

Lymphotoxin Is Required for Maintaining Physiological Levels of Serum IgE That Minimizes Th1-mediated Airway Inflammation

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Abstract

Although elevated levels of IgE in asthmatic patients are strongly associated with lung infiltration by activated T helper (Th) 2 cells, the physiological role of immunoglobulin E (IgE) in the airway remains largely undefined. Lymphotoxin-deficient α ($LT\alpha^{-/-}$) mice exhibit increased airway inflammation, paradoxically accompanied by diminished levels of IgE and reduced airway hyperresponsiveness in response to both environmental and induced antigen challenge. The severe lung inflammation in $LT\alpha^{-/-}$ mice is Th1 in nature and can be alleviated by IgE reconstitution. Conversely, depletion of IgE in wild-type mice recapitulates the lung pathologies of $LT\alpha^{-/-}$ mice. Therefore, this work has revealed that lymphotoxin is essential for IgE production, and a physiological role of IgE in the airway may consist of maintaining the balance of Th1 and Th2 responses to prevent aberrant inflammation.

Key words: bronchial hyperresponsiveness • cytokines • T helper cells • infiltration • allergen

Introduction

Several papers have shown that the development of bronchial asthma is associated with elevated IgE levels, the predominance of Th2 cytokine production, and eosinophilic inflammation of the airways (1–4). In addition to clinical correlations of asthma with high IgE levels, genetic analyses of families with asthma have also revealed a link between bronchial hyperresponsiveness (BHR) and high IgE levels (5, 6). Allergen-linked IgE signaling participates in the initiation of immediate hypersensitivity reactions and BHR by triggering mast cell degranulation (3, 7, 8). Allergens trigger mast cells by cross-linking surface Fc ϵ RI-bound IgE, resulting in the release of IL-4, IL-13, and other factors, and ultimately promoting a Th2 response and allergic airway inflammation (9–13). Moreover, reduced expression of T-bet, a transcription factor for Th1 cytokines, induces a phenotype of murine asthma analogous to both acute and chronic human asthma, thus providing further support for the necessity of an optimal balance between Th1 and Th2

to maintain lung homeostasis and prevent aberrant inflammation. (10). In contrast to the data that Th2-mediated inflammation is dominant in allergen-induced asthma, several papers have shown surprisingly that the addition of allergen-specific Th1 cells can exacerbate airway inflammation (11–13). To control moderate-to-severe allergic asthma, humanized monoclonal antibodies directed against IgE have been used for treatment, but their efficacy is rather limited (14, 15). Surprisingly, IgE may not be essential for allergen-induced BHR and airway inflammation (16); thus far, the physiological role of IgE in the airway has not been demonstrated.

Lymphotoxin (LT) α has been identified in two molecular forms as follows: a secreted form consisting of an $LT\alpha_3$ homotrimer that binds to TNF receptors and a membrane-associated heterotrimeric form with $LT\alpha\beta_2$ that binds to $LT\beta R$. $LT\alpha$ -deficient ($LT\alpha^{-/-}$) and $LT\beta R^{-/-}$ mice lack LNs but show a spontaneous accumulation of a diverse repertoire of immune cells in the lungs. The mechanisms underlying this spontaneous accumulation have not been

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Abbreviations used in this paper: BAL, bronchoalveolar lavage; BHR, bronchial hyperresponsiveness; i.t., intratracheally; LT, lymphotoxin; SEA, *Schistosoma mansoni* egg antigen.

adequately addressed (17–19). It is curious that $LT\alpha^{-/-}$ mice have a strong inflammatory response to antigen challenge in the airway, even in the absence of draining LNs (20, 21). Here, we report that the infiltrating cells are actively participating in airway inflammation, and their accumulation in the lung can be paradoxically attributed to diminished serum IgE levels in $LT\alpha^{-/-}$ mice. Therefore, our results uncover a hitherto unappreciated physiological role of IgE in the protection of the airway from inappropriate inflammation and BHR.

Materials and Methods

Mice. $LT\alpha^{-/-}$ and $LT\beta R^{-/-}$ mice were backcrossed to C57BL/6 mice and maintained under specific pathogen-free conditions as described previously (18, 22). $TNF^{-/-}$ and C57BL/6 mice were purchased from the Jackson Laboratory. $LT\beta R^{-/-}$ mice were obtained from Taconic Farms. Animal care and use were in accordance with institutional and National Institutes of Health guidelines.

Histology. Lung tissues for histological examination were fixed in 10% buffered formalin. Lung tissues of $IgE^{-/-}$ mice were

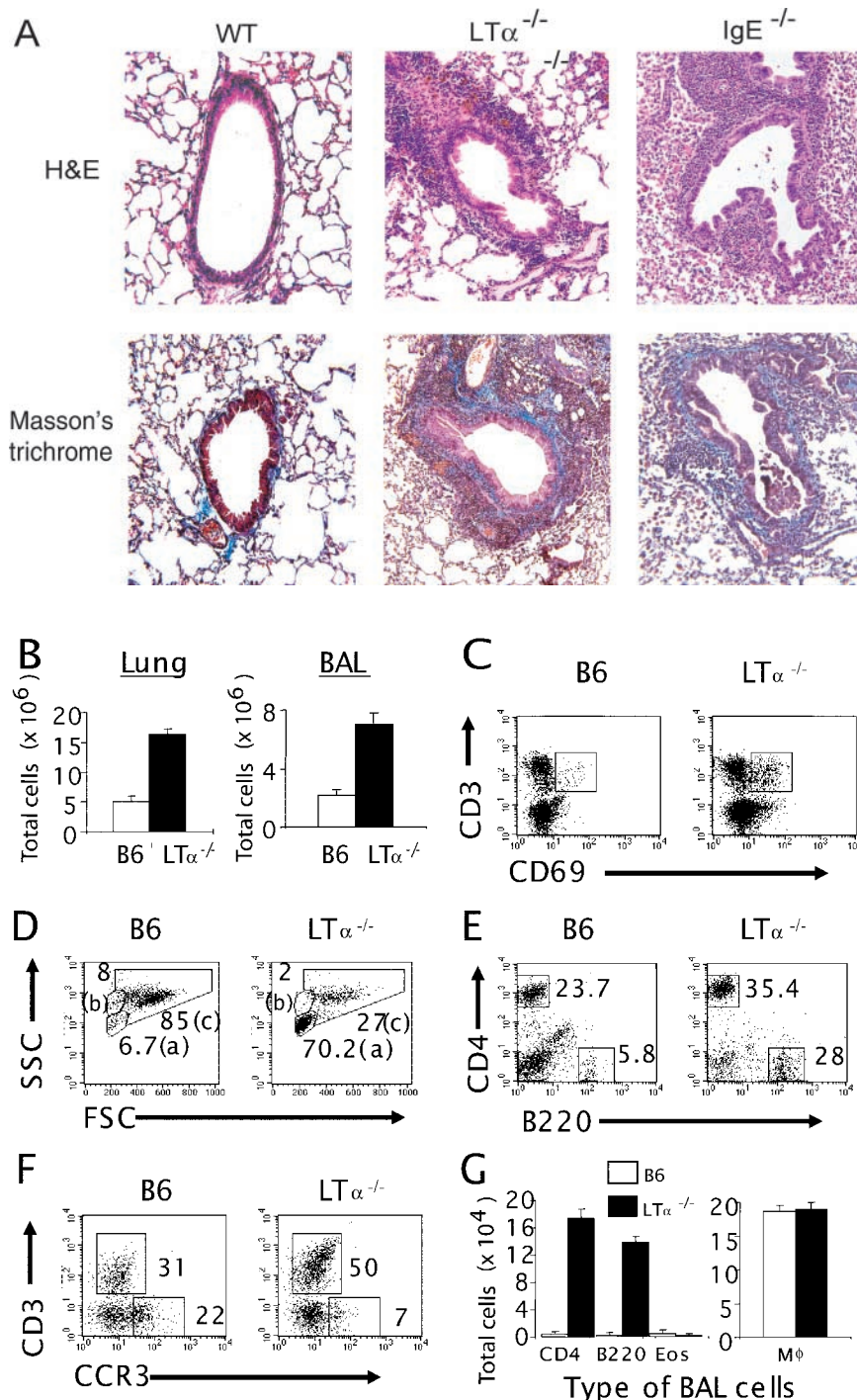
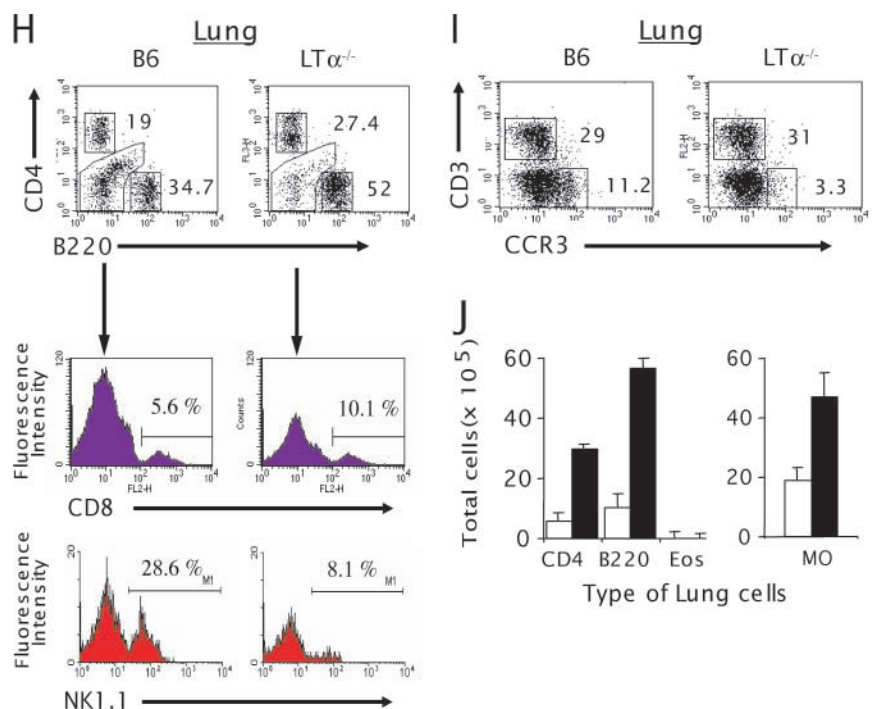


Figure 1. Increased inflammation and airway remodeling in $LT\alpha^{-/-}$ and $IgE^{-/-}$ mice. (A) Lung tissues from WT, $LT\alpha^{-/-}$, and $IgE^{-/-}$ mice (3 mo old) were fixed in 10% buffered formalin and embedded in paraffin. Hematoxylin and eosin staining (top, H&E) and Masson's trichrome staining (bottom) of B6, $LT\alpha^{-/-}$, and $IgE^{-/-}$ mice are presented. (B) The lung (left) and BAL cells (right) were isolated by collagenase digestion and lavage, respectively, from 3-mo-old B6 (white bars) and $LT\alpha^{-/-}$ (black bars) mice ($n = 5$ per group). The total number of cells was analyzed with trypan blue staining. The number of leukocytes in the lung of $LT\alpha^{-/-}$ mice is significantly higher than that of the WT ($P < 0.001$). BAL cells from five mice were pooled for further analysis by FACS[®]. (C) Lung cells were dually stained with FITC-conjugated anti-CD69 and PE-conjugated anti-CD3. The fluorescence intensity was analyzed from the gated lymphocyte population of B6 (left) and $LT\alpha^{-/-}$ (right) mice. The percentage of CD69⁺ cells among CD3⁺ cells is presented. (D) The forward/side scatter dot-plot profiles were analyzed from BAL cells of B6 (left) and $LT\alpha^{-/-}$ (right) mice; (a), (b), and (c) represent the lymphocyte, granulocyte, and macrophage population, respectively. (E) The gated lymphocyte population (a) from the forward/side scatter dot-plot profiles of BAL cells (D) was analyzed for CD4 and B220 expression by FACS[®] analysis. (F) CCR3 and CD3 expression was analyzed from both the gated lymphocyte (a) and granulocyte (b) populations. (G) The type of BAL cells was determined by calculating the absolute number of each cell type from the FACS[®] profiles and by the total number of cells ($P < 0.0001$; WT vs. $LT\alpha^{-/-}$).



(H) B6 and LT $\alpha^{-/-}$ mice were analyzed by three-color staining with FITC-anti-B220, cychrome-anti-CD4, and PE-anti-CD8 or PE-anti-NK1.1. The gated lymphocyte population (a) from the forward/side scatter dot-plot profiles of the lung cells was analyzed for CD4 and B220 ($P < 0.01$, WT vs. LT $\alpha^{-/-}$ mice). The percentage of CD8⁺ or NK1.1⁺ cells gated from B220⁻CD4⁻ was evaluated. (I) CCR3 and CD3 expression was analyzed from both the gated lymphocyte (a) and granulocyte (b) population. (J) The total number of cells was calculated. The type of lung cells was also determined by calculating the absolute number of each cell type from the FACS[®] profiles. The results are representative of five independent experiments.

provided by H. Oettgen (Boston Children's Hospital, Boston, MA). 3- μ m sections were obtained from the paraffin blocks and stained using both the hematoxylin and eosin and Masson's trichrome staining methods. All sections were examined qualitatively by an experienced pathologist in a blind fashion.

Flow Cytometric Analysis. All antibodies for flow cytometric analysis were purchased from BD Biosciences. Bronchoalveolar lavage (BAL) and lung cells were incubated in Fc-block, 2.4G2, for 20 min, stained in PBS containing 1% FBS (GIBCO BRL) plus 0.01% NaN₃ for 30 min on ice, and analyzed by flow cytometry on a FACScan[™] (BD Biosciences). For statistical analysis, p-values were determined by two-tailed Student's *t* test. For intracellular cytokine staining, lung cells were obtained by collagenase digestion and stimulated with 50 ng/ml PMA, 500 ng/ml ionomycin, and 10 μ g/ml Brefeldin A in complete RPMI 1640 media with 10% FCS for 6 h at 37°C, 5% CO₂. Extracellular staining was performed using anti-CD4-PE for 30 min on ice. The cells were fixed with 2% paraformaldehyde. For intracellular staining, cells were permeabilized with 0.5% saponin in PBS, stained with either IL-4-allphycocyanin or IFN γ -allophycocyanin in permeabilization buffer, and analyzed by FACS[®].

Isolation of Lung Leukocytes and BAL Cells. Lung tissues were digested three times by shaking (175 revolutions/min) for 30 min at 37°C in RPMI 1640 medium containing 1.5 mg/ml collagenase VIII and 2% FBS. Lung cells were passed through a nytex filter. Bronchial lavage was performed by delivering 1 ml of cold RPMI 1640 containing 2% FBS into the trachea and gently aspirating the fluid. The first lavage was centrifuged, and supernatant was stored at -20°C for cytokine analysis by ELISA. Lavage was repeated three times, and cells collected from each wash were pooled for FACS[®] analysis.

Cytokine and IgE ELISA. The lung tissue was homogenized in 500 μ l PBS containing protease inhibitors. The lysate was collected by centrifugation at 12,000 revolutions/min for 15 min. Cytokine production from the lung lysate and BAL fluid was measured by ELISA. The total IgE concentration from the serum

($n = 9$) was measured by ELISA according to the manufacturer's protocol (BD Biosciences).

Respiratory Mechanics. Assessment of cholinergic airway constrictor responsiveness was done with a computer-controlled small-animal ventilator (Flexivent; SCIREQ). In brief, the mice were anesthetized with 0.1 ml per 10 g body weight of a mixture containing 2 mg/ml xylazine and 40 mg/ml ketamine hydrochloride given i.p. Anesthesia was maintained by supplemental administration of 30% of the initial dose at ~25-min intervals, as required. Heart rate was monitored by EKG with needle electrodes. After tracheostomy, the trachea was cannulated with a blunted 18-gauge metal needle. The mouse was quasi-sinusoidally ventilated with a nominal tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H₂O. To determine the differences in airway response to methacholine between WT and LT $\alpha^{-/-}$ mice, each mouse was challenged with seven doses of methacholine aerosol (0, 0.1, 1, 5, 10, 20, and 40 mg/ml in saline) for 12 s. Before each aerosol challenge, the animal was given two deep inspirations to standardize volume history. After each methacholine challenge, respiratory system resistance was recorded during tidal breathing every 10 s for 2 min, and the peak resistance was selected as the bronchoconstrictor response to that methacholine dose. Analysis of variance is used to analyze the differences in airway response to methacholine between WT and LT $\alpha^{-/-}$ mice.

Schistosoma mansoni Egg Antigen (SEA) Sensitization and Challenge. Mice were sensitized i.p. on day 0 with 2.5×10^3 inactivated *S. mansoni* eggs. On day 7, the mice were challenged intratracheally (i.t.) with 50 μ g of soluble SEA. 4 d after challenge, mice were killed, BAL was collected, and lungs were dissected for digestion. Strong Th2-dominant airway inflammation with 70–90% eosinophils in BAL could be detected after challenge (23, 24). In IgE reconstitution experiments, LT $\alpha^{-/-}$ mice were injected i.p. on days -21, -14, and -7 with either mouse Ig or polyclonal IgE purified from WT mice. *S. mansoni* sensitization on day 0, SEA challenge on day 7, and harvest on day 11 were

performed as described in Fig. 4. In IgE depletion experiments, B6 mice were injected i.p. on days -21, -14, and -7 with 30 μ g of either rat Ig or anti-IgE antibodies as follows: EM95 or R1E4 (8, 25). All reagents were free of endotoxin (EU < 0.25).

Results

Increased Airway Inflammation and Remodeling in $LT\alpha^{-/-}$ Mice. To carefully determine the nature of the previously reported accumulation of leukocytes in the lungs of $LT\alpha^{-/-}$ mice (17–19), detailed histopathological analysis was performed. The lungs of $LT\alpha^{-/-}$ mice do not merely harbor leukocytes but gradually develop a chronic airway inflammation that worsens with age (Fig. 1 A). The infiltration consists of mainly mononuclear cells predominantly in the peribronchial and perivascular areas. Histopathological analysis of lung tissues in $LT\alpha^{-/-}$ mice also revealed significant airway remodeling. These changes include bronchial wall thickening, subepithelial fibrosis, increased goblet cell mass, myoblast–myocyte hyperplasia and hypertrophy, epithelial cell hypertrophy, and increased deposition of collagen below the basement membrane; these are all clear indicators of chronic airway inflammation. Furthermore, the thickness of this subepithelial collagen layer was visualized by Masson's trichrome staining, which stains collagen. In naive $LT\alpha^{-/-}$ mice, the collagen layer under the basement membrane was much thicker than that of WT mice (Fig. 1 A). To further determine the type and number of infiltrating cells in the lung tissue, lungs from WT and $LT\alpha^{-/-}$ mice were digested with collagenase. The suspension of cells was analyzed by flow cytometry and a three- to five-fold increase in total cell number was found in $LT\alpha^{-/-}$ mice (Fig. 1 B) with an increased proportion of activated T cells, as measured by CD69 expression, in $LT\alpha^{-/-}$ mice compared with WT mice (Fig. 1 C). Thus, $LT\alpha^{-/-}$ mice, without deliberate challenge, appear to develop a spontaneous, cumulative airway inflammation with active remodeling not seen in WT mice housed in the same specific pathogen-free facility.

To more closely define the inflammation in the airway, BAL cells were collected from WT and $LT\alpha^{-/-}$ mice and analyzed. An increase in total BAL cells was also found in $LT\alpha^{-/-}$ mice (Fig. 1 B), corresponding to escalating infiltration in the lung tissue. More impressively, $LT\alpha^{-/-}$ mice had a much higher percentage of BAL lymphocytes than WT mice as determined by flow cytometry (Fig. 1 D, area a, $6.7 \pm 0.8\%$ in WT mice, $70.2 \pm 1.6\%$ in $LT\alpha^{-/-}$ mice). These results were consistent with differential cell counts from cytopsin preparations of BAL (unpublished data). $CD4^+$ T and B cells were the major subsets of the increased lymphocyte population in $LT\alpha^{-/-}$ BAL (Fig. 1, E and G). The percentage of $CD8^+$ T cells was also increased, and $NK1.1^+$ cells were reduced (Fig. 1 H). The forward/side scatter dot-plot profile of WT and $LT\alpha^{-/-}$ BAL cells also showed a reduction in the granulocyte population in $LT\alpha^{-/-}$ mice (Fig. 1 D, area b, 8.3% in WT mice, 2.8% in $LT\alpha^{-/-}$ mice). CCR3 is a β chemokine receptor found predominantly, but not exclusively, on eo-

sinophils (26, 27). To determine whether $LT\alpha^{-/-}$ mice present with eosinophilia, cells from the BAL were stained with an anti-CCR3 antibody, and the number of CCR3 positive cells was reduced in airways of $LT\alpha^{-/-}$ (Fig. 1 F, $22 \pm 1.3\%$ in WT mice, $7 \pm 0.4\%$ in $LT\alpha^{-/-}$ mice). This result was consistent with the reduced percentage of eosinophils in $LT\alpha^{-/-}$ mice determined by cytopsin; however, the total number of eosinophils was not reduced (unpublished data). Although the absolute cell numbers of $CD4^-$ and $B220^-$ expressing cells were markedly increased in the BAL of $LT\alpha^{-/-}$ mice compared with WT mice ($\sim 30^-$ and 80^- -fold, respectively), the number of macrophages remained unperturbed (Fig. 1 G). Similarly to the BAL, the percentages of $CD4^-$, $CD8^-$, and $B220^-$ expressing cells in the lungs of $LT\alpha^{-/-}$ mice were increased (Fig. 1 H). Because the total number of leukocytes in the lung increased significantly ($P > 0.0001$), the actual total number of $CD4$, $CD8$, and $B220^+$ cells increased an impressive 8–14 fold. The percentage of NK cells and $CCR3^+$ cells was greatly reduced, and the absolute number of these cells was also slightly reduced in the lung of $LT\alpha^{-/-}$ mice (Fig. 1, I and J). Together, these observations implied that the profound increase of total cell numbers in the airways of $LT\alpha^{-/-}$ mice was largely due to the increased infiltration of lymphocytes, a feature of chronic inflammation.

Interaction between LT and Its Receptor Is Required for IgE Production. Increased airway inflammation is often associated with elevated serum IgE concentrations. Despite the increased airway inflammation in naive $LT\alpha^{-/-}$ mice, unexpectedly we found IgE concentrations reduced to nearly undetectable levels (Fig. 2). $LT\alpha^{-/-}$ mice lack both soluble and membrane LT. Either membrane or soluble LT could be required for IgE production; therefore, the sera from $LT\beta^{-/-}$ mice were collected. These mice, which lack only the membrane form of LT, also have low IgE levels. To better define the receptor involvement, serum from $LT\beta R^{-/-}$ mice was collected and found to share the IgE defect observed in $LT\alpha^{-/-}$ mice, thus identifying a new ligand and receptor pair required for IgE production. Interestingly, TNF, another closely related cytokine that shares the same receptors as soluble LT, is dispensable for IgE production (Fig. 2). These data revealed the presence

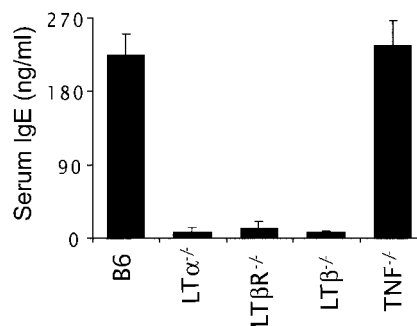


Figure 2. LT is required for IgE production. The sera from various 12–16-wk-old $LT\alpha^{-/-}$ mice ($n = 9$) were collected, and total IgE concentration was measured by ELISA. Data represent the mean \pm SE.

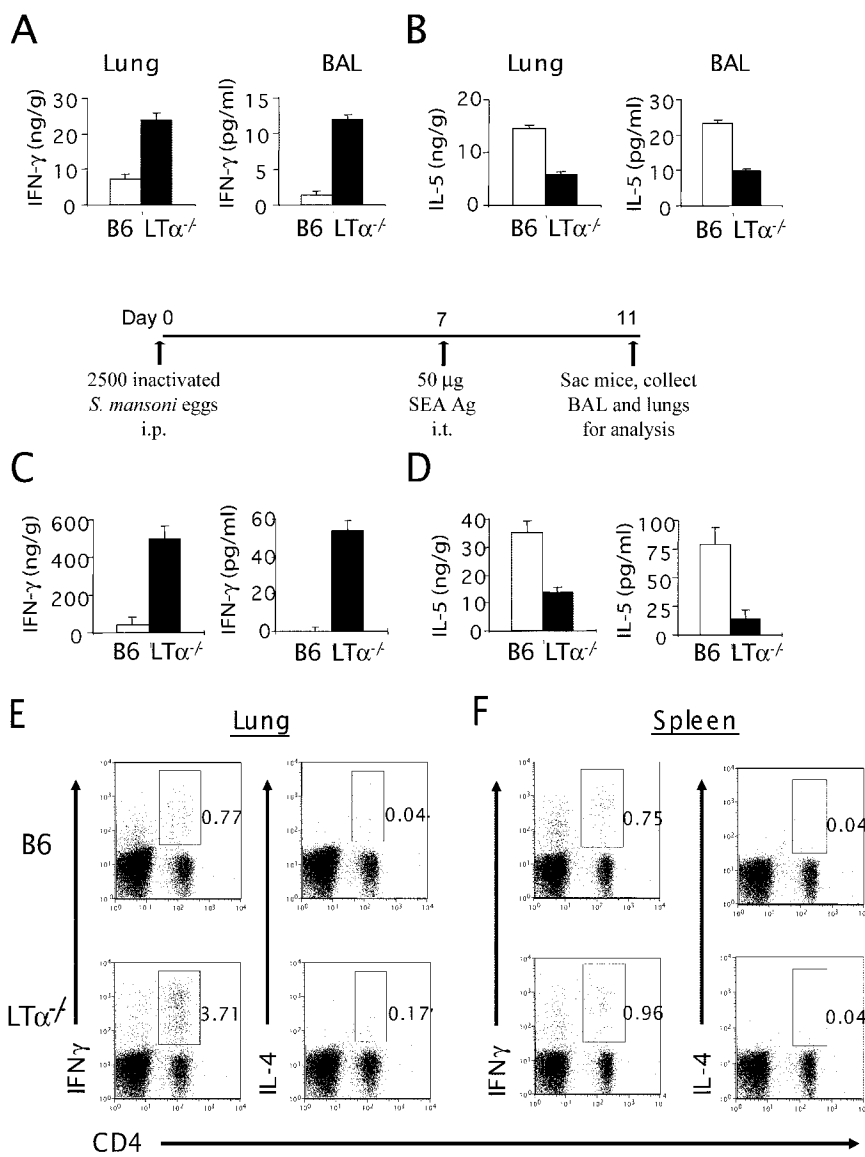


Figure 3. LTα^{-/-} airways display a Th1 phenotype even after a strong Th2 antigen challenge. The lung tissues from B6 and LTα^{-/-} mice (*n* = 3–5 per group) were homogenized in PBS containing proteinase inhibitors, and the supernatants were collected by centrifugation. Cytokines from the BAL were measured after a 1-ml flushing of the airways through the trachea. IFN-γ (A) and IL-5 (B) levels were measured by ELISA from the lung lysates (left) and BAL fluids (right). (C and D) B6 and LTα^{-/-} mice were sensitized i.p. on day 0 with 5 × 10³ inactivated soluble *S. mansoni* egg antigen (SEA). On day 7, the mice were challenged i.t. with SEA. On day 11, mice were killed, and cytokines from the lung and BAL were analyzed. The lung lysates and BAL fluids of those mice were subjected to ELISA. IFN-γ (C) and IL-5 (D) levels are shown (P < 0.05). Data represent the mean ± SD from a representative experiment. Experiments were repeated four times by different individuals with similar results. For intracellular staining, lung cells (E) and splenocytes (F) were collected and stimulated as described in Materials and Methods. Extracellular staining was performed using anti-CD4-PE, and intracellular staining was performed using either IL-4-allophycocyanin or IFN-γ-allophycocyanin.

of membrane LT and its receptor, LTβR^{-/-}, are critical for IgE production.

Mice Deficient in IgE Also Develop Airway Inflammation. Antigen-induced bronchial hyperreactivity and eosinophilic inflammation can occur in the absence of IgE in a mouse model of asthma, but whether there is an increase of airway inflammation without deliberate challenge had not been shown (16). Fixed lung tissues of IgE^{-/-} mice were obtained and showed a chronic airway inflammation by 3–4 mo of age (Fig. 1 A). The infiltration mainly consisted of mononuclear cells, predominantly in the peribronchial and perivascular areas in a pattern similar to LTα^{-/-} mice. Further histopathological analysis of IgE^{-/-} lung tissues revealed bronchial wall thickening, subepithelial fibrosis, increased goblet cell mass, myoblast–myocyte hyperplasia and hypertrophy, epithelial cell hypertrophy, and increased deposition of collagen below the basement membrane. In fact, 6-mo-old BCR^{-/-} mice have also increased infiltra-

tion in both the perivascular and peribronchial areas (unpublished data). Together, these data show that naive IgE^{-/-} mice, similar to the LTα^{-/-} mice, had increased airway inflammation compared with WT controls, which supports the importance of maintaining appropriate IgE levels for airway homeostasis.

The Airway Inflammation of LTα^{-/-} Mice Is Characterized by an Increased Th1 Response. To study the cytokine profile of the lung and BAL of LTα^{-/-} mice, first we measured the levels of IFN-γ, a prototypic cytokine for a Th1 response. The levels of IFN-γ, in both the lung and BAL of LTα^{-/-} mice were markedly higher than those of WT mice in the absence of active challenge (Fig. 3 A). Despite the increased T cell infiltration in their lungs, LTα^{-/-} mice have reduced Th2 cytokines, represented by a dramatic reduction of IL-5 on a per-T cell basis (Fig. 3 B). IL-4 levels in the BAL of LTα^{-/-} mice were also reduced profoundly (2.5 ± 0.8 pg/ml vs. 11 ± 1.8 pg/ml of WT

mice; $P < 0.001$). Together, these data suggest a deliberate Th1-type inflammation.

Stimulation of the airway with allergen may stimulate IgE-bound cells to release an initial burst of cytokines, such as IL-4, which bias the initial response to Th2 (9, 28). Intratracheal challenge of WT mice with SEA, subsequent to priming with *S. mansoni* eggs, results in a robust Th2-mediated lung inflammation (23, 24). To determine whether this Th2-skewing model of airway inflammation could deviate the T cell response in $LT\alpha^{-/-}$ mice, $LT\alpha^{-/-}$ mice were primed and challenged with SEA. $LT\alpha^{-/-}$ mice develop a much more severe airway inflammation in response to SEA challenge than WT mice ($4.6 \pm 0.4 \times 10^6$ cells in BAL and $52 \pm 4.3 \times 10^6$ cells in the lung of $LT\alpha^{-/-}$ mice vs. $1.8 \pm 0.2 \times 10^6$ in BAL and $16 \pm 3 \times 10^6$ in the lung of WT mice; $P < 0.001$). This response remains characterized by Th1 polarization including increased IFN- γ and reduced IL-4 or IL-5 in response to SEA (Fig. 3, C and D). To determine whether the altered cytokine patterns are associated with an increased Th1/Th2 ratio in the lung of naive $LT\alpha^{-/-}$ mice, intracellular staining of anti-IFN- γ and IL-4 in combination with T cell markers was performed (Fig. 3, E and F). Clearly, increased IFN- γ and reduced IL-4 from CD4 $^{+}$ T cells in $LT\alpha^{-/-}$ mice were also

demonstrated. Therefore, the preexisting milieu in the lungs of $LT\alpha^{-/-}$ mice, conditioned by reduced IgE levels, not only resists deviation to a Th2-mediated response, but also exacerbates the overall Th1 inflammatory reaction. To study whether such altered cytokine patterns were specific to the lung, intracellular cytokines of lymphocytes from the spleen of WT mice and $LT\alpha^{-/-}$ mice were studied. Interestingly, cytokine profiles in the spleen were unchanged (Fig. 3 F). These data suggest that the altered cytokine patterns might not be systemic but limited to the lung microenvironment.

IgE Reconstitution Reverses Th1-dominant Airway Inflammation in $LT\alpha^{-/-}$ Mice. $LT\alpha^{-/-}$ mice exhibit multiple defects in addition to IgE deficiency; for example, their absence of LNs, which may contribute to the increase of infiltrates in the lungs (17, 18, 29). To circumvent the numerous defects associated with $LT\alpha^{-/-}$ and define the relevant contribution of IgE, 12-wk-old $LT\alpha^{-/-}$ mice with spontaneous airway inflammation were reconstituted with polyclonal purified murine IgE. After three infusions (once a week for 3 wk), serum IgE concentrations in reconstituted $LT\alpha^{-/-}$ mice peaked at 30–50% of WT levels. When compared with control murine Ig treatments or no treatment, this short-term IgE reconstitution resulted in a

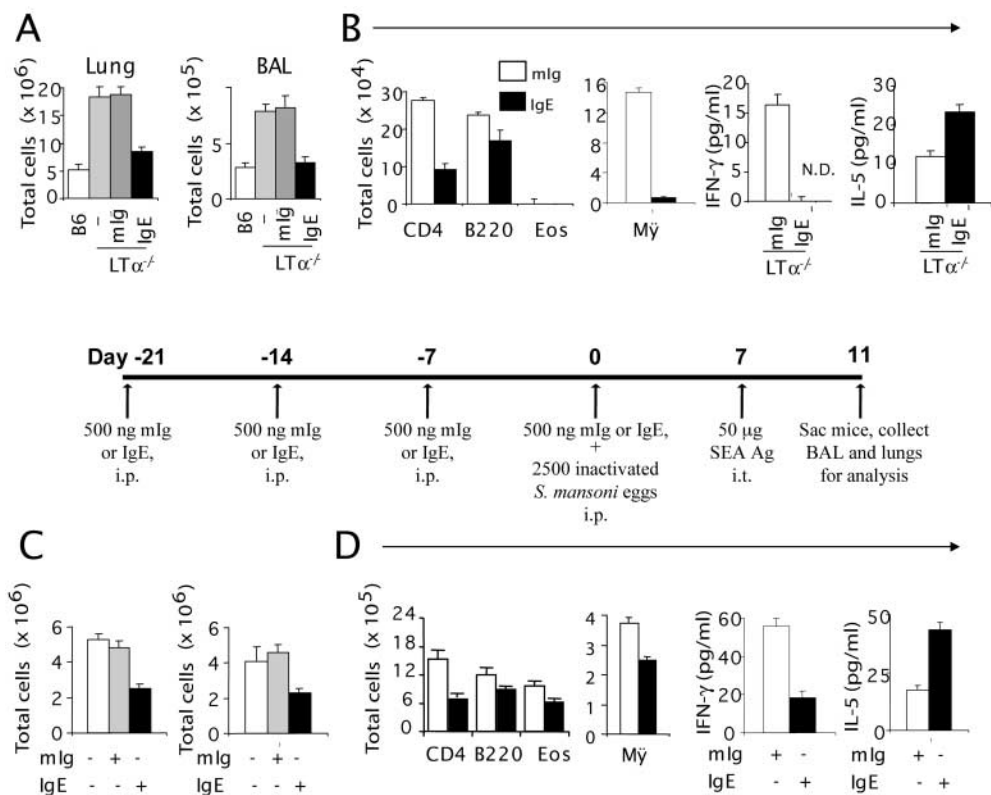


Figure 4. IgE reconstitution of $LT\alpha^{-/-}$ mice results in the reduction of the total cell number and a shift from a Th1 to Th2 cytokine profile in the lung and BAL cells. (A) $LT\alpha^{-/-}$ mice ($n = 3$) were treated i.p. with 500 ng of mouse Ig or purified IgE once per week for 3 wk. 1 wk after the last treatment, the total number of cells in the lung and BAL of B6, mIg-, or IgE-treated $LT\alpha^{-/-}$ mice were analyzed by trypan blue staining. IgE-reconstituted $LT\alpha^{-/-}$ mice have similar total cell numbers compared with control mice ($P < 0.01$). (B) Reconstituted $LT\alpha^{-/-}$ mice have reduced leukocytes in the BAL and lung compared with control treated mice ($P < 0.01$). The cell number was determined by calculating the total number of cells and the absolute number of each cell type from the FACS[®] profile. IFN- γ and IL-5 levels were determined by ELISA from BAL fluids of mIg- or purified IgE-treated $LT\alpha^{-/-}$ mice. ($P < 0.05$; IgE vs. mIg). (C) $LT\alpha^{-/-}$ mice ($n = 3$) were injected i.p. with either 500 ng mouse Ig or purified IgE on day -21, -14, and -7. On day 0, mice were injected with either mIg or IgE and sensitized i.p. with 2.5×10^3 inactivated *S. mansoni* eggs. On day 7, the mice were challenged i.t. with 50 μ g SEA. On day 11, mice were killed and analyzed for the total number of cells in BAL and digested lung ($P < 0.01$; IgE vs. mIg). (D) The type of cells present in the BAL of SEA-challenged and mIg- or IgE-reconstituted $LT\alpha^{-/-}$ mice was determined by calculating the total number of cells and the absolute number of each cell type from the FACS[®] profiles. IFN- γ and IL-5 levels were measured from BAL fluids of SEA-challenged and mIg- or IgE-reconstituted $LT\alpha^{-/-}$ mice ($P < 0.001$ for IFN- γ ; $P < 0.05$ for IL-5; IgE vs. mIg). Data represent the mean \pm SD from a representative experiment. Experiments were repeated four times by different individuals with similar results.

remarkable reduction of cell infiltration in the lung and BAL of $LT\alpha^{-/-}$ mice, predominantly in the $CD4^{+}$ and macrophage population (Fig. 4 A). To further test whether reconstitution of IgE in $LT\alpha^{-/-}$ mice depolarizes the established Th1 microenvironment in addition to alleviating the cellular infiltration, Th1/Th2 cytokine profiles were analyzed. IFN- γ levels in the BAL fluid of IgE-reconstituted $LT\alpha^{-/-}$ mice were reduced to undetectable levels, whereas IL-5 increased dramatically (Fig. 4 B). Consistently, IL-4 levels in BAL fluid in IgE-reconstituted $LT\alpha^{-/-}$ mice were also restored to the level of WT mice (8.6 ± 1.3 pg/ml in reconstituted group; 2.2 ± 0.8 pg/ml in control-treated group). After SEA challenge, the total cell number and cytokine profiles of IgE-reconstituted mice

matched that of WT mice, in both the lung and the BAL (Fig. 4, C and D). IgE reconstitution in $LT\alpha^{-/-}$ mice reduced their airway inflammation and restored the WT Th2 response, implicating IgE deficiency in $LT\alpha^{-/-}$ mice as the central defect responsible for the Th1 airway inflammation in the lungs.

Recapitulation of Th1-dominant Airway Inflammation in WT Mice by Depletion of IgE. To further define the importance of IgE in the airway inflammation, we determined whether reduced IgE in WT mice can sufficiently recapitulate the airway inflammation seen in $LT\alpha^{-/-}$ mice. To deplete IgE, WT mice were treated with mAb against mouse IgE (EM95), once a week for 3 wk. The serum, lung, and BAL cells of the IgE-depleted mice were collected and an-

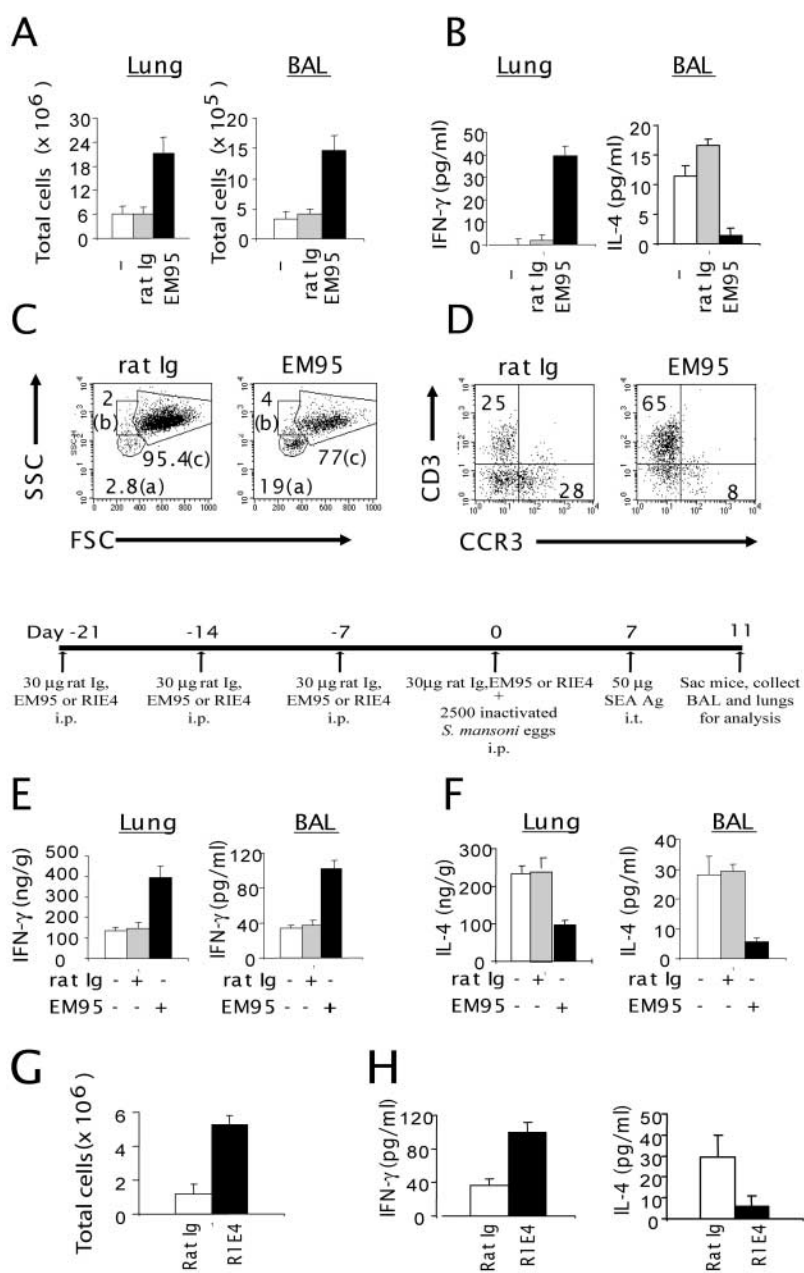


Figure 5. Increased cell number and altered cytokine production in the lung and BAL cells of IgE-depleted WT mice. (A) B6 mice ($n = 3$) were treated i.p. with 50 μ g of rat Ig or anti-IgE (EM95) once per week for 3 wk. 1 wk after the last treatment, the total number of cells in the lung and BAL was analyzed ($P < 0.01$; EM95 vs. control group). (B) IFN- γ and IL-4 levels were determined by ELISA in BAL fluids from B6 mice treated with either rat Ig or anti-IgE ($P < 0.05$; EM95 vs. control group). (C) The forward/side scatter dot-plot profiles were analyzed from B6 mice treated with either rat Ig (left) or EM95 (right). (D) The CCR3 and CD3 expression was analyzed from both the gated lymphocyte (a) and granulocyte population (b). (E) B6 mice were injected i.p. on day -21, -14, and -7 with 30 μ g rat Ig or EM95. On day 0, the mice were injected i.p. with 2.5 $\times 10^3$ inactivated *S. mansoni* eggs. On day 7, the mice were challenged i.t. with SEA. On day 11, mice were killed and analyzed. IFN- γ (E) and IL-4 levels (F) were measured from the lung lysates (left) and BAL fluids (right) by ELISA. Similar experiments were performed using a noncross-linking anti-IgE antibody, R1E4 (G and H). The number of BAL cells (G) and cytokine profiles (H) were determined as described in A and B ($P < 0.01$; R1E4 vs. control group). Data represent the mean \pm SD from a representative experiment. Experiments were repeated four times by different individuals with similar results.

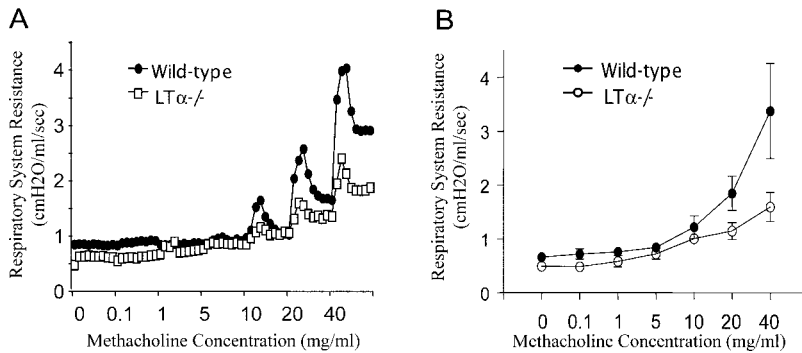


Figure 6. Methacholine dose–response curves for respiratory system resistance in a WT mouse versus an $LT\alpha^{-/-}$ mouse. Invasive measurement was used to determine respiratory system resistance in anesthetized, tracheostomized, mechanically ventilated mice. (A) Dose-dependent bronchoconstriction was induced by i.v. methacholine administration to WT mice versus $LT\alpha^{-/-}$ mice. (B) The peak resistance from each dose from pooled data from three experiments was analyzed to compare both groups ($n = 4$; $P < 0.005$).

alyzed 1 wk after the last treatment, when IgE concentrations were undetectable. The total number of leukocytes in the lung and BAL of EM95-treated WT mice increased to the level of $LT\alpha^{-/-}$ mice (Fig. 5 A). A severalfold increase of immune cells in both BAL and the lung was observed in IgE-depleted mice. Additionally, cytokine expression shifted from the prototypic Th2 to Th1 profile, with profoundly increased IFN- γ levels and reduced IL-4 in the BAL fluid (Fig. 5 B). The percentage of lymphocyte populations in IgE-depleted WT mice (19%) increased compared with mice treated with the control rat Ig (Fig. 5 C, area a, 2.8%). After treatment, the absolute number of CD4⁺, CD8⁺, and B220⁺ cells increased >20-fold, resulting in a profile similar to $LT\alpha^{-/-}$ mice (unpublished data). The granulocyte populations also increased after the anti-IgE treatment, whereas macrophage populations were reduced as shown (Fig. 5 C, areas b and c). Interestingly, the percentage of CCR3⁺ cells was reduced in the BAL of WT mice treated with EM95 (Fig. 5 D). After depletion of serum IgE (day -21, -14, and -7), mice were sensitized and challenged with SEA to test if antigens that favor a Th2 response can reverse the Th1 bias resulting from the lack of IgE. After SEA challenge, IgE-depleted mice had three- to fivefold more leukocytes in both the lung and BAL than WT mice or control Ig-treated mice recapitulating the inflammation in $LT\alpha^{-/-}$ mice ($5.8 \pm 1.8 \times 10^6$ in BAL and $74 \pm 6.3 \times 10^6$ in the lung of anti-IgE-treated mice vs. $1.8 \pm 0.2 \times 10^6$ in BAL and $16 \pm 3 \times 10^6$ in the lung of WT mice; $P < 0.001$). Furthermore, the elevated Th1 cytokine profile in IgE-depleted WT mice after SEA challenge reiterates the role of IgE in minimizing Th1-mediated inflammation (Fig. 5, E and F).

EM95 binds both soluble IgE and surface-bound IgE captured by Fc ϵ RI on mast cells. Cross-linking of the latter in vitro by anti-IgE antibody has been shown to induce mast cell degranulation and release of Th2 cytokines (8). This side effect of EM95 is unlikely to be a confounding factor in our system, as the treated mice show a Th1-skewed profile, including lower IL-4 levels, despite potential anti-IgE-triggered Th2 cytokines from mast cells or other Fc ϵ RI⁺ cells. To avoid mast cell degranulation during anti-IgE treatment, WT mice were treated with a different anti-IgE (R1E4) mAb. R1E4, which binds only serum IgE via the Fc portion, is incapable of causing mast cell

degranulation via IgE cross-linking, so that after sufficient receptor turnover and serum depletion, mast cells are incapable of eliciting IgE-mediated IL-4 production (25). The result from a short-term treatment of WT mice with R1E4 was consistent with our findings with the EM95 treatment as follows: an increase of Th1-skewed airway inflammation even with SEA challenge (Fig. 5, G and H). Consistently, the total number of leukocytes increased three- to fivefold (Fig. 5 H). T and B cells increased two- to threefold, and macrophages increased three- to fourfold, whereas NK cells remained unchanged (unpublished data). The data clearly underscore the importance of IgE in balancing Th1/Th2 responses and controlling airway inflammation in response to both environmental antigens and induced allergen.

Failure to Increase BHR to Nonspecific Challenge in $LT\alpha^{-/-}$ Mice. Increased spontaneous airway inflammation is often associated with increased BHR (3, 7, 8). To evaluate the severity of BHR in $LT\alpha^{-/-}$ mice, we used intravenous delivery of methacholine to assess airway constrictor responsiveness in anesthetized, tracheostomized, mechanically ventilated mice, using the Flexivent computerized mouse ventilator, which calculates respiratory system resistance automatically. Unexpectedly, $LT\alpha^{-/-}$ mice with severe airway inflammation failed to respond to methacholine challenge. $LT\alpha^{-/-}$ mice showed inert response to even a high dose of methacholine when compared with B6 mice (Fig. 6 A). The peak resistance in the bronchoconstrictor response to various methacholine doses from all WT mice and $LT\alpha^{-/-}$ mice are summarized in Fig. 6 B. The overall response in resting $LT\alpha^{-/-}$ mice is lower than that of WT mice (Fig. 6 B, $P < 0.001$ as determined by analysis of variance). In contrast to Th2-dominant airway inflammation with increased BHR seen in WT mice, $LT\alpha^{-/-}$ mice showed a rather distinct Th1 airway inflammation with reduced BHR.

Discussion

In this paper, we have demonstrated that LT is critical for IgE production, and that a physiological role of IgE in the airway is to minimize Th1-dominant airway inflammation. Remarkably, the spontaneous Th1-mediated airway inflammation phenotype of $LT\alpha^{-/-}$ mice, with or without challenge, can be rescued by short-term IgE reconstitution,

and is recapitulated in both IgE^{-/-} mice and IgE-depleted WT mice. These findings demonstrate the indispensable role of physiological levels of serum IgE in maintaining lung homeostasis and BHR as well as modulating a Th1/Th2 balance, thus providing a novel model by which to study how IgE affects airway inflammation.

There may be multiple mechanisms responsible for the control of the Th1/Th2 balance and airway inflammation exerted by IgE. The ability of IgE to enhance mast cell survival and FcεRI expression in vitro has been described previously (30, 31). It is possible that reduced IgE levels in LTα^{-/-} mice impair antigen-mediated mast cell degranulation and cytokine production, such as IL-4, that are required for a Th2 response. The resulting reduction in IL-4 may lead to a Th1-dominated inflammatory response. Whether the defect is due to mast cell maturation defects, reduced IgE avidity on the mast cell surface, or perhaps defects in other IgE binding cells remains to be investigated.

In this paper, we have demonstrated clearly that impaired IgE levels and increased Th1 responses occur in LTα^{-/-} mice with or without antigen challenge. However, recent works evaluated the role of LNs in the development of airway inflammation using LTα^{-/-} mice and found the generation of Th2-mediated airway inflammation could still occur in LTα^{-/-} mice (20, 21). Although baseline IgE levels have not been determined in these analyses, antigen-specific IgE responses can be detectable in OVA-GM-CSF-challenged LTα^{-/-} mice, and both Th1-associated IFNγ and Th2-associated IL-5/IL-13 were higher in OVA-GM-CSF-challenged LTα^{-/-} mice, suggesting that LNs are not essential for Th2-mediated airway inflammation (20). Several differences may contribute to the discrepancies between the analyses as follows. First, OVA-GM-CSF very strongly activates the Th2 pathway and may overcome the defect in LTα^{-/-} mice after repeated challenge. Second, it is possible that the relative increase in IFN-γ may be accountable for the dominating Th1 response in LTα^{-/-} mice. In fact, all three models have shown increased IFN-γ levels. Third, the age of the mice may also provide an explanation for the discrepancies between the two analyses. 5–7-wk-old mice were used in the previous two studies, whereas 12–16-wk-old mice were used in our study. We have found the level of baseline IgE is undetectable in 5–7-wk-old B6 mice but gradually elevates after 8 wk. We have also noticed that it is difficult to induce Th1-dominant airway inflammation in 5–6-wk-old LTα^{-/-} mice (unpublished data). Fourth, our work has used freshly isolated cells from the lung, whereas other works used restimulated splenocytes in vitro. Furthermore, different antigens/adjuvant, challenge route, and the assays for cytokine analysis may also account for potential discrepancies.

Our paper implies that a physiological role of IgE in the lung is to minimize airway inflammation via balancing Th1/Th2 responses. We hypothesize a possible mechanism in which physiological levels of IgE allow controlled degranulation and cytokine production of mast cells or other

cells at levels required to routinely clear airway antigens without provoking excessive inflammatory infiltration. When IgE concentrations are excessively low, IgE-mediated mast cell responses could be impaired, resulting in a poor ability to clear the inhaled environmental antigen. The accumulated antigens lead to the increase of infiltrates in the lung responding to retained antigen may actually cause tissue damage. Our paper has an impact on the recent administration of anti-IgE in the clinic. High IgE levels during inflammation or other pathological conditions that favor Th2 responses or asthmatic patients with pathologically high IgE concentrations may benefit from anti-IgE therapy in some cases. However, depletion of IgE resulting in levels below normal may trigger a destructive Th1 inflammation. Conversely, there may be a subset of yet unrecognized patients for whom lower IgE concentrations may cause a severe Th1-mediated airway inflammation. It will be interesting to determine whether such patients have low IgE levels and reduced BHR. Thus, a physiological role of IgE in the airway is required for balancing both Th1 and Th2 responses to prevent immoderate inflammation dominated by either.

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