

Leukotriene A₄ Hydrolase Inhibition Attenuates Allergic Airway Inflammation and Hyperresponsiveness

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Rationale: Allergic asthma is characterized by reversible airway obstruction, lung inflammation, and airway hyperresponsiveness (AHR). Previous studies using leukotriene B₄ (LTB₄) receptor 1-deficient mice and adoptive transfer experiments have suggested that LTB₄ plays a role in lung inflammation and AHR.

Objectives: In this study, we used a leukotriene A₄ hydrolase (LTA₄H) inhibitor as a pharmacological tool to directly examine the role of LTB₄ in a mast cell-dependent murine model of allergic airway inflammation.

Methods: We used the forced oscillation technique to test the effects of an LTA₄H inhibitor dosed during the challenge phase on AHR. Lung tissue and lavage were collected for analysis.

Measurements and Main Results: Treatment with an LTA₄H inhibitor improved multiple parameters encompassing AHR and lung function. Significant decreases in inflammatory leukocytes, cytokines, and mucin were observed in the lung lumen. Serum levels of antigen-specific IgE and IgG1 were also decreased. Labeled antigen uptake by lung dendritic cells and subsequent trafficking to draining lymph nodes and the lung were decreased on LTA₄H inhibitor treatment. Provocatively, inhibition of LTA₄H increased lipoxin A₄ levels in lung lavage fluid.

Conclusions: These data suggest that LTB₄ plays a key role in driving lung inflammation and AHR. Mechanistically, we provide evidence that inhibition of LTA₄H affects recruitment of both CD4⁺ and CD8⁺ T cells, as well as trafficking of dendritic cells to draining lymph nodes, and may beneficially modulate other pro- and anti-inflammatory eicosanoids in the lung. Inhibition of LTA₄H is thus a potential therapeutic strategy that could modulate key aspects of asthma.

Keywords: leukotriene B₄; leukotriene A₄ hydrolase; lung function; T cells; dendritic cells

Leukotriene B₄ (LTB₄) is a potent inflammatory mediator that is sequentially generated from arachidonic acid by cytosolic phospholipase A₂, 5-lipoxygenase (5-LO), and leukotriene A₄ hydrolase (LTA₄H) (1). LTB₄ is a potent chemotactic factor for numerous cells involved in airway inflammation and airway hyperresponsiveness (AHR), such as neutrophils, macrophages, eosinophils, monocytes, mast cell progenitors, and effector T cells (2–5). LTB₄ functions by binding to two G-protein-coupled receptors, leukotriene B₄ receptor 1 and 2 (BLT1 and BLT2) (6, 7).

Allergic asthma is characterized by reversible airway obstruction, lung inflammation, and AHR. Elevated levels of LTB₄ have been reported in a number of inflammatory disorders, including sputum and bronchoalveolar lavage (BAL) samples from patients with chronic obstructive pulmonary disease, allergy, and asthma (8–10). In addition, LTA₄H levels

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Leukotriene B₄ (LTB₄) is a key inflammatory mediator capable of attracting and activating multiple cell types. It is increased in allergic airway disease but its exact role in processes leading to airway hyperreactivity is not well defined.

What This Study Adds to the Field

Pharmacological inhibition of leukotriene A₄ hydrolase decreases LTB₄ in the airways in a mouse asthma model and attenuates airway inflammation and airway hyperreactivity through modulation of T cell and dendritic cell function.

are increased in circulating neutrophils and the airways of patients with asthma (11, 12). Evaluation of lung inflammation in BLT1-deficient mice on either the Balb/C or C57/B6 genetic background has yielded conflicting results. In Balb/C mice, no changes were seen in serum IgE or airway eosinophilia, whereas BLT1-deficient B6 mice showed a dramatic decrease in both parameters (13, 14). However, both studies reported attenuated AHR as well as decreased levels of IL-13 in BAL fluid. More recently mast cells were identified as the main source of LTB₄ that control the recruitment of IL-13-producing T cells (15).

Clinically, inhibition of cysteinyl leukotriene (CysLT) activity by agents such as montelukast has proved an effective treatment for various aspects of asthma, at least in a portion of patients (16), whereas attenuation of both CysLT and LTB₄ production through the upstream inhibition of 5-LO have suggested additional clinical benefit through this approach (17). In addition, genetic polymorphisms in both the 5-LO and LTA₄H gene are associated with asthma susceptibility (18) and variation in response to montelukast therapy (19), suggesting potential clinical usefulness for LTB₄ inhibition.

We have recently described a potent, orally bioavailable LTA₄H inhibitor (LTA₄H-I) that selectively blocks the production of LTB₄ (20). In the present study, we evaluated the effect of this LTA₄H inhibitor on allergen-induced airway responses in a mast cell-dependent murine model. In addition, we explored the mechanism of action of this inhibitor by examining effects on T-cell and dendritic-cell (DC) migration. Some of these results have been previously reported in the form of an abstract (21).

METHODS

Mice

Balb/c mice (female, 6–8 wk old) were purchased from Charles River Laboratories (Hollister, CA). The animal handling and study protocol was approved by the Institutional Animal Care and Use Committee. Mice were sensitized with seven intraperitoneal injections of ovalbumin (OVA) and challenged three times with intranasal OVA as

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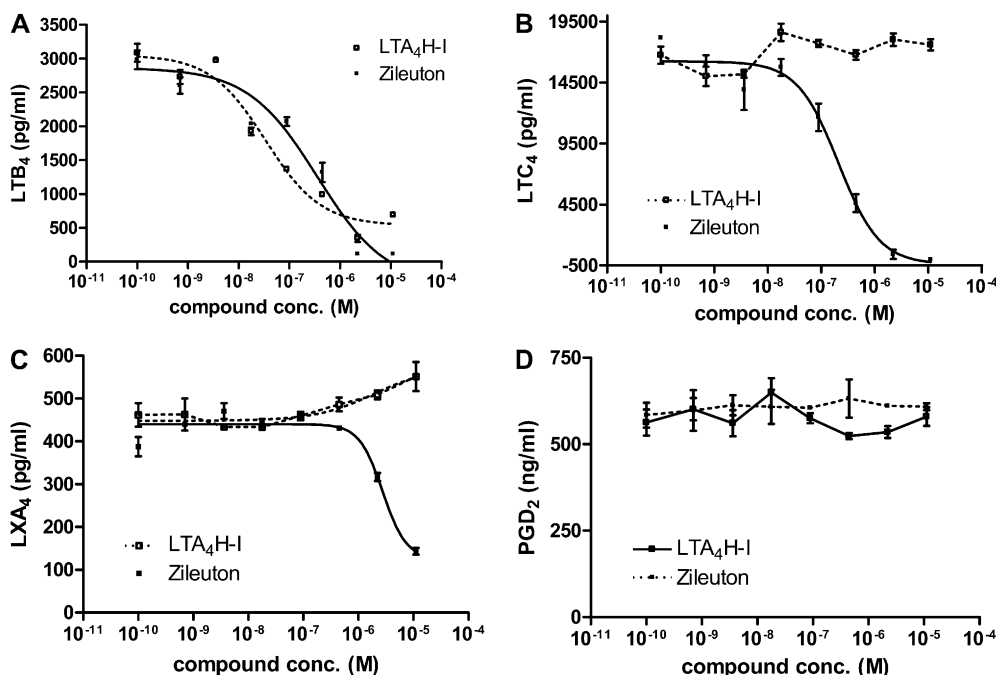


Figure 1. Effect of a leukotriene A₄ hydrolase inhibitor (LTA₄H-I) on murine bone marrow-derived mast cells (BMMCs). BMMCs were sensitized with antitrinitrophenyl IgE, preincubated with the indicated concentrations of an LTA₄H-I and zileuton, and then stimulated with TNP. (A) Leukotriene B₄ (LTB₄), (B) leukotriene C₄ (LTC₄), (C) lipoxin A₄ (LXA₄), and (D) prostaglandin D₂ (PGD₂) levels in culture supernatant were measured by enzyme immunoassay. The vehicle controls for each assay (mean ± SD of four replicates) were 2,911 ± 77 pg/ml (LTB₄), 15,433 ± 976 pg/ml (LTC₄), 480 ± 23 pg/ml (LXA₄), and 582 ± 40 ng/ml (PGD₂).

previously described (22); the study protocol is described in detail in the online supplement. Animals were orally dosed with a 20% cyclodextrin, vehicle (control), or JNJ 26993135, a selective LTA₄H-I (10, 25, and 50 mg/kg twice daily) during the challenge phase and 30 minutes before OVA challenge. JNJ 26993135 was formulated as previously described (20). Anti-mouse IL-13 monoclonal antibody, kindly provided by Dr. William Glass (Centocor Inc, Malvern, PA) was administered twice intravenously during the challenge phase (500 µg) (23).

Airway Function

Airway function was measured using a small animal ventilator (Scireq, Montreal, Canada) as previously described (24). The airway function protocol is described in detail in the supplemental data section.

BAL and Leukotriene Analysis

After killing, BAL samples were obtained as previously described (25). Cell-free supernatants were immediately frozen for subsequent cytokine analysis. Leukotriene C₄ (LTC₄) (Caymen 5205201), lipoxin A₄ (LXA₄) (Oxford Biomedical EA45), prostaglandin D₂ (PGD₂) (Caymen 512021), and LTB₄ (Assay Designs 901-068) levels were measured from mouse BAL samples, as previously described (26).

OVA-specific T-Cell Responses *Ex Vivo* and Cytokine Analysis

Peribroncholar lymph node (PBLN) cells and cells from phosphate-buffered saline (PBS)-perfused lungs were isolated and stimulated in quadruplicate as previously described (25, 27). Cytokine levels in cell culture supernatants were determined using Luminex multiplex system (Linco Research, Auburn, CA) as per manufacturer's protocol. Cytokine levels of interest were confirmed using individual ELISA assays (R&D, Minneapolis, MN).

Determination of Total and OVA-specific Serum Ig Levels

Serum samples were obtained from animals by cardiac puncture. Total IgE, IgG1, and IgG2a levels were measured as per manufacturer's instructions (BD Biosciences, San Jose, CA) and are described in detail in the online supplement. To ensure assay linearity, serial dilutions of serum were assayed as described previously (25).

Mucin Enzyme-linked Lectin Assay

Mucin levels were detected by enzyme-linked lectin assay as previously described (28) using *Ulex europaeus* agglutinin-1 (UEA-1; Sigma, St. Louis, MO) and is described in detail in the online supplement.

TABLE 1. RESPIRATORY MECHANICS IN MECHANICALLY VENTILATED MICE TREATED WITH LEUKOTRIENE A₄ HYDROLASE-I, DEXAMETHASONE, OR ANTI-IL-13

	R (cm H ₂ O · s/ml)	C (cm H ₂ O · s/ml)	R _N (cm H ₂ O · s/ml)	G (cm H ₂ O/ml)	H (cm H ₂ O/ml)
Naive	1.1 ± 0.2	0.0329 ± 0.0001	0.57 ± 0.15	5.97 ± 0.7	25 ± 2.2
Vehicle	10.5 ± 1.3	0.0066 ± 0.001	2.04 ± 0.41	92.3 ± 17	213 ± 26
10 mpk	9.39 ± 1.2	0.0051 ± 0.0009	1.71 ± 0.19	82.1 ± 28	199 ± 37
25 mpk	6.60 ± 1.2*	0.0084 ± 0.003	1.53 ± 0.22*	44.5 ± 17†	68.0 ± 26†
50 mpk	2.79 ± 0.8†	0.0131 ± 0.004*	0.70 ± 0.07‡	17.6 ± 5.5‡	69.0 ± 18†
Anti-IL-13	1.65 ± 0.3‡	0.0245 ± 0.003‡	0.84 ± 0.13‡	9.17 ± 1.5‡	29.8 ± 2.9‡
Dex	1.23 ± 0.7‡	0.033 ± 0.009‡	0.55 ± 0.3‡	7.2 ± 3.3‡	26.3 ± 8.5‡

Definition of abbreviations: C = airway compliance; Dex = dexamethasone; G = peripheral tissue damping; H = tissue elastance; R = airway resistance; mpk = mg/kg; R_N = Newtonian resistance.

Mean values with SD are listed (n = 5–8 mice/group) at the high dose of methacholine challenge (50 mg/ml). A one-way analysis of variance with a Bonferroni posttest was used to determine statistical significance.

* P < 0.05.

† P < 0.01.

‡ P < 0.001.

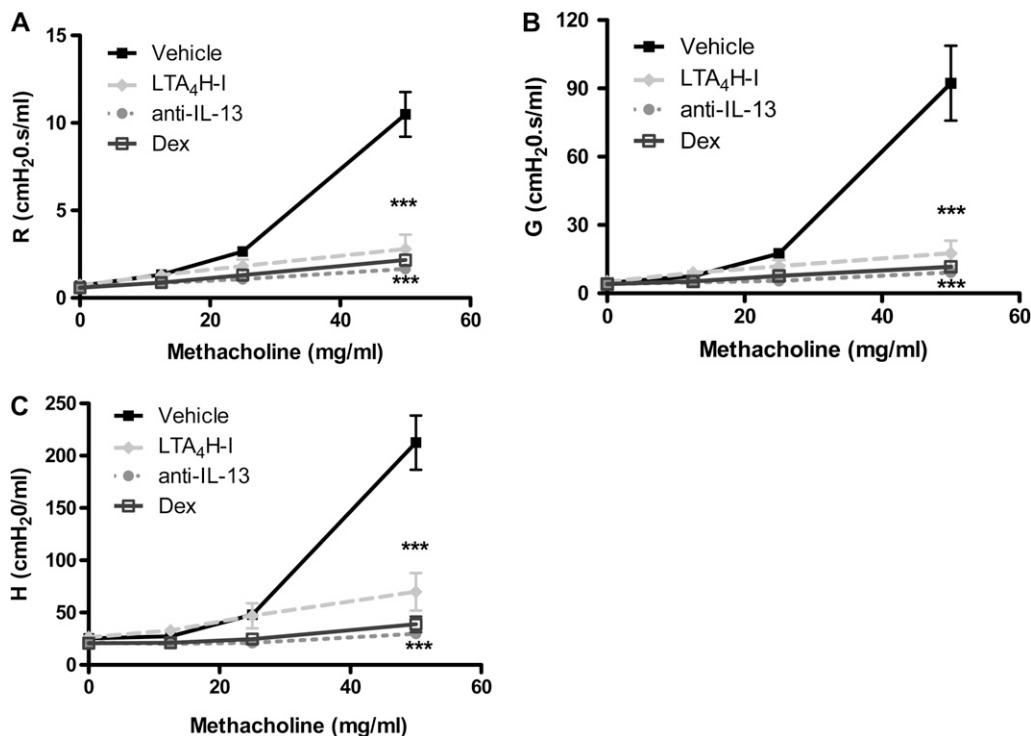


Figure 2. (A–C) Methacholine dose–response relationships for airway resistance (R), tissue damping (G), and tissue stiffness (H). Each plotted value reflects the mean values for each group of mice ($n = 6$ –10/group). Each animal's value reflects the mean of 12 sets of data captured over a 3-minute span after an aerosol methacholine challenge. Leukotriene A₄ hydrolase inhibitor (LTA₄H-I)-treated mice received a 50-mg/kg (mpk) dose, dexamethasone (dex)-treated mice received a 1-mpk dose, and anti-IL-13-treated mice received two 500- μ g intravenous doses. Statistical significance of each treatment group compared with control vehicle-treated animals was calculated using a one-way analysis of variance (* $P < 0.05$; *** $P < 0.001$).

Mast Cell Activation Assay

Bone marrow-derived mast cell (BMMC) preparation and antitrinitrophenyl assay conditions are described in detail in the online supplement.

Dendritic Cell Migration

Dendritic cell migration was assayed as previously described (27) and is described in detail in the online supplement.

Statistics

A one-way analysis of variance with a Bonferroni posttest or a Student t test was used to judge statistical significance as appropriate. In all cases the P values were calculated based on a comparison with vehicle-

treated animals. The error bars shown represent the SD. In all cases the experiments were repeated two to three times with similar results and representative data are shown.

RESULTS

An LTA₄H-I Blocks Mast Cell Production of LTB₄ and Suppresses AHR

Using a recently described LTA₄H inhibitor (20), we began by examining the efficacy of this compound and zileuton on primary, sensitized mast cells in response to IgE cross-linking

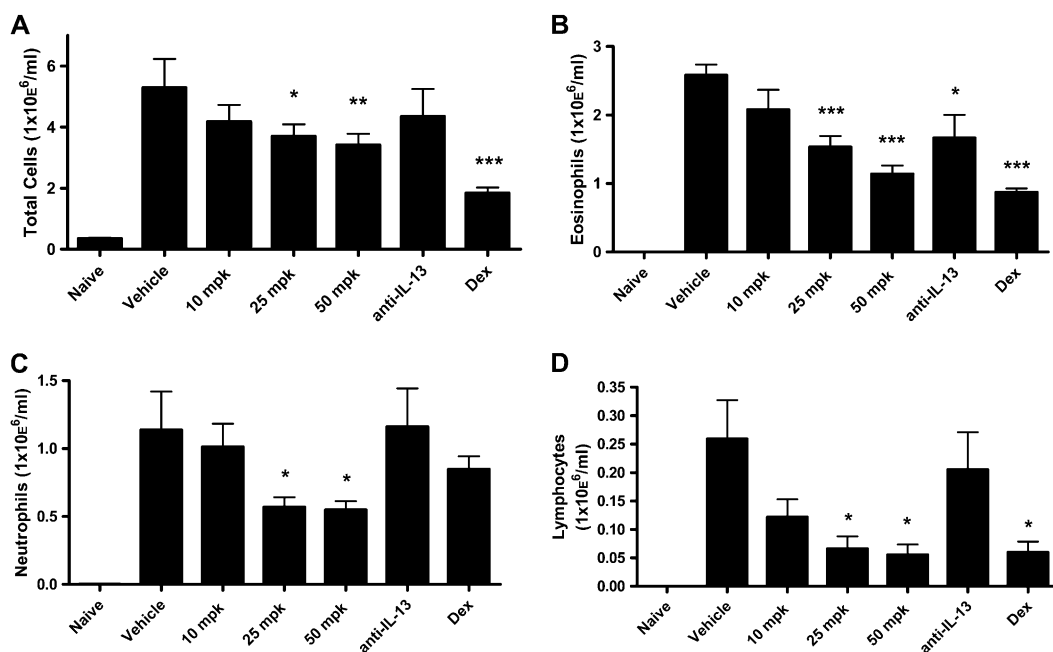


Figure 3. Dose–response relationship of a leukotriene A₄ hydrolase inhibitor, anti-IL-13, and dexamethasone (dex) on airway inflammation in Balb/C mice ($n = 8$ –10 animals/group). (A) The total number of cells and a differential cell count for (B) eosinophils, (C) neutrophils, and (D) lymphocytes were calculated from BAL fluid collected after the final ovalbumin challenge. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; one-way analysis of variance.) mpk = mg/kg.

TABLE 2. CYTOKINE AND CHEMOKINE LEVELS IN THE BRONCHOALVEOLAR LAVAGE FLUID

	IL-4	IL-5	IL-13	IFN- γ	MCP-1	Eotaxin
Naive	3 $\pm$ 4	6 $\pm$ 3	3 $\pm$ 2	2 $\pm$ 4	1 $\pm$ 1	0
Vehicle	95 $\pm$ 29	102 $\pm$ 42	538 $\pm$ 133	28 $\pm$ 11	130 $\pm$ 35	131 $\pm$ 54
10 mpk	78 $\pm$ 23	80 $\pm$ 51	295 $\pm$ 95*	27 $\pm$ 13	126 $\pm$ 52	126 $\pm$ 61
25 mpk	59 $\pm$ 32*	71 $\pm$ 36	153 $\pm$ 59†	33 $\pm$ 16	82 $\pm$ 36*	109 $\pm$ 50
50 mpk	35 $\pm$ 18‡	51 $\pm$ 19*	42 $\pm$ 53†	31 $\pm$ 13	41 $\pm$ 20†	63 $\pm$ 41*
Anti-IL-13	102 $\pm$ 39	68 $\pm$ 34	796 $\pm$ 298	36 $\pm$ 23	96 $\pm$ 44	80 $\pm$ 41

Definition of abbreviations: MCP-1 = monocyte chemoattractant protein-1; mpk = mg/kg.

Mean cytokine or chemokine values (pg/ml) with SD are listed (n = 9–10 mice/group). A one-way analysis of variance with a Bonferroni posttest was used to determine statistical significance.

* $P < 0.05$.

† $P < 0.001$.

‡ $P < 0.01$.

ex vivo. We observed potent inhibition of LTB₄ by both the LTA₄H-I and zileuton (calculated inhibitory concentration of 50% of 34 nM and 354 nM, respectively, Figure 1A). Zileuton inhibited both LTC₄ (IC₅₀ of 209 nM) and LXA₄ production (IC₅₀ of 260 nM), whereas the LTA₄H-I did not (Figures 1B and 1C). In fact, we observed a trend toward increased LXA₄ production when BMDCs were treated with the LTA₄H-I. We did not observe inhibition of PGD₂ production (Figure 1C) or mast cell degranulation as assessed by β -hexosaminidase release (data not shown).

To study the role of LTA₄H in an allergic lung inflammatory response, Balb/C mice were subjected to an OVA-induced lung inflammation protocol (26). Based on reports that BLT1-deficient mice have decreased levels of IL-13 in the BAL fluid (13, 14), we included an anti-mouse IL-13 antibody as well as the glucocorticoid steroid dexamethasone (dex) as a positive control in our studies. We used an invasive technique to examine lung function and AHR in a chronic, mast cell-dependent model (22). After methacholine provocation, OVA-challenged mice exhibited robust increases in all of the measured parameters compared with PBS-challenged animals

(Table 1). As previously described (29), anti-IL-13 significantly decreased airway resistance (Figure 2A). Treatment with an LTA₄H-I was statistically as efficacious as anti-IL-13 (Figure 2A) or dex, and exhibited a dose-dependent effect (Table 1).

To examine potential changes in the lung, we also gathered data derived from the constant phase model of respiratory mechanics. Tissue damping (G was also significantly decreased in mice treated with an LTA₄H-I, dex, or anti-IL-13 (Figure 2B). Other constant phase model parameters were also significantly decreased in mice treated with IL-13, dex, or LTA₄H-I (Table 1). These parameters included Newtonian resistance (R_N), a measure of resistance in the central airways, and tissue elastance (H), a measure of tissue stiffness comprising both the parenchymal and peripheral airways (Figure 2C).

LTA₄H Inhibition Blocks Lung Inflammation

Inflammatory leukocyte influx into the lung lumen was confirmed by dramatically increased cell counts in BAL fluid from OVA-challenged versus naive mice (Figure 3). Treatment with an LTA₄H-I significantly decreased total cells, neutrophils, and lymphocytes in the BAL fluid in a dose-dependent manner (Figures 3A–3D). Highly significant decreases in eosinophil numbers ($P < 0.001$) were also observed (Figure 3B). Treatment with anti-IL-13 antibody only modulated eosinophilia, whereas treatment with dex was generally antiinflammatory except for the neutrophilia. We observed no change in macrophage cell counts with either LTA₄H-I or anti-IL-13 treatment (data not shown).

Modulation of Th2 Cytokines and Mucin Levels

We next examined relevant chemokine and Th2 cytokine levels in the BAL samples. The LTA₄H-I dose-dependently decreased the levels of a panel of inflammatory cytokines and chemokines (Table 2). Significant reductions in IL-5, eotaxin, and monocyte chemoattractant protein-1 were observed, with highly significant reductions seen in IL-4 and IL-13 (Figure 4A). We did not observe a change in IFN- γ levels with inhibitor treatment, suggesting that there was no general skewing of the immune

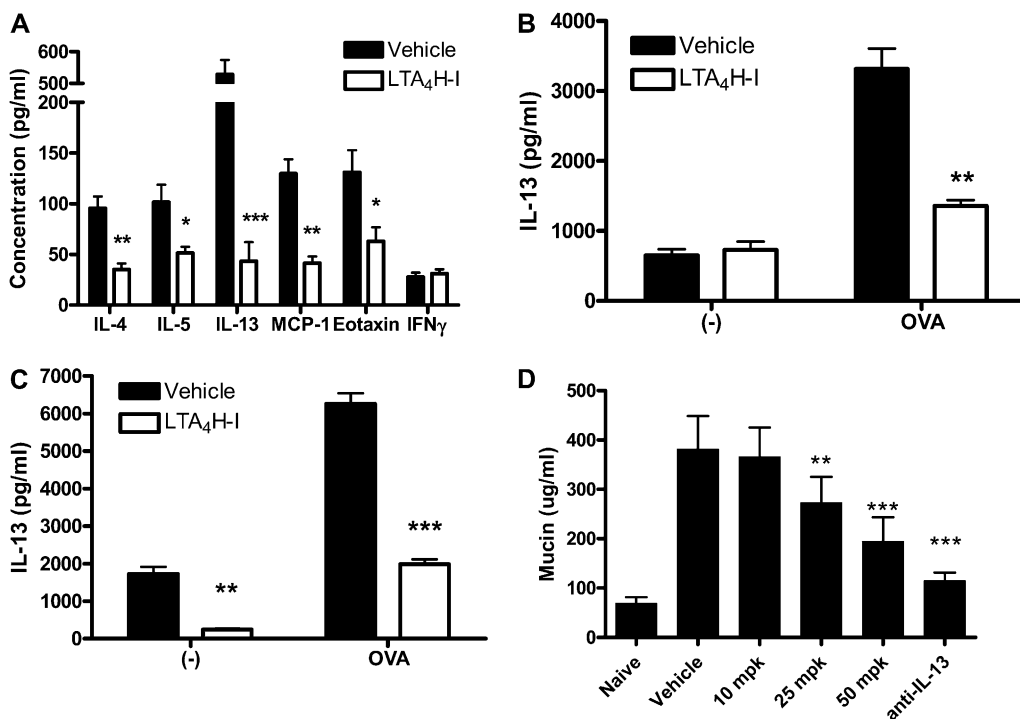


Figure 4. (A) A leukotriene A₄ hydrolase inhibitor (LTA₄H-I) decreases Th2-associated cytokines and chemokines in bronchoalveolar lavage (BAL) fluid. Cell-free BAL fluid from vehicle and inhibitor-treated mice (50-mg/kg [mpk] dose group) was assayed for the indicated cytokines (n = 8–10 animals/group). *Ex vivo* restimulation of T cells isolated from (B) lung or (C) peribronchiolar lymph nodes of vehicle or LTA₄H-I-treated mice. Cells were isolated and cultured without stimulation (–) or stimulated with ovalbumin (OVA) (+) as described in the METHODS. (D) Dose-response relationship of an LTA₄H inhibitor and anti-IL-13 on mucin levels in the BAL fluid. Mucin levels were quantified using an enzyme-linked lectin assay as described in the methods. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; one-way analysis of variance.)

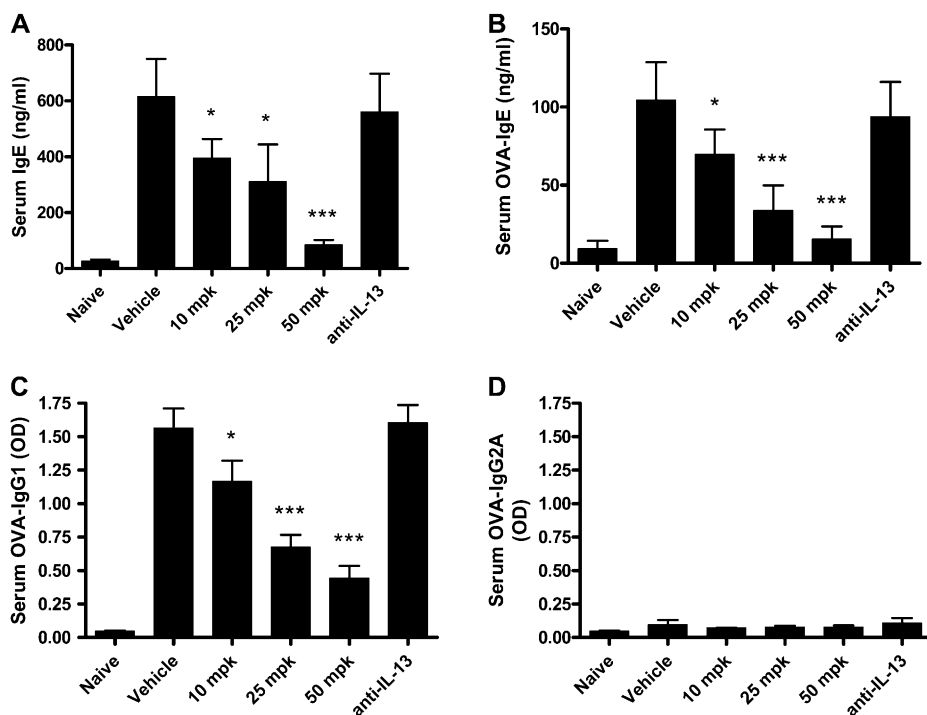


Figure 5. Dose-response relationship of a leukotriene A₄ hydrolase (LTA₄H) inhibitor and anti-IL-13 on antibody levels in serum from each treatment group (n = 6–10 mice). Serum samples were collected by cardiac puncture and assayed by ELISA for the presence of (A) total IgE, (B) ovalbumin (OVA)-specific IgE, (C) OVA-specific IgG1, and (D) OVA-specific IgG2A. (**P* < 0.05; ***P* < 0.01; ****P* < 0.001; one-way analysis of variance.) mpk = mg/kg.

system to a Th1 response. Given the dramatic decrease observed in BAL IL-13 levels on treatment with an LTA₄H-I, we next focused on T-cell production of IL-13 in response to *ex vivo* stimulation with OVA. Lung T cells produced high levels of IL-13 on restimulation, and LTA₄H-I treatment *in vivo* resulted in a significant decrease in IL-13 production *ex vivo* (Figure 4B). In the case of T cells from PBLN, both nonstimulated and OVA-stimulated T cells produced significantly decreased levels of IL-13 (Figure 4C). We observed no proliferative defect of these T cells to antigen *ex vivo* (data not shown).

Next, we used a lectin-based assay to measure levels of mucin in the lung lumen contents. We observed a significant

increase in mucin levels in the BAL fluid of vehicle-treated, OVA-challenged animals when compared with naive control animals (Figure 4D). Treatment with an anti-IL-13 antibody was extremely efficacious in decreasing mucin levels. Mucin levels were also significantly decreased in mice treated with an LTA₄H-I in a dose-dependent manner (Figure 4D).

An LTA₄H-I Decreases Serum IgE and IgG1

When compared with a traditional OVA model, the mast cell-dependent model results in much higher levels of total serum IgE (115 ng/ml vs. 665 ng/ml) and OVA-specific IgE (40 ng/ml vs. 311 ng/ml) (data not shown). Although extremely effective

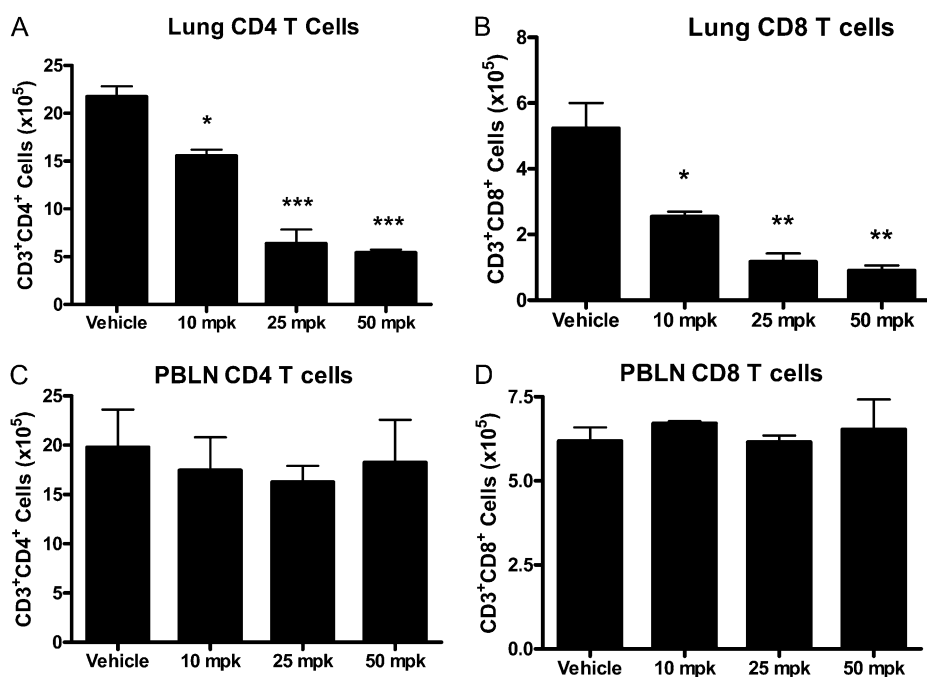


Figure 6. Dose-response relationship of a leukotriene A₄ hydrolase (LTA₄H) inhibitor on T cells in the lung and draining peribronchiolar lymph nodes. T cells were purified from lung and lymph nodes as described in methods (n = 6–8 animals/group). The number of T cells was measured using CD3, CD4, and CD8 surface staining by fluorescence-activated cell sorter. (**P* < 0.05; ***P* < 0.01; ****P* < 0.001; one-way analysis of variance.) mpk = mg per kg; PBLN = peribronchiolar lymph node.

at decreasing AHR, an anti-IL-13 antibody did not significantly decrease IgE and IgG1 levels (Figure 5). In contrast, treatment with an LTA₄H-I resulted in a statistically significant, dose-dependent decrease in total serum IgE as well as OVA-specific IgG1 and IgE (Figures 5A–5C). We did not observe a marked increase in IgG_{2A} in this model (Figure 4D). Collectively, these data indicated that an LTA₄H-I affected multiple features of allergic airway inflammation.

An LTA₄H-I Decreases Both CD4⁺ and CD8⁺ T Cells in the Lung and DC Trafficking to the Lung and Lymph Nodes

Based on the findings above, we decided to explore the mechanism by which an LTA₄H inhibitor might affect leukocyte trafficking in the allergic airways. Recent studies using BLT1-deficient mice have indicated that LTB₄ plays a key role in orchestrating lung inflammation by regulating effector T-cell recruitment (13, 30). Treatment of mice with an LTA₄H-I significantly reduced the numbers of both CD4⁺ and CD8⁺ T cells in the lung tissue (Figures 6A and 6B) and lumen (Figure 3D). However, we did not observe a change in the number of CD4⁺ and CD8⁺ T cells in PBLN or spleen (Figures 6C and 6D and data not shown).

Although we did not observe a decrease in total T cells in PBLN, *ex vivo* restimulation of T cells from either lung or PBLN resulted in decreased cytokine production, such as IL-13 (Figures 4B and 4C). These observations raised the possibility that blocking LTB₄ in the lung might regulate T-cell priming in the draining lymph node (31). To directly test whether inhibition of LTA₄H effects DC migration, we challenged OVA-sensitized mice with Alexa488-OVA via intranasal instillation and monitored the trafficking of Alexa488⁺ DC subsets to the lung-draining lymph nodes and the lung (Figure 7). Treatment with an LTA₄H-I decreased migration of CD8 α ⁺ DCs to the PBLNs and the lung (Figures 7B and 7C). As has been reported for BLT1-deficient DCs *in vitro* (32), blocking LTB₄ *in vivo* resulted in a decrease of CCR7⁺ DCs in the draining lymph nodes and the lung (Figures 7B and 7C).

An LTA₄H-I Decreases LTB₄ Levels in Lung Lavage Fluid while Increasing LXA₄

After challenge with antigen, BAL fluid from vehicle-treated mice contained detectable levels of LTB₄ (232 $\pm$ 26 pg/ml,

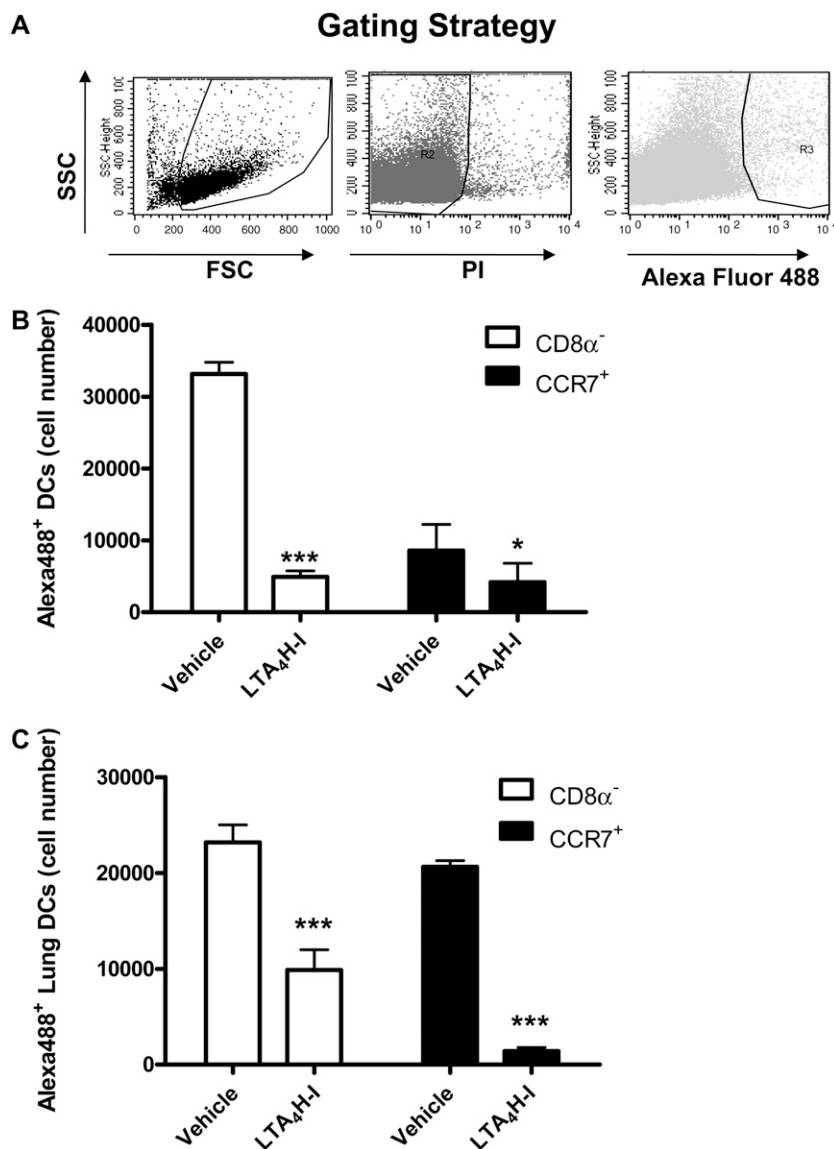


Figure 7. Inhibition of leukotriene B₄ (LTB₄) production decreases lung dendritic cell (DC) migration to draining lymph nodes. Mice were challenged three times with ovalbumin (OVA), the third time being with Alexa488-OVA. Lungs and draining lymph node cells were collected 18 hours later. Two groups (n = 6–8/group) consisting of vehicle or leukotriene A₄ hydroxylase inhibitor (LTA₄H-I)-treated mice (50-mg/kg dose) were analyzed. (A) Dead cells were excluded based on PI staining and Alexa488⁺ cells were gated for further analysis. (B) Total numbers of Alexa488⁺ DC subsets from (B) the lymph node and (C) the lung expressing CD8 α or CCR7 were calculated based on the percentage of positive cells and total number of cells analyzed. FSC = forward scatter, PI = propidium iodide, SSC = side scatter.

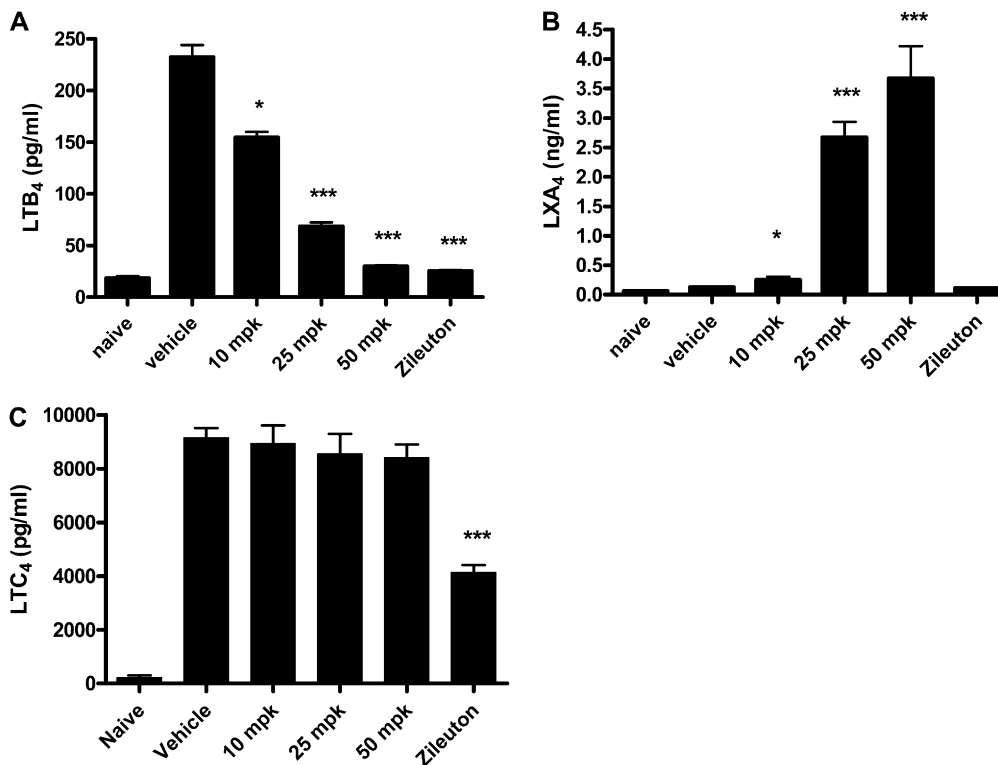


Figure 8. Inhibition of leukotriene A₄ (LTA₄) hydrolase decreases leukotrienes and increases lipoxins in the bronchoalveolar lavage (BAL) fluid. Mice were challenged with ovalbumin (OVA) and BAL fluid was collected 24 hours later. Cell-free BAL fluid from mice treated with vehicle, leukotriene A₄ hydrolase inhibitor (10, 25, and 50 mg/kg [mpk]), and zileuton (50 mpk) (*n* = 8 mice/group) were assayed for (A) leukotriene B₄ (LTB₄) levels, (B) lipoxin A₄ (LXA₄) levels, and (C) leukotriene C₄ (LTC₄) levels. (**P* < 0.05; ***P* < 0.01; ****P* < 0.001; one-way analysis of variance.)

mean $\pm$ SD) (Figure 8A). Treatment with an LTA₄H-I dose-dependently decreased the levels of LTB₄ detectable in cell-free BAL fluid, whereas BAL fluid from PBS-challenged mice had undetectable levels of LTB₄. Another potential mechanism by which LTA₄H-I might regulate AHR and lung inflammation involves increased production of lipoxins. We detected a dose-dependent significant increase in LXA₄ levels in the BAL fluid of mice treated with an LTA₄H-I. As expected, use of the 5-LO inhibitor zileuton blocked LTB₄ production but did not increase LXA₄ levels (Figure 8). As a control, we measured levels of LTC₄ and observed a significant decrease in zileuton-treated mice and no modulation with an LTA₄H-I (Figure 7C).

DISCUSSION

Inflammatory leukocytes, including mast cells, macrophages, and neutrophils, can produce LTB₄, a potent chemoattractant for multiple leukocytes. Elevated levels of LTB₄ have been reported in a number of inflammatory airway disorders, including sputum and BAL samples from patients with chronic obstructive pulmonary disease, allergy, and asthma (8–10).

Because of conflicting efficacy results obtained from acute lung inflammation studies using BLT1-deficient mice (13, 14), we chose to use a selective LTA₄H inhibitor to pharmacologically evaluate the contribution of LTB₄ to allergic lung inflammation. The LTA₄H-I, JNJ 26993135, was able to potently inhibit LTB₄ production by murine BMMCs triggered via the IgE receptor. Moreover, using this pharmacological inhibitor of LTB₄ production around the challenge phase of a mast cell-dependent OVA model to mimic a therapeutic situation, we observed dramatic decreases in airway inflammation and a reduction in markers of airway remodeling and function.

Previous work has demonstrated that individual airway function parameters obtained using the forced oscillation technique can be used to measure specific changes in lung mechan-

ics and pathology (33, 34). Herein, treatment with an LTA₄H inhibitor decreased all three parameters generated using the constant-phase model in response to methacholine challenge, central airways Newtonian resistance (*R_N*), peripheral tissue damping (*G*), and tissue rigidity (*H*). The highly significant effects on *G* suggest changes in the resistance of lung parenchyma or very small airways are decreased on inhibition of LTB₄ production. Likewise, effects on *H* suggest inhibition of LTB₄ production results in decreased airway closure. Together, these effects on both *G* and *H* suggest that LTB₄ inhibition results in decreased dysfunction of the peripheral airways. This hypothesis involving enhanced lung function due to reduced airway closure is further supported by our findings that inhibition of LTB₄ production results in dramatic reductions in Th2 cytokines, such as IL-13 and IL-4. Decreases in these cytokines would result in decreased goblet cell hyperplasia and the resulting production of mucin (35). Indeed, we observed significantly decreased levels of mucin in BAL fluid of mice treated with an LTA₄H-I or anti-IL-13. This mechanism of airway closure due to mucus hypersecretion is also reflected by clinical observations in the population with severe asthma (36).

A model has recently been proposed to explain how LTB₄ controls AHR (37); while this manuscript was in preparation, a role for LTA₄H in mast cell LTB₄ formation and allergic airway inflammation was reported using LTA₄H knockout mice (38). Antigen-triggered activation of mast cells via the IgE receptor results in localized production of LTB₄ in the lung. LTB₄ then recruits antigen-specific BLT1⁺ T cells that produce cytokines such as IL-4 and IL-13 (13, 30). Interestingly, our studies using a more chronic model of airway inflammation demonstrated that inhibition of LTB₄ production during the challenge phase affects multiple parameters of this model. We observed a dramatic decrease in antigen-specific IgE and IgG1 that would ultimately modulate the activation of mucosal mast cells and thus may further decrease the localized production of LTB₄ in the lung on subsequent antigen challenges.

Tager and colleagues (4) and Goodarzi and colleagues (39) demonstrated that effector T-cell recruitment into the inflamed lung is driven by LTB₄. Data using BLT1-deficient animals and adoptive transfer studies using BLT1-deficient T cells have indicated that both CD4⁺ and CD8⁺ effector T cells play a key role in inflammation and AHR (13, 30). In agreement with these observations, an LTA₄H-I in our studies significantly reduced the numbers of both CD4⁺ and CD8⁺ T-cell subsets in the lung tissue and lumen with a concomitant reduction in airway IL-4, IL-13, and AHR. Based on these findings, we hypothesize that blocking LTB₄ production with a pharmacological inhibitor results in decreased accumulation of both CD4⁺ and CD8⁺ T cells in the lung tissue and lumen, through a direct effect on mast cell production of LTB₄.

Despite similar numbers of T cells in PBLN, we did observe a dramatic decrease in cytokine production when PBLN T cells were restimulated *ex vivo*. This prompted us to examine the potential effects of LTB₄ on DCs. Lung DCs have been shown to play a key role in driving lung inflammation and AHR on allergen challenge (31). In addition, studies using DCs from BLT1-deficient mice have suggested that LTB₄ plays a role in DC migration to draining lymph nodes in a contact hypersensitivity model (32). We gated on CD11c⁺ DCs, a marker expressed by both airway and parenchymal DCs that are known to be more endocytic and efficient at presenting peptides to T cells (40). The use of an LTA₄H-I during the challenge phase effectively decreased the migration of CD11c⁺, Alexa488⁺ DCs into the PBLN. Decreased migration was observed with several subpopulations of lung DCs, including CD8α⁺ and CCR7⁺ DCs. These are the first data, to our knowledge, that demonstrate a role for LTB₄-induced CDα⁺ DC migration in the allergic lung. CD8α⁺ DCs have been demonstrated to be the dominant OVA-processing and presenting DC population in PBLNs after challenge with OVA (27). It has been previously demonstrated that CCR7 is an important chemokine receptor for pulmonary DC trafficking to draining lymph nodes (41), and its expression on DCs can be modulated by LTB₄ (32). These data suggest that blocking LTB₄ production in the lung could possibly decrease CD4⁺ and CD8⁺ T-cell activation in draining lymph nodes by modulating the migration of DCs that have taken up allergen in the lung. It remains to be determined whether this modulation results in a decrease in the precursor frequency of antigen-specific effector T cells or a defect in the ability of these T cells to produce Th2 cytokines.

Another potential mechanism by which LTA₄H-I might regulate AHR and lung inflammation involves increased production of lipoxins. We detected significantly increased LXA₄ levels in the BAL fluid of mice treated with an LTA₄H-I, a phenomenon that may result from shunting of LTA₄H substrates to the lipoxin pathway (20). LXA₄ and its analogs have been shown to compete for binding of LTD₄ with CysLT1 (42). In addition, LXA₄ has the potential to directly affect airway smooth muscle on antigen challenge, both *in vitro* to LTD₄-induced constriction in animal models of AHR and in patients with asthma challenged with LTC₄ (43–45). Conceivably, the observed increases in LXA₄ could therefore account for some of the results reported herein and enhance the therapeutic benefits of LTA₄H-I in the clinic. Previous studies with this LTA₄H-I have importantly demonstrated no such shunting to inflammatory LTC₄ production in ionophore-stimulated whole blood or in an acute peritonitis model in mice (20). Likewise, as one would expect from a selective LTA₄H-I, no inhibition of LTC₄ was observed here in the lung or previously in the peritoneum (20). In contrast, the 5-LO inhibitor zileuton was effective at inhibiting both LTB₄ and LTC₄ in the BAL fluid of OVA-challenged animals, although the effect on LTC₄ was less dramatic. This pharmacody-

amic effect has been reported clinically; in allergen-challenged patients, zileuton was able to inhibit LTB₄ in ionophore-stimulated whole blood, but only partially blocked LTE₄ in the urine, a predictive marker of LTC₄ synthesis (46).

A recent study with zileuton has reaffirmed its ability to significantly improve asthma symptoms and reduce exacerbations and concomitant drug usage (47). Whether this activity, or purported improved efficacy over CysLT1 antagonists in more severe asthma, is related to its inhibition of LTB₄ formation remains to be elucidated, but the preferential effect on LTB₄ production is provocative. Novel inhibitors of LTA₄H, dosed alone or in conjunction with CysLT1 antagonists, may help define the specific role of LTB₄ in asthma.

In summary, a novel and selective LTA₄H inhibitor can modulate airway inflammation and AHR through multiple mechanisms. Consistent with direct inhibitory effects of LTB₄, T-cell and DC chemotaxis were inhibited. Correspondingly, reductions in Th2 cytokine-driven pathologies, such as mucus secretion and AHR, were also attenuated. Additional, novel benefits of LTA₄H inhibition were also observed, including an increase in antiinflammatory lipoxin levels in the lung. These data suggest that LTA₄H inhibition might be useful in treating allergic airway diseases.

Conflict of Interest Statement: N.L.R. is an employee of Johnson & Johnson Pharmaceutical Research & Development LLC and owns stock in the company. J.P.R. is a full-time employee of Johnson & Johnson PRD, and owns stock in Johnson & Johnson. H.B. is an employee of Johnson & Johnson PRD. X.X. has been employed by Johnson & Johnson since November 2001. B.S. was employed by Johnson & Johnson, PRDUS from November 2005 to September 2008. S.C. has been employed by Johnson & Johnson since September 2001 and owns shares of Johnson & Johnson. K.A.L. has shares in Johnson & Johnson and is a former employee (2000–2009). F.Y. has been employed by Johnson & Johnson since 2007. L.K. is an employee of Johnson & Johnson Pharmaceutical Research and Development, is a named inventor on patents, and owns stock in Johnson & Johnson. A.M.F. is a full-time employee, an inventor on patent applications, and a stockholder of Johnson & Johnson. P.J.D. is a full-time employee, a named inventor on patents, and a stockholder of Johnson & Johnson PRD.

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Leukotriene A₄ Hydrolase Inhibition Attenuates Allergic Airway Inflammation and
Hyperresponsiveness

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Online Data Supplement

Supplemental Data Supplement

Compound Description

The LTA₄H-I, JNJ 26993135, was prepared as previously described (20). JNJ-26993135, dexamethasone and zileuton were formulated in 20% hydroxypropyl- β -cyclodextran and dosed orally as a solution.

Mouse Study Protocol

Balb/C mice (female, 6-8 weeks old) were sensitized and challenged with OVA as previously described (22). Mice were sensitized by intra-peritoneal injection seven times every other day with 10 μ g of OVA in 0.1 ml of sterile saline. On day 40 after sensitization, mice were challenged three times via intra-nasal instillation of 100 μ g of OVA in 50 μ l sterile saline, three days apart. Animals were dosed orally with vehicle (control), LTA₄H-I (10, 25 and 50 mpk bid) or dexamethasone (1 mpk qd) 30 min prior to OVA challenge. Anti-mouse IL-13 monoclonal antibody (500 μ g), was administered intravenously twice during the challenge phase just prior to the first OVA challenge (23). Mice were subjected to terminal procedures 48 hr after the last OVA challenge.

Airway function

Airway function was measured 48 hours after the third challenge using a small animal ventilator (Scireq, Montreal Canada) as previously described (24). Mice were anesthetized using an intra-peritoneal injection of 100 mg/kg sodium pentobarbital (Euthasol, ANADA#200-71). Once a surgical plane of anesthesia was established (indicated by lack of pedal and corneal reflex), a

tracheostomy was performed. A 19 g luer adapter was inserted in the trachea and secured with suture. Animals were immediately transferred to the flexi-Vent and mechanical respiration was initiated. Mice were ventilated with a tidal volume of 9 ml/kg at a rate of 150 breaths/min, with a positive end-expiratory pressure of 3 cm H₂O. Animals were allowed to acclimate to the respirator for approximately five minutes to establish a stable baseline. At this time a custom study script (AN-M1) was run using 0 mg/ml, 12.5 mg/ml, 25 mg/ml and 50 mg/ml of Mch. The mean of 12 sets of data after each Mch aerosol provocation was analyzed for individual animals. Individual data points were excluded from analysis if coefficient of determination values were lower than 0.9 (Snap-Shot) or 0.88 (Quick-Prime). The coefficient of determination is a quality control parameter that measures the fit of the experimental data to the model that generates the lung function parameters. The Snap-Shot technique is based upon the linear first-order, single compartment model and yields the dynamic resistance and compliance parameters. Quick-Prime is based upon the constant phase model offers distinction between central and peripheral respiratory mechanics and yields Newtonian Resistance, tissue elastance and tissue damping. Mean values for each treatment group were then plotted in Prism (GraphPad Software 4.02, San Diego, CA). Data was analyzed to compare drug-treated groups with vehicle-treated animals.

Determination of total and OVA-specific serum Ig levels

Serum samples were obtained from animals at experiment end by cardiac puncture. Total IgE, IgG1, and IgG2a levels were measured as per manufacturer's instructions (BD Pharmingen). OVA-specific IgE, IgG1 and IgG2a was determined using plates coated overnight with 2 µg/ml OVA in sodium carbonate buffer (pH 9.5; Sigma-Aldrich) and detection Abs for total IgE

(55248), IgG1 (559626), and IgG2a (553391). The Ab levels were quantified by measurement of the absorbance at 415 nm. OVA-specific IgE was measured as described (25).

Levels of serum immunoglobulin isotypes were quantified by ELISA. High binding, 96 well plates (Costar 3590) were coated with 2 µg/ml of ovalbumin in 0.1M carbonate buffer pH 9.5 at 4°C overnight. Plates were washed three times on an automatic plate washer with PBS + 0.05% tween, then blocked for 1 hr with assay diluent (PBS + 10% fetal bovine serum). Thawed serum samples were diluted (1/25, 1/50, 1/100 and 1/200) with assay diluent and 100 µl was added in duplicate for 2 hr. Plates were washed three times prior to the addition of 100 µl diluted detection antibody (1:250 diluted biotin-anti-mouse IgE with 1:250 avidin-HRP; 1:1000 diluted anti-mouse IgG1-HRP; and 1:1000 diluted anti-mouse IgG2a-HRP). The plates were washed three times, after an hour, prior to adding 100 µl of substrate. ELISA plates were incubated at RT for 20 min and the reaction was stopped by adding 50 µl of 2 N H₂SO₄. The plates were read at 450 nm, and the mean of each duplicate sample was plotted as serum isotype readings for each animal.

Dendritic Cell Migration

Mice were challenged three times with intra-nasal OVA (50 µg). For the third challenge, mice were given an intra-nasal challenge of 75 µg of Alexa 488 conjugated OVA prepared in 50 µl of endotoxin-free saline. Mice were dosed with vehicle or 50 mpk LTA₄H-I 30 min prior to OVA challenge. Mice were euthanized 18 hr after challenge, and pooled PBLN (consisting parathymic and posterior mediastinal nodes) were collected. In addition, lungs were harvested for dendritic cell isolation. Lungs and lymph nodes were sliced with a scalpel then digested with 1.5–2 mg/ml Liberase Blendzymes 3 (Roche Diagnostics Corporation, Indianapolis, IN) and 0.1

mg/ml DNase I (Sigma chemicals Co, St Louis, MO) in media at 37°C for 30 min. Total lung cells were further purified by ficoll gradient. For FACS analysis, dead cells were excluded by PI staining and Alexa Fluor 488 OVA labeled cells were gated and further analyzed for expression of specific DC markers. Dendritic cells were identified as cells staining positive for CD11c as well CD8 α . Percent positive cells of relevant populations represented in Panel C are indicated in the relevant quadrant. Antibodies against CD8 α PE, CD11c APC, and CCR7 PE were purchased from BD Pharmingen (San Diego, CA).

Mucin enzyme linked lectin assay (ELLA)

Mucin levels were detected by ELLA as previously described (28) using *Ulex europaeus* agglutinin-1 (UEA-1; Sigma, St. Louis, MO). High-binding microtiter plates (Costar) were coated with UEA-1 then blocked with buffer containing Tween 20 and gelatin. Both a purified human mucus and porcine mucin standard was used. Bound mucin was detected using HRP-conjugated UEA-1 (Sigma) and orthophenylenediamine dihydrochloride substrate (Sigma) detected at 492 nm.

Mast cell Activation Assay

Mast cell precursors were isolated from bone marrow of 6-wk-old Balb/C female mice, and maintained in RPMI 1640 medium (Invitrogen Life Technologies) containing 10% ultra-low IgG FBS (Invitrogen Life Technologies), penicillin and streptavidin, glutamine and 20 ng/ml recombinant mouse stem cell factor, and 20 ng/ml IL-3 (PeproTech) for at least 4 wk and with culture times not exceeding 8 wk. Mast cells were sensitized overnight by incubation with 1 μ g/ml IgE-TNP (Pharmingen) at 37°C. The next day, cells were resuspended in Tyrode's buffer

at 2×10^6 cells/ml. 10^5 cells were plated in 96-well plates, preincubated for 20 min with inhibitor or 0.1% DMSO Vehicle, followed by stimulation for 20 min with 50 ng/ml TNP-human serum albumin (Sigma-Aldrich), in a final volume of 100 μ l. Cell supernatant and cellular pellets were harvested by 5 min centrifugation at 1500 rpm. To measure β -hexosaminidase activity, 50 μ l of supernatant or cell pellet (lysed in 100 μ l of Tyrode's buffer containing 0.5% Triton X-100) were transferred to 96-well flat-bottom plates containing 50 μ l of 3.7 mM p-nitrophenol-N-acetyl- β -D-glucosaminide (Sigma-Aldrich) in 100 mM Na-acetate (pH 4.5) and further incubated for 1 h at 37°C. The reaction was stopped by addition of 100 μ l of 2 M NaOH, followed by measurement of absorbance at 405 nm.