The following reasons persuaded us to undertake this study. (1) The transcription factors c-Maf (Ho et al., 1996, 1998) (also Th2-specific) and NF-ATc (Ranger et al., 1998; Yoshida et al., 1998) have been shown to be critical for *IL-4* gene expression. However, there was no evidence that either of these transcription factors was important for *IL-5* or *IL-13* gene expression in effector T cells. Indeed, in recent studies, *c-Maf*$^{-/-}$ animals lacked IL-4 production (Kim et al., 1999). However, exogenously supplied IL-4 supported both IL-5 and IL-13 production in *c-Maf*-deficient CD4^{+} T cells (Kim et al., 1999). (2) It has been unclear from already published work whether GATA-3 is a key regulator of *IL-4*, *IL-5*, and *IL-13* gene expression. For example, contrary to previous reports (Zheng and Flavell, 1997), we and others have shown that GATA-3 expression alone is sufficient for optimal *IL-5* but not *IL-4* gene expression in a non-Th2 environment (Ouyang et al., 1998; Ranganath et al., 1998; Zhang et al., 1998). Also, the transgenic mice data of Zheng and Flavell showed minimal effects of GATA-3 on *IL-5* and *IL-13* gene expression (Zheng and Flavell, 1997). (3) Studies of mice lacking NF-κB showed differential control of *IL-4* and *IL-5* gene expression; NF-κB-deficienct mice displayed a more pronouced defect in *IL-5* gene expression (Yang et al., 1998). (4) There was limited information on regulation of *IL-13* gene expression which has been shown to be a critical regulator of asthma pathogenesis. (5) Recently, increased GATA-3 expression was documented in the lungs of asthmatic subjects correlating with *IL-5* mRNA expression and AHR (Nakamura et al., 1999). The data presented here show that GATA-3 plays an important role in the expression of all three cytokine genes, *IL-4*, *IL-5*, and *IL-13*, and plays a critical regulatory role in promoting eosinophilia in vivo. Inhibition of GATA-3 activity causes a severe blunting of Th2 effects both locally in the lung (eosinphil influx, mucus production) as well as systemically (IgE production).

GATA-3 belongs to the GATA family of transcription that bind to the WGATAR (W = A/T; R = A/G) DNA sequence through a highly conserved C4 zinc finger domain (Orkin, 1992). GATA-3 is expressed primarily in T lymphocytes and in the embryonic brain (George et al., 1994). Targeted disruption of the *GATA-3* gene in mice results in embryonic death on day 12, with a failure of fetal hematopoiesis and defects in the central nervous system (Pandolfi et al., 1995). GATA-3 was shown to be an essential component in the earliest steps of T cell

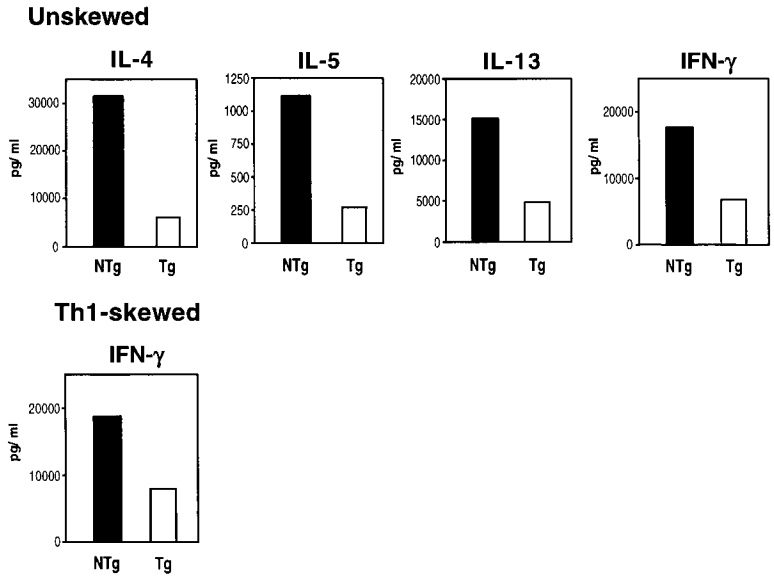

Figure 7. KRR Inhibits IFNγ Production

Splenic $CD4^+$ T cells (cells were pooled from spleens of three mice in each group) were isolated from transgenic mice (line 4) and nontransgenic littermates and stimulated with anti-CD3 and anti-CD28 antibodies under neutral or Th1-skewing conditions. After 5 days in culture, cells were restimulated, and after 72 hr culture supernatants were harvested for ELISA. Shown is a representative experiment of two.

development in the thymus using antisense oligonucleotides for GATA-3 in fetal thymus organ cultures (Hattori et al., 1996) and by using *GATA-3*$^{-/-}$/*RAG-2*$^{-/-}$ chimeric mice (Ting et al., 1996). Thus, GATA-3 is not a functionally redundant GATA-family protein in that it is a critical regulator of both early T cell development and Th2 cytokine gene expression in vivo.

The GATA-3 mutant KRR was shown to have dominant negative effects on the activation of minimal TCR α and β enhancers in Jurkat cells, which have modest endogenous levels of wild-type GATA-3 (Smith et al., 1995). However, recently, both wild-type and KRR were shown to support low-level IL-4 expression in Th1 cells (Ouyang et al., 1998). Activation of *IL-4* gene expression in Th1 cells probably involves mechanisms that are distinct from those operative in Th2 cells, since even the wild-type protein is incapable of supporting optimal expression of the gene in Th1 cells. Either the low-level IL-4 expression in Th1 cells is mediated through binding of GATA-3 to single GATA elements, to which both the wild-type and mutant protein bind with equal affinity, or it may be due to cooperation with proteins with which GATA-3 does not normally cooperate in Th2 cells. Although the binding of KRR to single GATA elements is indistinguishable from the binding of the wild-type protein (Smith et al., 1995), its binding to a GATA doublet is weak (Figure 1). Our studies have shown the importance of the GATA doublet in activation of the IL-5 promoter; mutation of either element completely eliminates activation of the promoter in Th2 cells (Zhang et al., 1998). Since the single GATA elements in the IL-4 promoter have little or no role in GATA-3 responsiveness of this gene in Th2 cells, it is possible that GATA-3 activates the *IL-4* and *IL-13* genes through binding to GATA doublets elsewhere in the genes. Interestingly, studies of Ko and Engel show a preference for GATA double sites for the binding of GATA proteins (Ko and Engel, 1993). Several regions around the *IL-4*/*IL-13* locus were shown to bind GATA-3, but the functional implications of these interactions remain to be determined (Ranganath et al., 1998). Thus, while GATA-3 directly activates the IL-5 promoter, GATA-3 may directly or indirectly control the expression of IL-4 and IL-13.

Several studies show the importance of the Th2 cytokines IL-4, IL-5, and IL-13 in the development of eosinophilic inflammation and AHR. In initial studies, *IL-4*$^{-/-}$ animals displayed reduced Th2 responses including airway eosinophilia and AHR in a model of allergic inflammation (Kopf et al., 1993; Brusselle et al., 1995; Corry et al., 1996). However, adoptive transfer of IL-$4^{-/-}$ $CD4^+$ T cells to IL-$4^{-/-}$ (or wild-type) recipient mice resulted in AHR and mucus production (Cohn et al., 1998). IL-13, which also binds to the IL-4Rα subunit and thus has overlapping functions with IL-4, was also shown to be important for the development of Th2 cells in vivo, although unlike IL-4, a direct role for IL-13 in Th2 differentiation has yet to be demonstrated (McKenzie et al., 1998). To sort out the specific functions of IL-4 and IL-13 in the generation of allergic inflammation, Th2 cells were transferred into IL-4R$\alpha^{-/-}$ mice (Cohn et al., 1999a). When these cells were activated in the respiratory tract, no eosinophilia or mucus production was detected (Cohn et al., 1999a). When viewed in combination, these results suggested that blockade of both IL-4 and IL-13 is critical for the reduction of the inflammatory pathology in asthma. In other studies, use of a specific inhibitor of IL-13 abolished mucus production and AHR (Grunig et al., 1998; Wills-Karp et al., 1998) but did not reduce eosinophilia (Wills-Karp et al., 1998). All studies reported to date suggest a critical role for IL-5 in eosinophilia (Coffman et al., 1989; Dent et al., 1990; Foster et al., 1996; Kopf et al., 1996; Cohn et al., 1999a). When IL-$5^{-/-}$ cells were transferred into IL-$5^{-/-}$ recipients, no eosinophilia was observed, although mucus production was unaffected (Cohn et al., 1999a). Taken together, these studies show that inhibition of all the key features of asthma—airway eosinophilia, mucus production, and AHR—requires inhibition of function of all three cytokines. Our studies suggest that this can be achieved by inhibition of GATA-3 function.

While the reduction in eosinophilia, AHR, and mucus production in *IL-4*/*IL-13* (or *Stat6*)-deficient animals is

due to the lack of direct effects of these cytokines, it may also be a consequence of a shift to the development of Th1 cells in the absence of the Th2-driving cytokines that cross-regulate Th1 responses. Indeed, IFNγ production by Th1 cells reduces both Th2-induced airway inflammation and mucus production (Cohn et al., 1999b). However, in the present study, expression of KRR did not result in a shift to increased Th1 responses despite inhibition of IL-4 and IL-13 production. This can be explained by our results and by those of Ouyang et al. showing the ability of KRR, like the wild-type protein, to inhibit IFNγ production in Th1 cells (Figure 7; Ouyang et al., 1998). This suggests that distinct regions in the GATA-3 protein are involved in the inhibition of IFNγ production and activation of Th2 cytokine gene expression.

To date, no asthma therapy can selectively target Th2 lymphocyte-mediated inflammation in asthmatics. Corticosteroids, the mainstay of asthma therapy, cause general immunosuppression and prolonged use is associated with multiple side effects. We have provided evidence that inhibition of activity of a Th2-specific factor blunts an asthma-like phenotype in an animal model. Since GATA-3 is not ubiquitous and is expressed predominantly in T cells in adults and furthermore is not required for Th1 responses, inhibition of GATA-3 activity by using antisense RNA or pharmacological approaches will have one clear advantage over current treatment modalities for asthma—selectivity. Thus, our results establish GATA-3 as a potential therapeutic target for the treatment of asthma and allergy.

Experimental Procedures

Generation of Transgenic Mice

The *lck-rtTA* expression vector was made by inserting a 1.0 kb EcoRI/BamHI *rtTA* fragment isolated from the plasmid 172–1 *neo* (a gift from Drs. H. Bujard and M. Gossen) into the plasmid pTLC containing the *lck* proximal promoter, *hGH* intronic, and polyadenylation sequences and the CD2 LCR at the 3′ end (a gift from Dr. R. Bravo). The purified recombinant plasmid was digested with SpeI, and the fragment containing the *lck-rtTA-hGH* minigene was purified and used for microinjection. The KRR mutation (amino acids KRR in positions 304–306 changed to AAA) was introduced into the murine GATA-3 cDNA by polymerase chain reaction (PCR) techniques. The tet O/P-KRR expression vector was made by inserting the following fragments in pBluescript II (KS+) plasmid (Stratagene): a 0.49 kb XhoI/ClaI fragment consisting of heptamerized tet operator sequences linked to the CMV minimal promoter (isolated from pUHC-13–3 [Gossen and Bujard, 1992; Gossen et al., 1995]) was inserted into pBluescript II (KS+) plasmid. A BamHI/NotI fragment containing *hGH* poly(A) and intronic sequences was next inserted into the polylinker region of the plasmid. An EcoRI fragment containing the *KRR* cDNA was blunt ended with Klenow polymerase and inserted into the EcoRV site of the plasmid, and the orientation was determined by digestion with HindIII and further confirmed by sequencing. The *tet O/P-KRR-hGH* fragment was isolated by digestion of the plasmid with BssH2, purified, and used for microinjection. The two constructs were coinjected into the pronuclei of C57BL/6×SJL F2 zygotes. Transgene positive animals were identified initially by PCR and confirmed by Southern blotting techniques. Tail DNA from the animals was digested with BamHI and resolved by gel electrophoresis. Hybridization with the rtTA probe and the GATA-3 probe was carried out by overnight incubation in a buffer containing 6×SSPE, 0.5% SDS, 50% formamide, 50 mg/ml salmon sperm DNA, and the probe at 5×10^6 cpm/ml of the hybridization buffer. For the *rtTA* probe, a 1 kb EcoRI/BamHI fragment of *rtTA* isolated from the plasmid 172–1 *neo* (Gossen and Bujard, 1992; Gossen et al., 1995) was used. For the *GATA-3* probe, a 360 bp ClaI/BglI *GATA-3* cDNA fragment was used that hybridized with a 700 bp fragment from the transgene. The blots were washed twice for 15 min each at room temperature in 6×SSPE/0.2% SDS, then twice for 15 min each at 55°C in 0.2×SSPE/1.0% SDS, dried, and subjected to autoradiography. The founders were crossed to BALB/c mice to establish transgenic lines.

RPA

For RPA, a 309 bp ^{32}P-labeled antisense RNA specific for the transgene was derived by T7 polymerase-mediated in vitro transcription (MaxiScript kit; Ambion) of a template derived by PCR using a primer derived from the *GATA-3* cDNA and a second primer derived from *hGH* poly(A) sequences, which was tagged with a T promoter sequence. The β-*actin* antisense RNA was derived using a template that was obtained from Ambion. Total RNA (40 μg) from each mouse was used in RPA. Conditions for RPA were essentially as described previously (Yang et al., 1998), except that only RNase T1 (20 U/ml) was used for digestion of RNA duplexes. After digestion with RNase T1, the protected RNA duplexes were purified by phenol/chloroform extraction and ethanol precipitation and analyzed by electrophoresis on 5% polyacrylamide/8 M urea gels. The gels were dried and subjected to autoradiography.

Transfections

Rested D10 cells were washed once in serum-free RPMI 1640 medium and resuspended in the same medium. The cells (5×10^6) were first stimulated with the antigen conalbumin and mitomycin C–treated and T cell–depleted APCs in complete medium containing 5 U/ml of IL-2 for 72 hr. The cells were incubated with 5 μg of reporter plasmid, 5 μg of vector alone or tet O/P KRR plasmid, and 2 μg of CMV-β-galactosidase plasmid (a monitor for tranfection efficiency) with or without 2 μg of lck-rtTA construct for 10 min at room temperature, and electroporation was carried out using Gene Pulser (Bio-Rad) at 0.27 kV and 960 μFD. The cells were left on ice for 10–30 min, diluted to 5 ml with fresh medium, and incubated at 37°C. Cells were harvested for reporter gene assays as described previously (Zhang et al., 1997, 1998).

Sensitization and Challenge of Mice

Mice were sensitized and challenged essentially as described previously (Yang et al., 1998). Mice were sensitized with 10 μg of ovalbumin (OVA; Sigma Chemical) and 1 mg of alum (Resorptar; Intergen) intraperitoneally on days 0 and 5. Sham-immunized mice received alum alone. On day 12, mice were challenged by exposing to an aerosol of 1% OVA in PBS twice for 1 hr each at an interval of 4 hr. BAL and lung histology were as described previously (Yang et al., 1998).

Cell Proliferation Assays

Mice were sensitized as above by intraperitoneal injections of OVA plus alum on days 0 and 5. Spleens were harvested on day 12 and $CD4^+$ T cells were prepared by positive selection using monoclonal antibodies to CD4 coupled to magnetic beads (MACS Miltenyi) and a MACS separation column. Syngeneic T cell–depleted splenocytes were used as antigen-presenting cells (APCs), which were prepared by depletion of CD4 and CD8 T cells using antibody-coupled magnetic beads (MACS Miltenyi) and were treated with mitomycin C as described previously (Cohn et al., 1997). $CD4^+$ T cells (5×10^5; pooled from two mice in each group) together with 5×10^5 APCs were cultured with OVA (0.1–100 μg/ml) in Bruff's medium supplemented with 5% fetal calf serum (FCS). Cultures were incubated for 72 hr, supernatants were collected for cytokine analyses, and cultures were pulsed with 1 μCi of [methyl-^{3}H]thymidine/well. After incubation at 37°C for 24 hr, triplicate wells were harvested onto glass filters and incorporated radioactivity was measured in a β counter. The background was subtracted from the results.

Cytokine Assays

For determination of cytokine expression under Th2-skewing conditions, mice were sensitized with Ova plus alum on days 0 and 5. The spleens of the mice were harvested on day 12, and $CD4^+$ T cells were purified as described above. The cells were incubated

under Th2-skewing conditions with dox (10 μg/ml), IL-4, antigen, anti-IFNγ antibodies, and T cell–depleted and mitomycin C–treated APCs as described previously (Zhang et al., 1997). After 5 days, cells were restimulated with antigen and APC and incubated for 72 hr. IL-2, IL-4, and IL-5 protein levels in culture supernatants and in BAL (also IFNγ levels in BAL) were determined by ELISA. For cytokine evaluations under Th1-skewed or unskewed conditions, purified $CD4^+$ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies (each at 2 μg/ml) with either dox (10 μg/ml), IL-2 (50 U/ml), IL-12 (7.5 ng/ml), and anti-IL-4 antibody or with dox and IL-2 only, respectively. After 5 days in culture, cells were restimulated with the antibodies in the presence of dox and IL-2. Culture supernatants were harvested after 72 hours for ELISA. The lower limits of detection for the cytokines were: IL-4, 5 pg/ml; IL-5, 5 pg/ml; IFNγ, 15 pg/ml (Endogen); and IL-13, 1.5 pg/ml (RandD).

Ova-Specific IgE Assay

Ova-specific IgE was estimated in the sera of the antigen-challenged mice. 96-well microtiter plates were coated with Ova (Sigma) (50 μg/ml) in 0.1 M $NaHCO_3$ at 4°C overnight. Plates were washed 3X with PBS-Tween (0.05%) between each of the following assay incubations: (1) blocking with 1% BSA in Borate saline (BSA-BS) for 1 hr at 37°C, (2) serial dilutions of sera in 1% BSA-BS-0.05% Tween for 2 hr at 37°C, (3) biotin-labeled secondary rat anti-mouse antibody (anti-IgE [2379–04S; Biosource]) for 1 hr at 37°C, (4) streptavidin-horseradish peroxidase for 30 min at 37°C, and (5) TMB substrate solution (Dako Corp) and termination of peroxidase reaction with 6N HCl. Color development was read at 405 nm in an ELISA plate reader. Serum IgE concentrations were calculated by comparison to a standard: monoclonal anti-Ova mouse IgE (kindly provided by Dr. E. Gelfand).

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