

Absence of Leukotriene B₄ Receptor 1 Confers Resistance to Airway Hyperresponsiveness and Th2-Type Immune Responses¹

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Bronchial asthma is an increasingly common disorder that remains poorly understood and difficult to manage. The disease is characterized by airway hyperresponsiveness, chronic inflammation, and mucus overproduction. Based on the finding that leukotriene B₄ receptor 1 (BLT1) is expressed highly in Th2 lymphocytes, we analyzed the roles of BLT1 using an OVA-induced bronchial asthma model. BLT1-null mice did not develop airway hyperresponsiveness, eosinophilic inflammation, and hyperplasia of goblet cells. Attenuated symptoms were accompanied by reduced IgE production, and accumulation of IL-5 and IL-13 in bronchoalveolar lavage fluid, suggesting attenuated Th2-type immune response in BLT1-null mice. Peribronchial lymph node cells of sensitized BLT1-null mice showed much attenuated proliferation and production of Th2 cytokines upon re-stimulation with Ag in vitro. Thus, LTB₄-BLT1 axis is required for the development of Th2-type immune response, and blockade of LTB₄ functions through BLT1 would be novel and useful in the effort to ameliorate bronchial asthma and related Th2-biased immune disorders. *The Journal of Immunology*, 2005, 175: 4217–4225.

Bronchial asthma is globally prevalent, affecting several million people's daily lives. The disease is defined as a reversible airway obstruction with airway inflammation and mucus overproduction (1). Bronchial hypersensitivity and airway remodeling following inflammatory response are hallmarks of this disease (2). Whereas smooth muscle constriction is reversed by β_2 agonists, chronic and persistent infiltration of inflammatory cells, accompanied by edema, is a major therapeutic target to improve pulmonary function and decrease morbidity (3). Anti-inflammatory steroids in combination with 5-lipoxygenase inhibitors, or cysteinyl leukotriene antagonists are currently used to control and treat bronchial asthma.

Studies using genetically engineered mice and various inhibitors/antagonists suggest that cytokines, chemokines, cell adhesion molecules, and lipid mediators play roles in the pathogenesis of asthma (4–9). We previously reported that cytosolic phospholipase A2 plays a pivotal role in developing airway hyperrespon-

siveness (AHR)⁴ (10). However, downstream lipid mediators and detailed cellular events remained totally elusive. Among several lipid mediators, prostaglandin D₂ (11) and leukotrienes (LTs) (3) are potent proinflammatory mediators derived from arachidonic acid by cyclooxygenases and 5-lipoxygenase, respectively (12–14). Importantly, mice deficient in 5-lipoxygenase exhibited reduction of AHR, airway eosinophilia, and Ig production in OVA-induced asthma model (15). Among downstream lipid mediators of 5-lipoxygenase, cysteinyl LTs (LTC₄, D₄, and E₄), originally termed slow-reacting substance of anaphylaxis have been reported to increase vascular permeability, and bronchial smooth muscle constriction, through two distinct types of receptors, CysLT1 and CysLT2. Although several CysLT1 antagonists such as zafirlukast, montelukast, and pranlukast are currently used for treatment of asthmatic patients, the effect is heterogeneous, and a significant number of patients are resistant to the CysLT antagonist treatment (16). Leukotriene B₄ (LTB₄), another eicosanoid derived from 5-lipoxygenase pathway of arachidonic acid, has well-documented potent chemotactic activity toward granulocytes (17). This lipid mediator exerts its biological activities through two distinct LTB₄ receptors: BLT1, a high-affinity type, and BLT2, a low-affinity type (18, 19). Recently, LTB₄ was reported to recruit early effector helper T cells and effector cytotoxic T cells to inflamed tissues (20, 21). Mast cells release LTB₄ upon stimulation, leading to a prominent infiltration of effector T cells (22). These in vitro and ex vivo data prompted us to define the roles of LTB₄ in the pathogenesis of asthmatic Th2 responses in vivo. Here, using a mouse model in which BLT1 is genetically ablated, we provide in vivo evidence that LTB₄-BLT1 interaction is necessary for production of Th2 cytokines and the development of AHR. Based on our new finding

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⁴ Abbreviations used in this paper: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; EPO, eosinophil-specific peroxidase; LT, leukotriene; LTB₄, 5(S),12(R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid; MCh, methacholine; MPO, myeloperoxidase; PAS, periodic acid-Schiff; WT, wild type.

that LTB₄ is a principal immunomodulator raised a novel therapeutic possibility for prevention and treatment of asthmatic patients.

Materials and Methods

Targeting construct and generation of BLT1^{-/-} mice

Clones containing the mouse BLT1 gene were isolated by screening a 129/Sv genomic library in Lambda fix II (Stratagene) using an expressed sequence tag clone for mouse BLT1 cDNA (GenBank accession number AA028322) as a probe. An 18.6-kb fragment was subcloned into pBlue-scriptII SK (Stratagene). The BLT1 genomic locus was modified by inserting a LacZ-neo cassette into *Pst*I site in the open reading frame in exon 2 of the BLT1 gene. Diphtheria toxin A fragment (23) was used as a negative selection marker. This construct was induced by electroporation into the TT-2 line of embryonic stem cells from an F₁ embryo derived from a cross of C57BL/6 and CBA mice (24), and transfected cells were selected by addition of the G418 at 300 ng/ml. Four clones, which showed evidence of homologous recombination by Southern blotting, were injected into blastocysts by standard techniques. Southern blot analysis was performed with 10 µg of genomic DNA using probe a (0.63-kb *Bam*HI-*Dra*I fragment; Fig. 1A) external to the 5' end of the knockout construct, and probe b (0.5-kb *Xho*I-*Mlu*I fragment; Fig. 1A) as an internal probe. After implantation into pseudopregnant C57BL/6 mice, these clones resulted in chimeric mice that transmitted the disrupted allele to the offspring. The offspring of these founders were identified by Southern blot analysis with the same probes or more routinely by PCR from tail snips with the following three primers: 5'-ATGGGTGTGTCCACTTTCACCTCTC-3', 5'-GCACACAGATGAAACGCCGAGTT-3', and 5'-CCGGTATGGCCAGCA GAAAGACA-3'. In all experiments, BLT1^{-/-} and BLT1^{+/-} mice were generated from mating between heterozygous (BLT1^{+/-}) mice, and raised in identical specific pathogen-free conditions. The sex, age, and generation of mice used in the experiments are shown in the figure legends. All studies and procedures were approved by International Guiding Principles for Biomedical Research Involving Animals.

[³H]LTB₄ binding assay

BLT1^{+/-} and BLT1^{-/-} mice were injected i.p. with 2 ml of 2% casein, and after 4 h, peritoneal cells were collected with ice-cold PBS. Diff-Quik staining revealed >90% of the cells as granulocytes. The [³H]LTB₄ binding assay was performed as described previously (25) with some modifications. Granulocytes at a density of 1 × 10⁶ cells/ml were incubated with 2 nM [³H]LTB₄ in HBSS containing 0.49 mM MgCl₂ and 0.95 mM CaCl₂. For determination of nonspecific binding, 10 µM unlabeled LTB₄ was used. Mixtures were incubated at 4°C for 60 min in a total volume of 100 µl. The assay was performed in quadruplicates, followed by rapid filtration through GF/C filters (Packard Instrument) and washing with 3 ml of binding buffer. The radioactivity of the filters was determined with a scintillation counter (Top Count; Packard Instrument). Lungs and spleens were dissected from BLT1^{+/-} and BLT1^{-/-} mice. Pooled tissue was minced and homogenized twice with a Physcotron homogenizer in sonication buffer (20 mM Tris-HCl, (pH 7.4), 0.25 M sucrose, 10 mM MgCl₂, 1 mM EDTA) containing a proteinase inhibitor mixture (Complete; Roche) at 3 ml/g (v/w) tissue at 4°C. The homogenate was centrifuged at 12,000 × g for 10 min at 4°C, and the resulting supernatant was centrifuged at 105,000 × g for 60 min at 4°C. The final pellet was resuspended in sonication buffer and stored at -80°C until use. Protein concentrations were determined by the Bradford method (26) with BSA as a standard. [³H]LTB₄ binding to membrane fraction of mouse lung and spleen was determined according to a method by Cheng et al. (27) with some modifications. [³H]LTB₄ binding was conducted in a volume of 100 µl containing membrane preparation (80 µg of protein) in binding buffer (50 mM Tris-HCl, (pH 7.4), 10 mM MgCl₂, 10 mM NaCl, and 0.05% BSA) and 2 nM [³H]LTB₄ for 60 min at room temperature. The reaction was terminated, and the radioactivity was determined as described above.

Calcium mobilization in mouse granulocytes

Mouse peritoneal granulocytes were incubated with 3 µM fura-2/AM (Dojindo) in modified HEPES-Tyrodé's BSA buffer (25 mM HEPES-NaOH, (pH 7.4), 140 mM NaCl, 2.7 mM KCl, 1.0 mM CaCl₂, 12 mM NaHCO₃, 5.6 mM D-glucose, 0.37 mM NaH₂PO₄, 0.49 mM MgCl₂, and 0.1% (w/v) BSA (fraction V) at 37°C for 1 h, washed, and resuspended in HEPES-Tyrodé's BSA buffer at a density of 2 × 10⁶/ml. One-half milliliter of the cell suspension was applied to a CAF-100 system (Jasco), and 5 µl of LTB₄ in DMSO or fMLP in water was added. Intracellular Ca²⁺ con-

centration was measured by the ratio of emission fluorescence at 500 nm, after excitation at 340 and 380 nm.

Release of myeloperoxidase (MPO) from mouse granulocytes

MPO release from mouse granulocytes was measured as previously reported (28) with some modifications. Aliquots (50 µl) of the cells (2 × 10⁷ cells/ml) in HBSS containing 0.49 mM MgCl₂, 0.95 mM CaCl₂, 0.05% BSA, and 5 µg/ml cytochalasin B were preincubated at 37°C for 5 min, and 50 µl of the ligand solution was added to initiate the reaction. After incubation for 10 min at 37°C, the reaction was stopped by placing the plate on ice. After centrifugation at 2300 × g for 5 min at 4°C, 20 µl of the supernatants were transferred to a new 96-well plate. Remaining cells were lysed by adding 20 µl of lysis buffer (1 × HBSS, 5% Triton-X). The MPO activities of the supernatants and cells were measured in MPO assay buffer (50 mM potassium phosphate buffer, (pH 6.0), 0.157 mg/ml *o*-dianisidine (Sigma-Aldrich), and 0.0005% hydrogen peroxide). Commercially available MPO (Calbiochem) in serial dilution was used as a standard.

AHR model

Sensitization and challenge protocol. Mice used in this model were backcrossed on the C57BL/6 background for 5–8 generations. Heterozygous mice were intercrossed, and resulting male littermates were genotyped and used in all experiments. Mice were sensitized by i.p. injection of 0.5 ml of solution containing 0.1 mg of OVA (Sigma-Aldrich) complexed with aluminum hydroxide (Wako Chemical) (2 mg/ml) in saline. On day 8, the mice were subsequently boosted with the same mixture. On day 12 and 13, these sensitized mice were challenged for 30 min with 1% OVA in saline aerosol generated with ultrasonic nebulizer. Control mice received i.p. injection of saline/aluminum hydroxide and saline aerosols in the same manner. Mice were studied 2 days after the last aerosol challenge.

Measurement of airway hyperresponsiveness. Animals were anesthetized with sodium pentobarbital (25 mg/kg, i.p.) and ketamine hydrochloride (25 mg/kg, i.p.) in combination, and then paralyzed with pancuronium bromide (0.3 mg/kg, i.p.). After tracheostomy, an endotracheal metal tube (1 mm inside diameter, 8 mm in length) was inserted into the trachea. Animals were mechanically ventilated (model 683, Harvard Apparatus) with tidal volumes of 10 ml/kg at a frequency of 2.5 Hz. The thorax was widely opened by means of midline sternotomy, and a positive end expiratory pressure of 2 cm H₂O was applied by placing the expiration line under water. During the experiments, oxygen was continuously supplied to the ventilatory system. Under these ventilatory conditions, arterial pH, PaO₂, and PaCO₂ were 7.35–7.45, 100–180 mm Hg and 30–45 mm Hg, respectively, at the end of experiments (blood gas analyzer; Compact 3; AVL Medical Systems). A heating pad was used to maintain body temperature. Tracheal pressure was measured with a piezo-resistive microtransducer (8510B-2; Endevco) placed in the lateral port of the tracheal cannula. Tracheal flow was measured with a Fleisch pneumotachograph (Metabo SA). All signals were amplified, filtered at a cutoff frequency of 100 Hz, and converted from analog to digital format with a converter (DT2801-A; Data Translation). The signals were sampled at a rate of 200 Hz and stored on an IBM-AT compatible computer. Lung resistance (R_L) and elastance (EL) were measured as described previously (29). Saline and methacholine (MCh) (Sigma-Aldrich) were administered at a positive end expiratory pressure of 2 cm H₂O to mice. At the beginning of the experiments, two deep inhalations (3 times tidal volume) were delivered to standardize volume history. All animals were then challenged with saline aerosol for 2 min. Aerosols were generated with an ultrasonic nebulizer (Ultra-Neb100; DeVilbiss) and delivered through the inspiratory line into the trachea. Measurements of 10 s duration were sampled during tidal ventilation beginning at 1 min after administration of saline aerosol. This represented the baseline measurement. Following this, each dose of MCh aerosol was administered for 2 min in a dose-response manner (0.3125–20 mg/ml). Airway responsiveness was assessed using the concentration of MCh required to increase lung resistance to 200% of baseline values (EC₂₀₀R_L).

Bronchoalveolar analyses. In other experiments, blood was taken from the abdominal vein using heparinized syringes. Blood samples were collected in a plasma separator tube (Microtina; BD Bioscience), and plasma was obtained by centrifugation. After blood sampling, the trachea of a tracheostomized mouse was cannulated with an 18-gauge metal cannula. After opening of the thorax by a wide incision of the diaphragm, the lung was lavaged once with 1 ml of Ca²⁺- and Mg²⁺-free PBS. Eighty-five percent (0.85 ml) of the total injected volume was consistently recovered. Bronchoalveolar fluid (BALF) was centrifuged at 450 × g for 5 min; the pellet was resuspended in 500 µl of PBS. Aliquots (100 µl) were used for cell counting with a flow cytometer EPICS XL (Beckman Coulter) using Flow-count particles (Beckman Coulter) as internal standards. Aliquots (300 µl) were centrifuged onto slide glasses using a Cytospin (Thermo

Shandon). Cells were stained using Diff-Quik (International Reagents), and differential cell counts were obtained. The supernatant was stored at -80°C until assays were performed. The BALF contents of CysLTs, LTB₄, PGD₂, PGE₂, IL-13, eotaxin, and MCP-1 α were determined by ELISA using commercially available kits (Amersham Biosciences for CysLTs and LTB₄, Cayman Chemicals for PGD₂ and PGE₂, and R&D Systems for IL-13, eotaxin, and MCP-1 α). For quantification of OVA-specific IgG1, ELISA plates were coated with 100 $\mu\text{g}/\text{ml}$ OVA, and BALF (undiluted), or plasma (100-fold dilution) was applied. Bound IgG was detected by HRP anti-mouse IgG1 Ab (clone X56; BD Pharmingen) and Sigma FAST OPD (Sigma-Aldrich). For IgE determination, ELISA plates were coated with 1 $\mu\text{g}/\text{ml}$ IgE capture Ab (clone R35-72; BD Pharmingen) and BALF (undiluted), or plasma (10-fold dilution) was applied. Captured IgE was detected by biotin anti-mouse IgE Ab (clone R35-118; BD Pharmingen), avidin-APC (Sigma-Aldrich), and Sigma FAST OPD (Sigma-Aldrich). Cytokines were measured using mouse Th1/Th2 CBA kit (BD Pharmingen).

Lung histology. After the blood collection, the lungs were removed and fixed in 10% phosphate-buffered formalin. From the paraffin-embedded right and left lobes of lung, sections of 5- μm thickness were prepared from the middle position of each lobe, and stained with H&E or periodic acid-Schiff (PAS). A semiquantitative scoring system was used to grade the size of lung infiltrates in the H&E-stained sections, where +5 signifies widespread infiltrates around the majority of vessels and bronchioles, and +1 signifies a small number of inflammatory foci (30). The goblet cell hyperplasia in the PAS-stained sections was graded by a semiquantitative scoring system (0 = <5% goblet cells in airway epithelium; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; 4 = >75%) as described by McMillan et al. (31). The sum of the airway scores from each section was divided by the number of airways examined (6–8 per section), and expressed as PAS score in arbitrary units. For both quantitative scoring, randomized and blinded slides were graded. The EPO activity in the lung was measured as described (32).

Restimulation of lymph node cells in vitro. Mice were sensitized and challenged with OVA as described above. Cells isolated from peribronchial lymph nodes were pooled from nine mice per group, and stimulated in vitro (1.5×10^5 cells per well in a 96-well plate) with increasing doses of OVA or plate-bound anti-CD3 ϵ Ab (10 $\mu\text{g}/\text{ml}$, clone 145-2C11; BD Bioscience). [^3H]-Labeled methyl-thymidine (1 $\mu\text{Ci}/\text{well}$; Amersham Biosciences) was pulsed for the last 8 h of 72 h of culture. We collected the supernatants after 96 h and assayed them for cytokines.

Statistical analysis

All experiments were performed at least twice with similar results, and representative results are shown. All statistical calculations were performed with ANOVA using Prism 4 (GraphPad Software). A value of $p < 0.05$ was considered statistically significant.

Results

Generation of BLT1-deficient mice

The BLT1 gene was disrupted by a conventional method using an embryonic stem cell line, TT-2 (23, 24). The frequency of homologous recombination attained was 2.5% (6 of 240 G418-resistant colonies). The design of the targeting is shown in Fig. 1A. Resulting chimeric mice were mated with C57BL/6 females to generate heterozygous mice. Mice were genotyped by Southern blot of *Kpn*I- or *Eco*RI-digested genomic DNA (Fig. 1B). Three-primer PCR was routinely used for genotyping (Fig. 1C). BLT1 $^{-/-}$ mice were born at the expected Mendelian distribution, were fertile, and showed no developmental or morphological abnormalities. Heterozygous mice used in this study were backcrossed with C57BL/6 mice for at least four to seven generations depending on the experiments in specific pathogen-free environment. Blood cell counts, biochemical analyses of serum, the populations of CD3-, CD4-, CD8-, B220-, Thy-1-, MHC class II-, and CD11c-positive cells in spleen, thymus and inguinal lymph nodes were similar between naive BLT $^{+/+}$ and BLT1 $^{-/-}$ mice (data not shown). To confirm disruption of the BLT1 gene, RT-PCR was performed. mRNA isolated from lungs or spleens of BLT1 $^{-/-}$ mice did not produce mBLT1 transcript (data not shown).

LTB₄ binding assay

The binding of 2 nM [^3H]LTB₄ to peritoneal granulocytes from BLT1 $^{-/-}$ mice was less than one third the capacity of that of BLT1 $^{+/+}$ granulocytes (Fig. 2A). Membrane fractions of lung and spleen from BLT1 $^{-/-}$ mice also exhibited about one third of the binding to 2 nM [^3H]LTB₄ compared with that of BLT1 $^{+/+}$ mice (Fig. 2, B and C). The remaining binding activity might be due to BLT2 or other LTB₄-binding proteins.

Calcium mobilization and MPO release induced by LTB₄

LTB₄ binding to BLT1 and BLT2 increases intracellular calcium concentrations (18, 19). To examine the effects of BLT1 disruption on LTB₄-induced calcium mobilization, casein-induced peritoneal cells (>90% granulocytes) were analyzed. Although LTB₄ dose-dependently induced calcium mobilization in peritoneal cells from BLT1 $^{+/+}$ mice, no calcium response was observed even at 10 μM

FIGURE 1. Generation of BLT1 $^{-/-}$ mice. **A**, Schematic representation of endogenous BLT1 locus (*top*), targeting vector for BLT1 (*middle*), and targeted BLT1 locus after recombination (*bottom*). **B**, Southern blot analyses of mouse tail DNA. DNA from WT and mutant mice (indicated by numbers) was digested with *Kpn*I (*top*) and *Eco*RI (*bottom*). Blotted DNA was hybridized with either probe a or probe b seen in panel A. Probe a is a 5'-flanking probe, not present in the targeting vector. Probe b is an internal probe. **C**, Genotyping using mice tails. Results from WT (+/+), heterozygous mice (+/-), homozygous mice (-/-), and negative control (N, no template) are indicated.

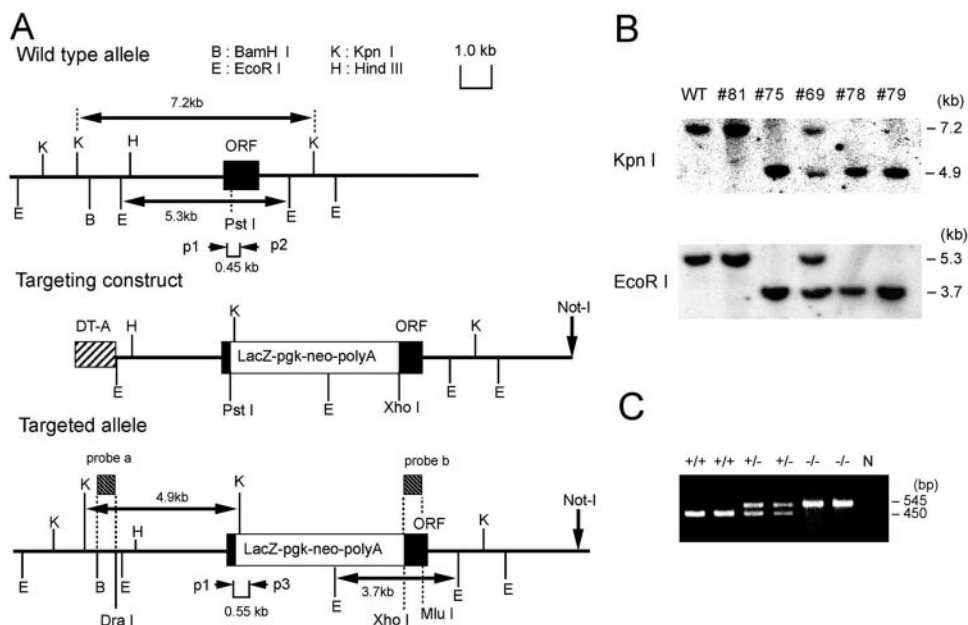
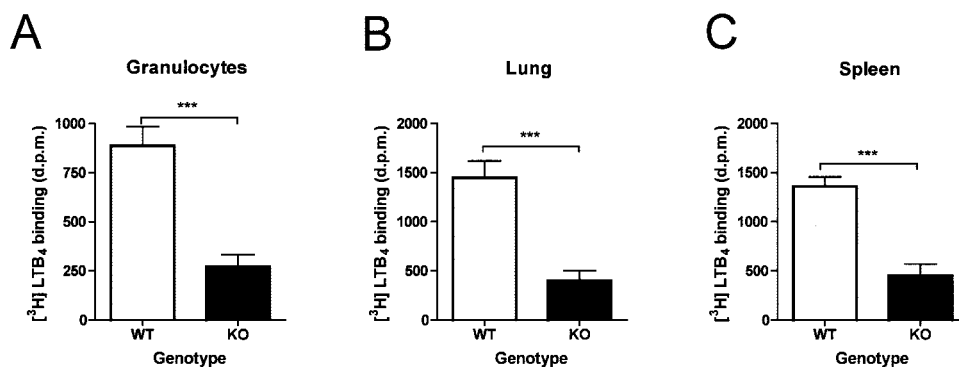


FIGURE 2. Reduced LTB₄ binding in BLT1^{-/-} mice. *A*, Specific binding of 2 nM [³H]LTB₄ to mouse peritoneal granulocytes. *B* and *C*, Specific binding of 2 nM [³H]LTB₄ to the membrane fractions of mouse lung and spleen. Nonspecific binding was determined by incubation with 10 μM unlabeled LTB₄. F3 male mice were used for cell and membrane preparations. Values are mean ± SD of triplicate determinations from three independent experiments. ***, *p* < 0.001, unpaired *t* test.



LTB₄ in BLT1^{-/-} cells (Fig. 3A). Peritoneal cells from BLT1^{+/+} and BLT1^{-/-} mice exhibited almost similar calcium mobilization in response to 1 μM fMLP. LTB₄ is reported to cause degranulation in granulocytes and macrophages, and the requirement of high concentrations of LTB₄ for degranulation has led to the suggestion that this response is mediated by signaling through the low-affinity LTB₄ receptor BLT2 in this process (33). However, when we analyzed the effect of BLT1 deficiency on LTB₄-dependent degranulation using mouse granulocytes by measuring released MPO, LTB₄ dose-dependently released MPO in peritoneal cells from BLT1^{+/+} mice, whereas in BLT1^{-/-} mice, LTB₄-dependent MPO release was completely lost (Fig. 3B), showing that BLT1 is required for LTB₄-dependent degranulation. Peritoneal cells from both BLT1^{+/+} and BLT1^{-/-} mice exhibited a similar response to 1 μM platelet-activating factor.

AHR model

To understand the pathophysiologic roles of BLT1 *in vivo*, we established a murine model of AHR that mimics human bronchial asthma. Mice were sensitized by i.p. injection of OVA in alum adjuvant twice, and challenged with OVA aerosol twice as de-

scribed in *Materials and Methods*. Airway contraction was induced by inhalation of increasing doses of MCh, and airway responsiveness was evaluated by determining the concentration of MCh required to double the lung resistance above the basal level (EC₂₀₀R₁). Airway hyperresponsiveness in BLT1^{-/-} mice was significantly attenuated compared with BLT1^{+/+} mice and was similar to that of mice sensitized with saline alone (Fig. 4A). We analyzed the numbers and population of the cells present in BALF. Although significant increase in total cell counts was observed in the BALF of BLT1^{+/+} mice challenged with OVA compared with BLT1^{+/+} and BLT1^{-/-} mice treated with saline, total cells counts in BLT1^{-/-} mice challenged with OVA was not changed significantly (Table I). Differential cell counts revealed the prominent recruitment of eosinophils into BALF in BLT1^{+/+} mice challenged with OVA, which was almost completely abolished in BLT1^{-/-} mice with same treatments (Table I, Fig. 4, B–D). BALF contents of lymphocytes and granulocytes were also reduced in BLT1^{-/-} mice challenged with OVA compared with BLT1^{+/+} mice, but the difference was not statistically significant (Table I). The number of macrophages was not changed between BLT1^{+/+} and BLT1^{-/-} mice. Histological examination of lung revealed drastic difference between BLT1^{+/+} and BLT1^{-/-} mice (Fig. 5). Sensitization and challenge with OVA caused dense peribronchial and perivascular accumulation of inflammatory cells as well as the gross integrity of the airway walls in BLT1^{+/+} mice (Fig. 5, A and C). These changes were minimal in BLT1^{-/-} mice challenged with OVA (Fig. 5, B and D). Apparent accumulation of eosinophils to the peribronchial and perivascular inflammatory lesion and attachment of eosinophils to the vascular endothelial wall were observed in OVA-challenged BLT1^{+/+} mice (Fig. 5E), which was not apparent in BLT1^{-/-} mice (Fig. 5F). To confirm the reduced eosinophil accumulation in BLT1^{-/-} mice, we measured EPO activities in the lung homogenates (32). The EPO activity in OVA-challenged BLT1^{-/-} lung was lower than that in OVA-challenged BLT1^{+/+} lung, showing that BLT1-deficiency reduced eosinophil accumulation in asthmatic lung (Fig. 5I). Blood eosinophil counts were not changed between OVA-challenged BLT1^{+/+} and BLT1^{-/-} mice (12.7 ± 4.0 and 10.3 ± 5.3% of total leukocytes, respectively; *n* = 5), showing that reduced eosinophil accumulation in the airway and lung of BLT1^{-/-} mice is not due to the decreased number of total eosinophils in the circulation. Blinded semiquantitative grading of the lung sections revealed significant difference in the degree of airway inflammation between BLT1^{+/+} and BLT1^{-/-} mice (*p* < 0.05, Kruskal-Wallis with Dunn's test; Fig. 5G). Excessive production of airway mucus glycoproteins by goblet cells in airway epithelium is a consistent finding in the lungs of asthmatics. Blinded semiquantification of staining of the goblet cells stained with PAS also revealed attenuated mucus scores in BLT1^{-/-} mice compared with BLT1^{+/+} mice (*p* < 0.01,

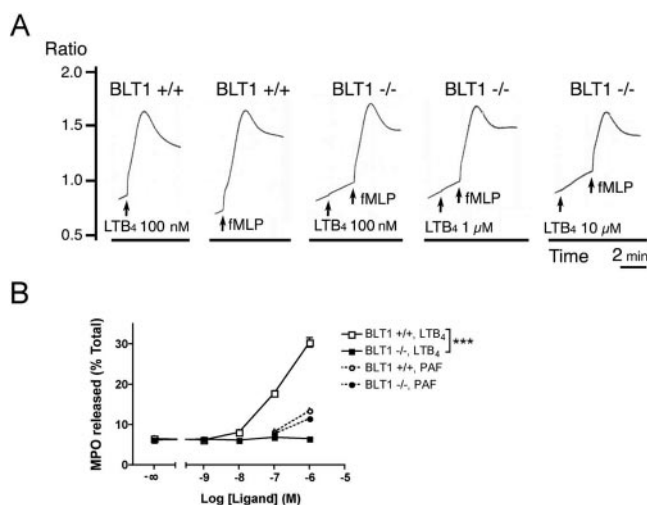
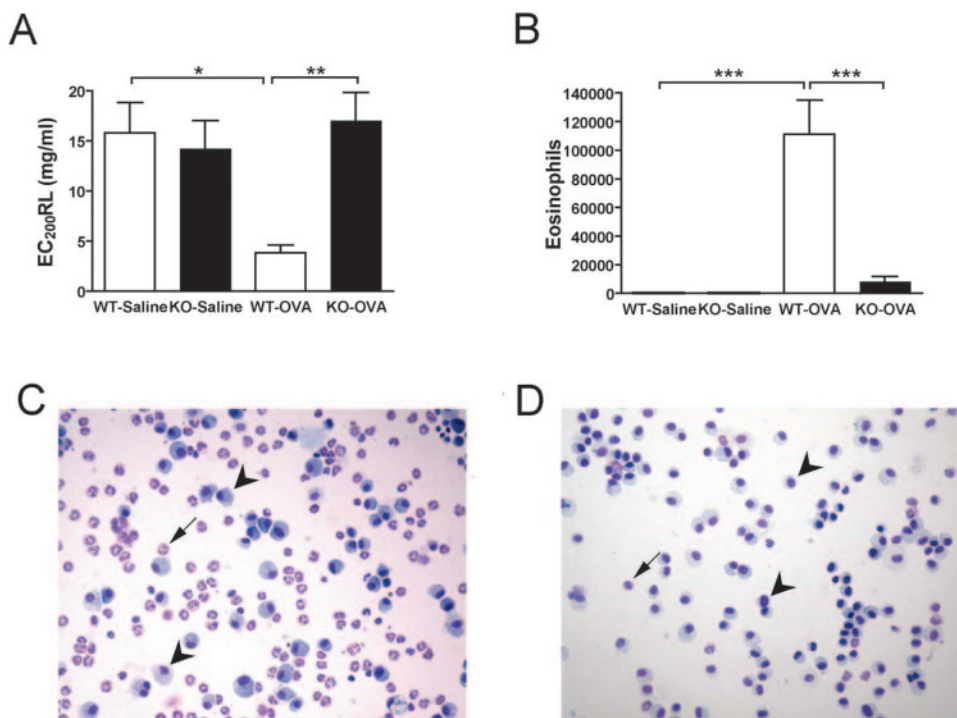


FIGURE 3. Ca²⁺ mobilization and MPO release in mouse peritoneal granulocytes. *A*, Ca²⁺ mobilization in granulocytes. Peritoneal granulocytes of F3 male mice loaded with fura-2/AM were stimulated with various concentrations of LTB₄ and 1 μM fMLP. *B*, MPO release. Peritoneal granulocytes of F4 male mice were stimulated with various concentrations of LTB₄ or platelet-activating factor (PAF) for 10 min at 37°C. The released MPO activities were measured against the synthetic substrate *o*-dianisidine and presented as percentages of total cellular MPO activities. A representative result from three independent experiments with similar results (values are mean ± SD, *n* = 3). ***, *p* < 0.001, two-way ANOVA.

FIGURE 4. Role of BLT1 in AHR. *A*, Airway responsiveness to MCh. MCh-induced AHR is expressed as the concentration of MCh required to double lung resistance ($EC_{200}RL$). Values are mean $\pm$ SEM ($n = 8$). *B*, Eosinophil counts in BALF. BALF cells were cytopun, stained by Diff-Quik, and differential cell counts were obtained. Eosinophil numbers were calculated from total numbers of BALF cells and differential cell counts. Values are mean $\pm$ SEM ($n = 6$ – 12). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, ANOVA with Bonferroni's test. *C* and *D*, Representative images of inflammatory cells in BALF obtained from OVA-treated- BLT1^{+/+} mice (*C*) and from OVA-treated-BLT1^{-/-} mice (*D*). Arrowheads and arrows represent macrophages and eosinophils, respectively. Age-matched (10–12 wk at initial sensitization) F6–8 male mice were used for these experiments.



Kruskal-Wallis with Dunn's test; Fig. 5*H*). All these data suggest that airway inflammation and goblet cell hyperplasia are reduced in BLT1^{-/-} mice.

To assess the mechanism for the reduced AHR and inflammation in BLT1^{-/-} mice, we measured Ig contents in BALF and plasma, and accumulation of Th1/2 cytokines and eicosanoids in BALF. Sensitization and challenge with OVA caused significant increase in OVA-specific IgG1 production in BALF and plasma in BLT1^{+/+} mice, and this was not attenuated in BLT1^{-/-} mice (Fig. 6, *A* and *C*). Sensitization and challenge with OVA also increased the contents of total IgE in BALF and plasma in BLT1^{+/+} mice, which were significantly reduced in BLT1^{-/-} mice (Fig. 6, *B* and *D*). We next analyzed the concentrations of Th1/Th2 cytokines in BALF and plasma from OVA-sensitized BLT1^{+/+} and BLT1^{-/-} mice. IL-5 and IL-13 contents in BALF were increased by OVA sensitization/challenge in BLT1^{+/+} mice, but this increase was significantly reduced in BLT1^{-/-} mice (Fig. 7, *A* and *B*). IFN- γ and IL-4 contents in BALF were below the detection limit (2.5 pg/ml) in all groups. We also measured eicosanoid contents in BALF. PGE₂ content in BALF was increased by OVA sensitization/challenge, and there was no difference seen between BLT1^{+/+} and BLT1^{-/-} mice challenged with OVA (Fig. 7*C*). In contrast, LTB₄ in BALF was increased only in OVA-sensitized BLT1^{+/+} mice compared with the saline-sensitized

mice, but not in OVA-sensitized BLT1^{-/-} mice (Fig. 7*D*). CysLTs, eotaxin, and MCP-1 α were below the detection limits (0.75, 15.6, and 4.7 pg/ml, respectively) in all BALF samples.

We next hypothesized that the reduced Th2 cytokines in BALF are due to the attenuated Th2 response in BLT1^{-/-} mice, thus performed in vitro stimulation experiments using lymph node cells. Cells from peribronchial lymph nodes of sensitized and challenged BLT1^{-/-} mice exhibited attenuated proliferation (Fig. 8*A*) and production of Th2 cytokines, IL-5 (Fig. 8*C*), and IL-13 (Fig. 8*D*) by re-stimulation with OVA in vitro compared with those of sensitized and challenged BLT1^{+/+} mice. These cells exhibited similar proliferation by stimulation with plate-bound anti-CD3 ϵ Ab (Fig. 8*B*), suggesting that the TCR signaling is not impaired in BLT1^{-/-} mice. The ratios of the CD4⁺, CD8⁺, or CD3⁺ T cells, and CD11c- and MHC class II-double positive dendritic cells were not changed between wild-type (WT) and BLT1^{-/-} lymph node. These data suggest that induction of Th2 lymphocytes relies significantly on signals through BLT1.

Discussion

LTB₄, known as a potent chemoattractant and activator for granulocytes and macrophages, has been intensively studied and implicated in various inflammatory diseases such as psoriasis and

Table I. Total and differential cell counts obtained from bronchoalveolar lavage fluid^a

Genotype	Exposure	n	Total Counts ($\times 10^5$ cells)	Differential Cell Counts ($\times 10^4$ cells)			
				Macrophages	Eosinophils	Lymphocytes	Granulocytes
BLT1 ^{+/+}	SAL	7	1.27 $\pm$ 0.05	12.5 $\pm$ 0.45	0 $\pm$ 0.0	0.15 $\pm$ 0.04	0.02 $\pm$ 0.02
BLT1 ^{-/-}	SAL	6	1.24 $\pm$ 0.10	12.1 $\pm$ 0.94	0 $\pm$ 0.0	0.21 $\pm$ 0.04	0.05 $\pm$ 0.03
BLT1 ^{+/+}	OVA	12	2.16 $\pm$ 0.29	10.3 $\pm$ 1.26	11.1 $\pm$ 2.4	0.68 $\pm$ 0.17	0.10 $\pm$ 0.05
BLT1 ^{-/-}	OVA	12	1.44 $\pm$ 0.14	13.2 $\pm$ 1.14	0.84 $\pm$ 0.3	0.41 $\pm$ 0.14	0.03 $\pm$ 0.03

^a Values are the means $\pm$ SEM. BLT1^{+/+}, wild-type mice; BLT1^{-/-}, BLT1-deficient mice; SAL, saline-immunized and saline-aerosolized treatment; OVA, ovalbumin-immunized and ovalbumin-aerosolized treatment. After bronchoalveolar lavage, the recovered cells were counted and a portion of the cells was centrifuged onto a microscope slideglass using Cytospin. The slides were stained with Diff-quick, and differential cell counts were obtained. There was no significant difference between total cell counts of BLT1^{+/+} OVA and that of BLT1^{-/-} OVA mice. The numbers of total BALF cells and eosinophils of BLT1^{-/-} mice given OVA were significantly reduced compared with those of BLT1^{+/+} mice given OVA ($p < 0.001$, One-way ANOVA).

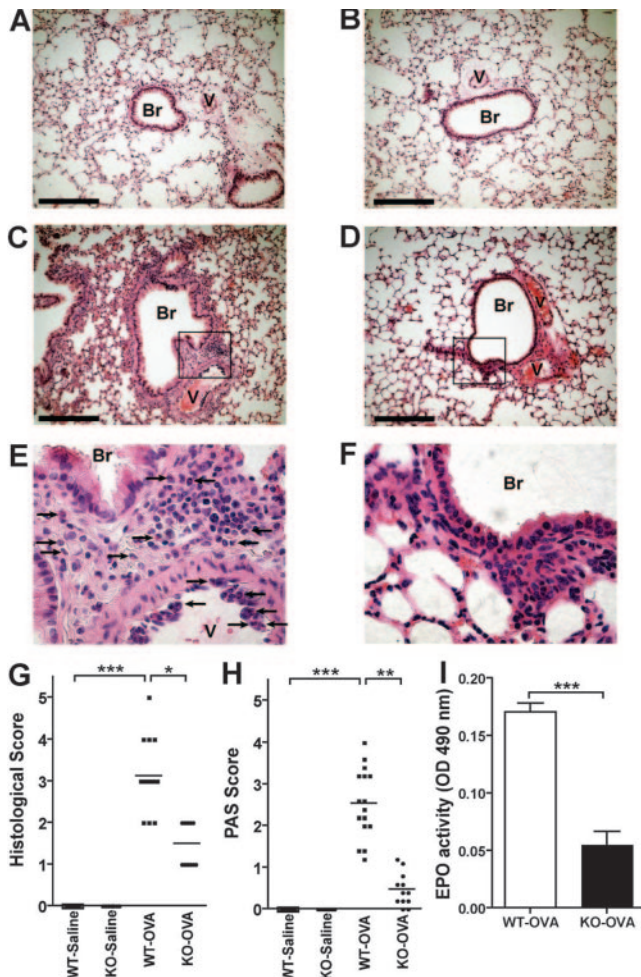


FIGURE 5. Histological analysis of lung sections. Nonsensitized BLT1^{+/+} (A) and BLT1^{-/-} (B) mice were exposed to an aerosol of saline, and sensitized BLT1^{+/+} (C) and BLT1^{-/-} (D) mice exposed to an aerosol of 1% OVA/saline. The H&E-stained sections shown are representative of two lung sections per mouse, from four or six mice in each saline-treated or OVA-treated group, respectively. Scale bar, 200 μ m. E and F, the higher magnification images ($\times 1000$) of squares shown in C and D, respectively. Representative eosinophils are shown by arrows. Br, bronchus; V, vessels. G and H, Assessment of lung inflammation. The stained sections were semiquantitatively scored as described in *Materials and Methods*, and scores for individual sections are presented. Bars depict means of groups. After aeroallergen challenge, BLT1^{-/-} mice developed milder lung inflammation than BLT1^{+/+} mice, as determined in sections stained with H&E (G). The levels of OVA-induced mucus production in BLT1^{-/-} mice were milder than in BLT1^{+/+} mice, as determined in sections stained with PAS (H). Age-matched (10–12 wk at initial sensitization) F8 male mice were used for these experiments. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, Kruskal-Wallis with Dunn's test. I, EPO activity in the lung homogenate from sensitized/challenged mice. Values are mean $\pm$ SEM ($n = 9$). ***, $p < 0.001$, unpaired t test.

inflammatory bowel diseases. BLT1, a high-affinity G protein-coupled receptor for LTB₄, was first isolated in our laboratory and shown to be expressed in granulocytes, macrophages, monocytes, eosinophils, and to a lesser extent in naive lymphocytes (34–36). These findings seemed consistent with the classical notion that LTB₄ is a local inflammatory mediator. We, thus, generated targeted BLT1 homozygous mutant mice to clarify the in vivo functions of BLT1.

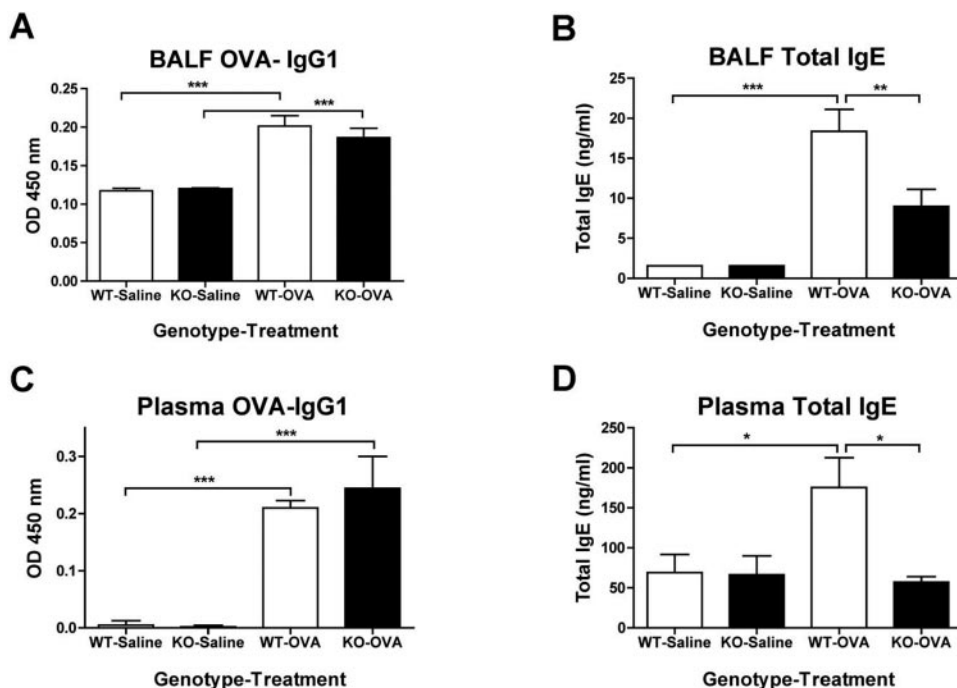
Recently, several papers suggested that LTB₄ functions through BLT1 not only as a local inflammatory mediator, but also acts as

an important attractant for differentiated T cells. BLT1 expression is greatly enhanced in CD4⁺ T cells differentiated into Th0, Th1, and Th2 in vitro (21). Accordingly, Th1- and Th2-differentiated T cells showed robust chemotaxis toward LTB₄, but naive CD4⁺ cells did not (20, 37). LTB₄-dependent T cell chemotaxis was dependent on BLT1, as the cells derived from BLT1-null mice did not show any chemotactic activity toward LTB₄ either in vitro or in vivo (20, 37). Thus, LTB₄ is now considered to control immunological reactions by attracting both CD4⁺ and CD8⁺ T cells with the effector phenotypes. Mast cells, recognized for their involvement in allergic responses, release LTB₄ upon stimulation and recruit effector CD8⁺ cells in vitro (22). Despite these attracting ex vivo models, no prominent in vivo phenotype has been so far reported in BLT1^{-/-} mice. Thus, we adopted a mouse AHR model using OVA sensitization to reveal the functions of BLT1 in immunological disorders in vivo, as it is an allergic inflammatory animal model where mast cells, dendritic cells, Th2 cells, and eosinophils play dominant roles.

After systemic sensitization of mice with i.p. injection of OVA in alum adjuvant, mice were challenged with inhalation of aerosolized OVA. Sensitization/challenge with OVA caused hyperresponsiveness to MCh (Fig. 4A) accompanied by prominent accumulation of eosinophils into airway (Fig. 4, B and C), peribronchovascular accumulation of inflammatory cells (Fig. 5, C, E, and G), and by excessive production of airway mucus glycoproteins by goblet cells (Fig. 5, C and H) in WT mice. However, EC₂₀₀R_L values of MCh in BLT1^{-/-} mice were not changed by sensitization/challenge with OVA (Fig. 4A). Airway eosinophilia and histological change in lung by sensitization/challenge with OVA were almost completely abolished in BLT1^{-/-} mice (Figs. 4, B and D, and 5, D, F–I). All these data show that BLT1 is required for the development of airway inflammation, eosinophilia, and AHR in this mouse model that mimics human bronchial asthma.

It is widely accepted that mast cells, eosinophils, and T-lymphocytes are important inflammatory cells in the onset and progression of bronchial asthma (38–40). Recently, effector CD4⁺ T cells are recognized to play dominant roles in initiating allergic pulmonary inflammation, because adoptive transfer of Ag-specific Th2 cells, not Th1 cells, into naive mice followed by aerosol challenge of Ag caused AHR, eosinophilia, and mucus secretion with recruitment of Th2 cells into the allergic lung (41). Tager et al. (21) elegantly showed the requirement of BLT1 in trafficking of Th2 cells as well as Th1 cells into allergic airway in active immunization model. In our model, a relatively small number of lymphocytes accumulated into the allergic airway, and no significant difference was observed between WT and BLT1^{-/-} mice (Table I). Although IgE content and Th2 cytokines IL-5 and IL-13 in BALF were increased in BLT1^{+/+} mice after sensitization/challenge with OVA, this was not observed in BLT1^{-/-} animals (Figs. 6 and 7), suggesting the impaired development of Th2 cells in BLT1^{-/-} mice. Impaired proliferation and production of Th2 cytokines in peribronchovascular lymph node cells from BLT1^{-/-} mice (Fig. 8) showed that not only recruitment of Th2 cells into airway, but also the differentiation of naive T cells into Ag-specific Th2 cells are also impaired in BLT1^{-/-} mice. Enhanced production of Th2 cytokines after Ag exposure is important for the initiation of bronchial asthma. In asthmatic state, IL-4 drives IgE synthesis from B cells (42), IL-5 plays important roles in differentiation, recruitment, and activation of eosinophils (43), and IL-13 is considered to activate B cells, eosinophils, and airway smooth muscle cells (44). It is reasonable to speculate that reduced IL-5 production resulted in the attenuated activation of eosinophils leading to the reduced airway eosinophilia in BLT1^{-/-} mice (Fig. 4). The importance of LTB₄-BLT1 axis is reported in relation to Th2 cytokines in

FIGURE 6. Reduced IgE production in BLT1^{-/-} mice. Levels of OVA-specific IgG1 (A and C) and total IgE (B and D) were measured in mouse BALF (A and B) and plasma (C and D). Undiluted BALF and diluted plasma (100-fold dilution for IgG1, and 10-fold dilution for IgE) were subjected to ELISA. Values are mean $\pm$ SEM ($n = 5-6$). Age-matched (10–12 wk at initial sensitization) F8 male mice were used for these experiments. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, ANOVA with Bonferroni's test.



eosinophils. BLT1 expression was greatly enhanced in eosinophils of IL-5 transgenic mice (36). In thioglycollate-induced peritonitis models, eosinophil migration into peritoneal cavity was greatly reduced in BLT1^{-/-} mice (45). Although we have observed a marked reduction in the accumulation of eosinophils (Figs. 4 and 5I) and LTB₄ (Fig. 7D) in BALF of BLT1^{-/-} mice, we have no evidence showing that LTB₄ functions as a direct attractant for eosinophils in our AHR model. Because we could not detect measurable amounts of other possible eosinophil attractants, eotaxin, MCP-1 α , and CysLTs in BALF of both BLT1^{+/+} and BLT1^{-/-} mice even after sensitization/challenge

with OVA, LTB₄ might be a principle attractant for eosinophils under Th2 environments. This does not exclude the involvement of eotaxin, MCP-1 α and CysLTs in this asthma model. We should carefully examine the roles of these possible eosinophil chemoattractants at various time points. Cells responsible for LTB₄ production in allergic lung are not clear now, but mast cells, dendritic cells, and eosinophils are all candidate cells. Considering the previous report showing that expression of 5-lipoxygenase, a responsible enzyme for biosynthesis of LTB₄, is enhanced in BLT1 transgenic mice (46), there is a positive feedback system between BLT1 activation and LTB₄ production in

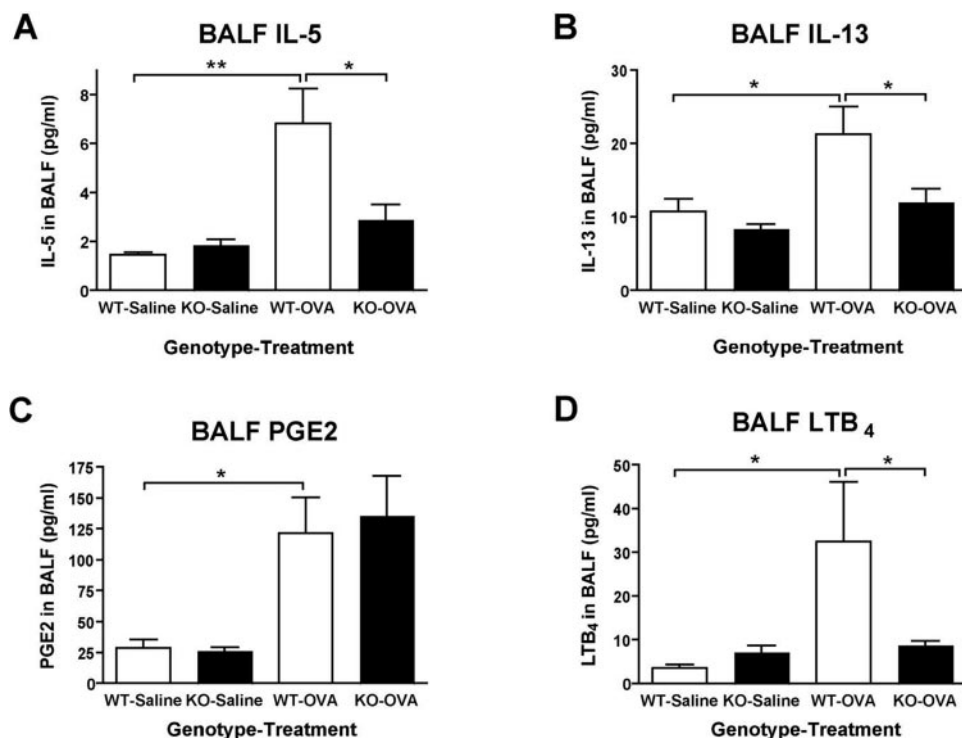
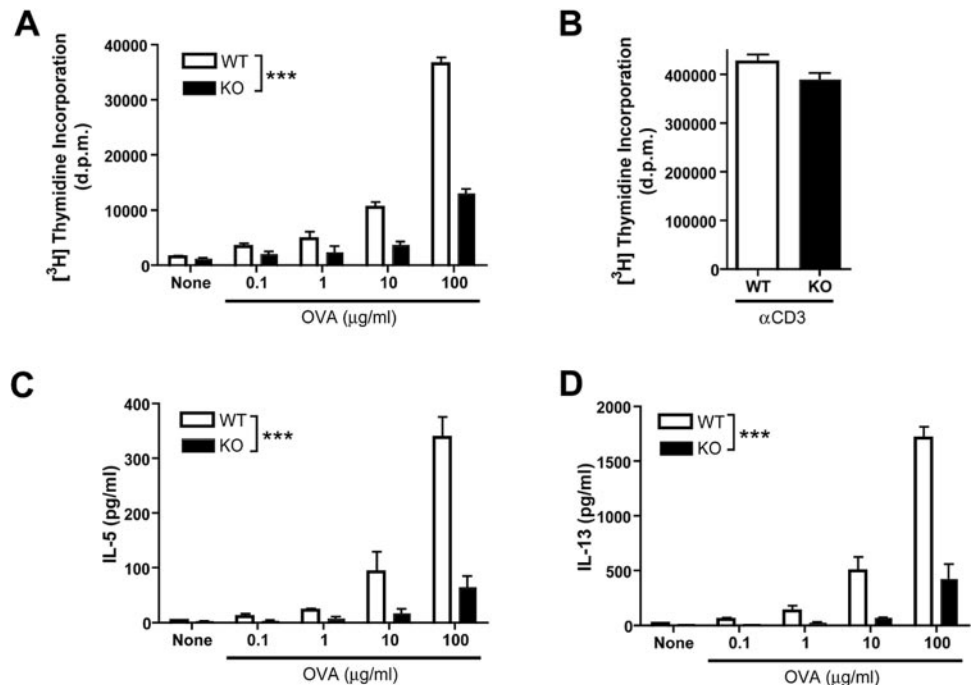


FIGURE 7. Reduced IL-5, IL-13, and LTB₄ in BALF in BLT1^{-/-} mice. Cytokines (A and B), PGE₂ (C) and LTB₄ (D) in BALF were measured. Values are mean $\pm$ SEM ($n = 6-12$). IL-2 and IL-4 in BALF were not detectable. Age-matched (10–12 wk at initial sensitization) F8 male mice were used. *, $p < 0.05$, **, $p < 0.01$, ANOVA with Bonferroni's test.

FIGURE 8. Reduced Th2 response in BLT1^{-/-} mice. Cells isolated from peribronchial lymph nodes of OVA-sensitized/challenged mice were restimulated in vitro with OVA (A, C, D) or plate-bound α CD3 Ab (B). [³H]Thymidine was pulsed for the last 8 h of 72 h of culture (A, B). Accumulation of IL-5 (C) and IL-13 (D) in culture supernatants by OVA stimulation. Age-matched (10–12 wk at initial sensitization) F8 male mice were used. Values are mean $\pm$ SD ($n = 3$). ***, $p < 0.001$, two-way ANOVA.



inflammation. Indeed, the increased production of LTB₄ was observed in OVA-sensitized and challenged BLT1^{+/+} mice (Fig. 7D). IL-13 activates B cells, eosinophils, and airway smooth muscle cells (44), more importantly activates mucus metaplasia (47), and is now considered an important drug target in human bronchial asthma. In good relations to the reduced hyperplasia of PAS-positive goblet cells in the airway, BLT1^{-/-} lymph node cells produced much less IL-13 than BLT1^{+/+} cells (Fig. 8D).

Recently, CD8⁺ T cells in the lung were reported as a possible source of IL-13 and required for the development of allergen-induced AHR (48). During preparation of this manuscript, Miyahara et al. (49) reported the attenuated allergen-induced airway hyperresponsiveness in BLT1-deficient mice, which were established independently from ours. In their study, BLT1-deficient mice on BALB/c background exhibited reduced AHR, goblet cell hyperplasia, and IL-13 production in LN cells after sensitization and challenge with OVA. In contrast to our results, airway eosinophilia and productions of IL-4 and IL-5 in peribronchial LN cells were not attenuated by BLT1 deficiency, and this could be due to the different genetic backgrounds of mice and inhalation protocols used in the experiments. They also addressed the mechanism and showed that adoptive transfer of in vivo primed CD8⁺ BLT1^{+/+} T cells or in vitro generated OVA-specific effector CD8⁺ BLT1^{+/+} T cells from OT-I mice fully restored the reduced AHR, airway eosinophilia, and IL-13 production in CD8-null mice (50). Thus, LTB₄-BLT1 interaction plays an important role in initiating and developing Th2-type immune response by attracting CD8⁺ effector T cells into the lung to produce IL-13, and stimulating T cells to produce IL-4 and -5 in the LN, providing a novel idea about how LTB₄ plays immunological roles. Blocking BLT1 by specific antagonists may be beneficial for the prevention and therapy of various immunological disorders including bronchial asthma, and BLT1^{-/-} mice will be useful in the identification of the diseases for which BLT1 antagonists could be effective as novel types of anti-inflammatory and immunosuppressive drugs.

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Disclosures

The authors have no financial conflict of interest.

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