

Leukotriene B₄ receptor BLT2 negatively regulates allergic airway eosinophilia

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ABSTRACT Leukotriene B₄ (LTB₄) has been implicated in the pathogenesis of allergic diseases. BLT2, a low-affinity LTB₄ receptor, is activated by LTB₄ and 12(S)-hydroxyheptadeca-5Z,8E,10E-trienoic acid (12-HHT). Although the high-affinity LTB₄ receptor BLT1 has been shown to exert proinflammatory roles, the role of BLT2 in allergic inflammation has not been clarified. To study the function of BLT2 in development of asthma, we used mice model of ovalbumin (OVA)-induced allergic airway disease. The 12-HHT levels were elevated in bronchoalveolar lavage (BAL) fluids of OVA-sensitized/challenged wild-type mice. BLT2-deficient mice exhibited enhanced eosinophilia in BAL fluids after OVA exposure. Interleukin (IL)-13 levels in BAL fluids and IL-13-producing CD4⁺ T cells in the lungs were elevated in BLT2-deficient mice compared to wild-type mice, whereas the levels of IL-4, IL-5, and interferon (IFN)- γ in BAL fluids and serum OVA-specific IgE were comparable. Transfection of BLT2-specific small interfering RNA enhanced IL-13 production in CD4⁺ T cells *in vitro*. Expression of BLT2 mRNA in CD4⁺ T cells was significantly reduced in patients with asthma compared to healthy control subjects. These findings indicate that BLT2 has a protective role in allergic airway inflammation and that diminished BLT2 expression in CD4⁺ T cells may contribute to the pathophysiology of asthma.—Matsunaga, Y., Fukuyama, S., Okuno, T., Sasaki, F., Matsunobu, T., Asai, Y., Matsumoto, K., Saeki, K., Oike, M., Sadamura, Y., Machida, K., Nakanishi, Y., Kubo, M., Yokomizo, T., Inoue, H. Leukotriene B₄ receptor BLT2 negatively regulates allergic airway eosinophilia. *FASEB J.* 27, 000–000 (2013). www.fasebj.org

Key Words: asthma • CD4⁺ T cell • interleukin-13 • 12(S)-hydroxyheptadeca-5Z,8E,10E-trienoic acid

ASTHMA IS A CHRONIC inflammatory disease of the airways characterized by infiltration of eosinophils, mast cells, and T lymphocytes. These inflammatory cells and structural cells releasing proinflammatory cytokines and lipid mediators contribute to the development of the pathophysiology of asthma (1). In addition to an overabundance of proinflammatory mediators, persistent airway inflammation in asthma can also result from defects in counterregulatory signaling (2).

Leukotriene B₄ (LTB₄) is a potent lipid mediator derived from arachidonic acid by the consequent action of 5-lipoxygenase, 5-lipoxygenase-activating protein, and leukotriene A₄ hydrolase (3). LTB₄ mediates a diverse array of biological activities through its interaction with two distinct G-protein-coupled 7-transmembrane domain receptors: a high-affinity receptor, BLT1, and a low-affinity receptor, BLT2 (4). BLT1 is expressed mainly on granulocytes both in humans and mice (5) and controls cell migration of the sites of inflammation (6–9). BLT2 is widely distributed across multiple organ systems in humans (10).

LTB₄ has been widely implicated in the pathogenesis of several inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and asthma (11). Elevated levels of LTB₄ were detected in the airways of patients with asthma (12). Studies using BLT1-deficient mice demonstrated an important role for BLT1 in asthma (7, 9). LTB₄-BLT1 signals are responsible for an early response to antigen-induced inflammation. BLT1

Abbreviations: 12-HHT, 12(S)-hydroxyheptadeca-5Z,8E,10E-trienoic acid; BAL, bronchoalveolar lavage; BMMC, bone marrow-derived mast cell; BSA, bovine serum albumin; [Ca²⁺]_i, intracellular Ca²⁺ concentration; CHO, Chinese hamster ovary; DNP, dinitrophenyl; IFN, interferon; IL, interleukin; LTB₄, leukotriene B₄; OVA, ovalbumin; PBLN, peribronchial lymph node; WT, wild type

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doi: 10.1096/fj.12-217000

This article includes supplemental data. Please visit <http://www.fasebj.org> to obtain this information.

is required for the recruitment of effector CD4⁺ and CD8⁺ T cells to the sites of inflammation at the early stages of antigen stimulation (8, 13). However, the BLT2 receptor has not been well characterized, and its potential role in the pathogenesis of asthma remains unclear. BLT1 and BLT2 share a distinct repertoire of ligands. BLT2 was originally identified as a low-affinity LTB₄ receptor; however, a cyclooxygenase metabolite, 12(*S*)-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid (12-HHT), has subsequently been shown to activate BLT2 at a lower concentration than LTB₄. Considering the abundance of 12-HHT in serum, 12-HHT most likely functions as an endogenous ligand for BLT2 *in vivo* (14).

Based on the difference in those pharmacological properties and different distribution among tissues, we speculated that different functions might exist between BLT1 and BLT2 in the pathogenesis of bronchial asthma. We thus investigated the role of BLT2 on allergic asthma using *BLT2*-deficient mice. Interleukin (IL)-4, IL-5, IL-13, and interferon (IFN)- γ cytokine levels were measured as a primary output along with eosinophil levels. These parameters were selected due to their association with the asthma-related immune response. We show here that *BLT2*-deficient mice exhibited increased production of IL-13 from CD4⁺ T cells and exaggerated allergen-induced eosinophilia in an allergic asthma model. These findings indicate some novel inhibitory functions of BLT2 in allergic airway inflammation. Some of the results of these studies have been reported previously in an abstract form (15).

MATERIALS AND METHODS

Mice

BLT1^{-/-} mice and *BLT2*^{-/-} mice were generated as described previously (9, 16) and backcrossed onto BALB/c mice over 10 generations. Male mice were used for experiments. All studies and procedures with mice were approved by the Ethics Committees for Animal Experiments of Kyushu University and of Kagoshima University. Approval for experiments with human samples was provided by the Human Ethics Committee of Kyushu University.

LTB₄-induced airway inflammation

LTB₄ (0.1 μ g; Cayman Chemicals, Ann Arbor, MI, USA) in 50 μ l saline was administered intratracheally. Control mice were treated with 50 μ l of saline. At 6 h after instillation, mice were euthanized, and bronchoalveolar lavage (BAL) was performed using 1 ml saline.

Sensitization and challenge

BLT2^{-/-} mice or their wild-type (WT) littermates were sensitized with intraperitoneal injection of 20 μ g ovalbumin (OVA; grade 5; Sigma-Aldrich, St Louis, MO, USA) with 2.25 μ g aluminum hydroxide (Thermo Scientific, Bremen, Germany) on d 1 and 14. On d 26–28, the mice received an aerosol challenge containing either saline or 1% OVA for 20 min/day, as described previously (17). On d 30, the mice were anesthetized, and their tracheas were cannulated *via* tracheostomy. The animals were ventilated mechanically

(tidal volume, 0.3 ml; frequency, 120 breaths/min). Airway opening pressure was measured with a differential pressure transducer and recorded continuously. Stepwise increases in intratracheal administration of the acetylcholine dose (0.6 to 20 mg/ml) were given using an ultrasonic nebulizer.

BAL and cytokine measurement

After measurement of the airway responsiveness, blood was drawn from the inferior vena cava; then, BAL was performed with 1 ml saline, as described previously (17). Total cell counts and differential cell counts on BAL fluid were performed. The levels of IL-4, IL-5, IL-13, and IFN- γ in the supernatants were quantified using ELISA kits (R&D Systems, Minneapolis, MN, USA).

The serum OVA-specific IgE was measured using ELISA (Shibayagi, Gunma, Japan).

Bone marrow-derived mast cell (BMMC) culture, BLT2-siRNA transfection, and IL-13 production

WT BALB/c background BMDCs were cultured in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Carlsbad, CA, USA). BMDCs (4 $\times$ 10⁶/sample) were nucleofected with either 2.2 mM BLT2-siRNA (Ambion, Austin, TX, USA) or control siRNA using the Amaxa Nucleofector II or Amaxa Nucleofector 96-well shuttle system with the X-005 program (Amaxa, Gaithersburg, MD, USA). The cells were cultured for 24 h per the manufacturer's instructions. BLT2-siRNA transfection efficiency was 40–60%, as determined using a quantitative real-time PCR system (Roche Diagnostics, Mannheim, Germany). BMDCs (1 $\times$ 10⁶/sample) were sensitized using 1 μ g/ml monoclonal dinitrophenyl (DNP)-IgE (Seikagaku, Tokyo, Japan) for 24 h. BMDCs (5 $\times$ 10⁵/sample) were stimulated with 100 ng/ml DNP-bovine serum albumin (BSA; LSL Ltd., Tokyo, Japan) for 24 h.

The supernatant IL-13 level was analyzed using ELISA (R&D Systems). BMDCs were also cultured from *BLT2*^{-/-} or WT mice. BMDCs (1 $\times$ 10⁶/sample) were sensitized and stimulated using DNP-IgE and DNP-BSA, respectively, and supernatant IL-13 was analyzed.

Intracellular Ca²⁺ concentration ([Ca²⁺]_i) measurement (CD4⁺ T cells)

CD4⁺ T cells were isolated from peribronchial lymph nodes (PBLNs) using the MACS separation method (Miltenyi Biotec, Gladbach, Germany). Cells were loaded with Fura-2-acetoxymethyl ester (Fura-2-AM) and stimulated with either 0.3% ethanol or 1 μ M 12-HHT dissolved in 0.3% ethanol, and the changes in [Ca²⁺]_i were measured using a fluorescence microscopy system as described previously (18).

Calcium assay [Chinese hamster ovary (CHO) cells]

Chinese hamster ovary (CHO) cells were transfected with BLT1 or BLT2 expression vector using the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol, as described previously (19). A calcium assay was performed as described previously (20). Both cell strains are pretreated with the BLT2 antagonist LY255283 for 30 min and stimulated with LTB₄ or 12-HHT at the indicated concentration at 37°C. The FLEXstation was used for the fluorometric [Ca²⁺]_i measurement.

Determination of lipid fraction by mass spectrometry analysis of BAL fluids

BAL fluids (100 μ l) from OVA-sensitized/challenged WT mice were mixed with 200 μ l methanol, followed by dilution with distilled H₂O to the methanol concentration of 20%. Samples were subjected to solid-phase columns. The lipids absorbed in the column were eluted with 200 μ l methanol. Elutions (50 μ l; equal to 25 μ l of BAL fluid) were subjected to electrospray ionization-mass spectrometry by flow injection. Mass spectrometry analyses were performed using a TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific) as described previously (21).

Flow cytometry

Lung cells were stimulated with anti-mouse CD3 mAb (145-2C11) plus anti-mouse CD28 mAb (37.51) for 48 h. Surface staining was performed using FITC-labeled anti-CD4 (RM4-5) or anti-CD8a mAbs (53-6.7) (22). After fixation and permeabilization, cells were stained with allophycocyanin-labeled anti-IL-13 mAb (JES5-16E3). Flow cytometry was performed using a FACS Calibur (Becton-Dickinson, San Jose, CA, USA) with CellQuest software (BD Biosciences, San Jose, CA, USA).

Quantitative real-time PCR

Quantitative real-time PCR for mBLT2 or hBLT2 was performed with a LightCycler system (Roche Diagnostics) using specific primers (10). The levels of mBLT2 mRNA were normalized to the level of GAPDH mRNA. hBLT2 was quantified using the TaqMan LightCycler system (Roche Diagnostics). For hBLT2, human β -actin was used as a standard.

Isolation of CD4⁺ T cells, transfection of siRNA, and induction of Th2 cells

Several murine BLT2 siRNAs and scrambled control siRNA were purchased from Ambion. Optimal BLT2 siRNA was chosen according to the results of each siRNA experiment. CD4⁺ T cells were isolated from splenocytes of DO11.10 Tg mice using the MACS negative-selection method. CD4⁺ T cells were transfected with 2.2 mM of control or BLT2 siRNA by electroporation (Amaxa Nucleofector II or Amaxa Nucleofector 96-well shuttle system, program X-001). Scrambled control siRNA were purchased from Ambion. Scrambled siRNAs contained the same nucleotide content as the selected siRNAs but in a random sequence and had no calculated target gene specificity.

After being cultured in the Nucleofector medium for 24 h, CD4⁺ T cells were activated with irradiated splenocytes of naive BALB/c mice as antigen-presenting cells, an OVA peptide (pOVA 323–339; 1 μ g/ml; Bachem, Torrance, CA, USA), recombinant IL-4 (100 ng/ml; Pepro Tech, Inc., Rocky Hill, NJ, USA), anti-IL-12 mAb (10 μ g/ml; R&D Systems), and anti-CD28 mAb (1 μ g/ml, BD PharMingen, San Diego, CA, USA). On d 3 and 5, the cells were fed recombinant IL-2 (2 ng/ml; Pepro Tech). On d 8, Th2-differentiated CD4⁺ T cells were restimulated with antigen-presenting cells and pOVA (1 μ g/ml) for 48 h. The cytokine profiles of cultured supernatants were analyzed using ELISA.

Collection of human blood samples

Blood samples from normal control individuals were collected from 12 healthy volunteers aged 24–64 yr old who had total IgE < 100 IU/ml, eosinophils < 5%, mite-specific IgE <

0.34 IU/ml, and no history of asthma, atopic dermatitis, or allergic rhinitis. The samples from adult asthma patients with or without inhaled corticosteroid treatment were collected from 11 and 15 volunteers aged 27–65 and 25–78 yr old, respectively. CD4⁺ T cells were isolated using Lymphoprep (Axis-Shield Poc AS, Oslo, Norway) and a MACS positive-selection method.

Statistical analysis

Values are expressed as means $\pm$ SEM. Differences among groups were analyzed using the Student's *t* test or ANOVA with a Scheffe *post hoc* test.

RESULTS

Neutrophilic inflammation induced by LTB₄ was not impaired in *BLT2*^{-/-} mice

Although the genes for BLT1 and BLT2 are closely clustered on the same chromosome (4), *BLT2* disruption did not affect the expression of BLT1 in granulocytes (16) in our line of *BLT2*^{-/-} mice. In the present study, we first excluded the possibility of impairment of BLT1 function in *BLT2*^{-/-} mice *in vivo*. LTB₄ was administered intratracheally in WT, *BLT1*^{-/-}, and *BLT2*^{-/-} mice to evaluate the inflammatory responses. LTB₄-administered WT mice showed a higher number of neutrophils in BAL fluids than saline-administered mice (Fig. 1). *BLT1*^{-/-} mice failed to induce neutrophilia in BAL fluids following LTB₄ administration. However, LTB₄-instilled *BLT2*^{-/-} mice showed marked neutrophilia in BAL fluids that was comparable to that of WT mice (Fig. 1). These results confirmed that BLT1 is necessary for the LTB₄-induced recruitment of neutrophils into the airways and that BLT1 function is not impaired in our line of *BLT2*^{-/-} mice.

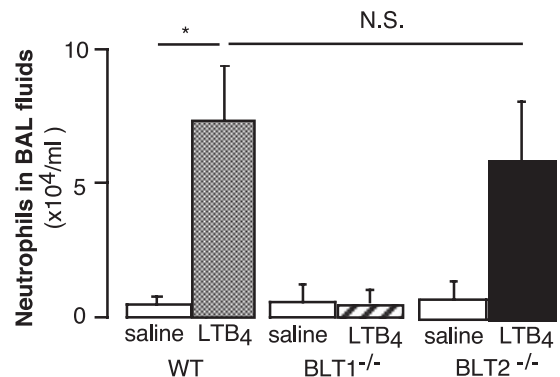


Figure 1. Airway response to LTB₄ in *BLT1*^{-/-} and *BLT2*^{-/-} mice. LTB₄ (0.1 μ g/50 μ l/body) was administered intratracheally to WT-, *BLT1*^{-/-}-, and *BLT2*^{-/-}-BALB/c male mice. At 6 h after administration, BAL was performed using 1 ml saline. *n* = 4 for WT-saline and *BLT1*^{-/-}-saline mice, *n* = 3 for *BLT2*^{-/-}-saline mice; *n* = 7 for WT-LTB₄ mice and *BLT2*^{-/-}-LTB₄ mice; *n* = 6 for *BLT1*^{-/-}-LTB₄ mice. N.S., not significant. **P* < 0.05.

Enhanced allergic airway inflammation in $BLT2^{-/-}$ mice

To clarify the roles of BLT2 in allergic airway responses, we investigated the effects of $BLT2$ deficiency in a mouse model of OVA-induced asthma. OVA sensitization and challenge resulted in an increased number of eosinophils in BAL fluid in WT mice. $BLT2^{-/-}$ mice after OVA challenges displayed a further increase in eosinophils in BAL fluids compared to WT mice, although no significant difference was found in the number of lymphocytes and neutrophils (Fig. 2A, left panel). Female $BLT2^{-/-}$ mice after OVA challenges also showed enhanced eosinophilia in BAL fluids compared to female WT mice (see Supplemental Fig. S1). WT mice showed hyperresponsiveness to acetylcholine after OVA sensitization and challenge, and $BLT2^{-/-}$ mice also showed hyperresponsiveness comparable to that of WT mice (Fig. 2A, right panel). In the absence of sensitization and challenges, no substantial differences were apparent in inflammatory cells in BAL fluids and airway responsiveness between $BLT2^{-/-}$ and WT mice.

Next, we directly measured cytokine levels in BAL fluids. The concentrations of IL-4, IL-5, and IL-13 significantly increased in OVA-treated WT mice compared to those of naive WT mice. The concentration of IL-13 in OVA-treated $BLT2^{-/-}$ mice was significantly higher than that in WT mice (Fig. 2B). However, no difference was found in the concentration of IL-4, IL-5, or IFN- γ between OVA-treated $BLT2^{-/-}$ and WT mice. Compared to their naive mice, the titers of OVA-specific IgE in the sera were significantly increased after OVA challenges in WT mice and $BLT2^{-/-}$ mice, and there was no significant difference in the titers between the OVA-treated groups (Fig. 2C).

We then assessed the development of Th1 and Th2 cells from naive $CD4^{+}$ T cells in WT and $BLT2^{-/-}$ mice. T cells were stimulated with anti-TCR plus anti-CD28 mAbs. IL-4 and IFN- γ levels in the culture supernatant were also comparable between $BLT2^{-/-}$ and WT mice (data not shown). These data suggest that $BLT2$ deficiency affects IL-13 production in the airways but not Th2 differentiation.

IL-13 was overproduced in $CD4^{+}$ T cells of $BLT2^{-/-}$ mice

We next evaluated the source of IL-13 production. When whole cells were collected from the PBLNs of OVA-treated mice and then stimulated *ex vivo* with OVA, the concentration of IL-13 in the cultured supernatant was significantly higher in the supernatants from $BLT2^{-/-}$ mice than in that from WT mice (Fig. 3A). This finding suggests that T cells may be a potent source of IL-13. To determine which T-cell subset is responsible for the overproduction of IL-13 in the lungs, IL-13-producing $CD4^{+}$ and $CD8^{+}$ T-cell subsets were evaluated by intracellular cytokine staining. OVA sensitization and challenge resulted in an increased number of IL-13-producing $CD4^{+}$ T cells in WT mice. The number of IL-13-producing $CD4^{+}$ T cells obtained from the lungs of OVA-challenged $BLT2^{-/-}$ mice was significantly higher than that obtained from those of OVA-challenged WT mice (Fig. 3B). No significant difference was found in the number of IL-13-producing $CD8^{+}$ T cells between WT and $BLT2^{-/-}$ mice. The results suggest that the excessive IL-13 is at least produced from $CD4^{+}$ T cells in $BLT2^{-/-}$ mice.

Mast cells are also known to produce IL-13 (23–25), and recent studies suggest that BLT2 is expressed on mast cells (26, 27). We assessed the effect of BLT2

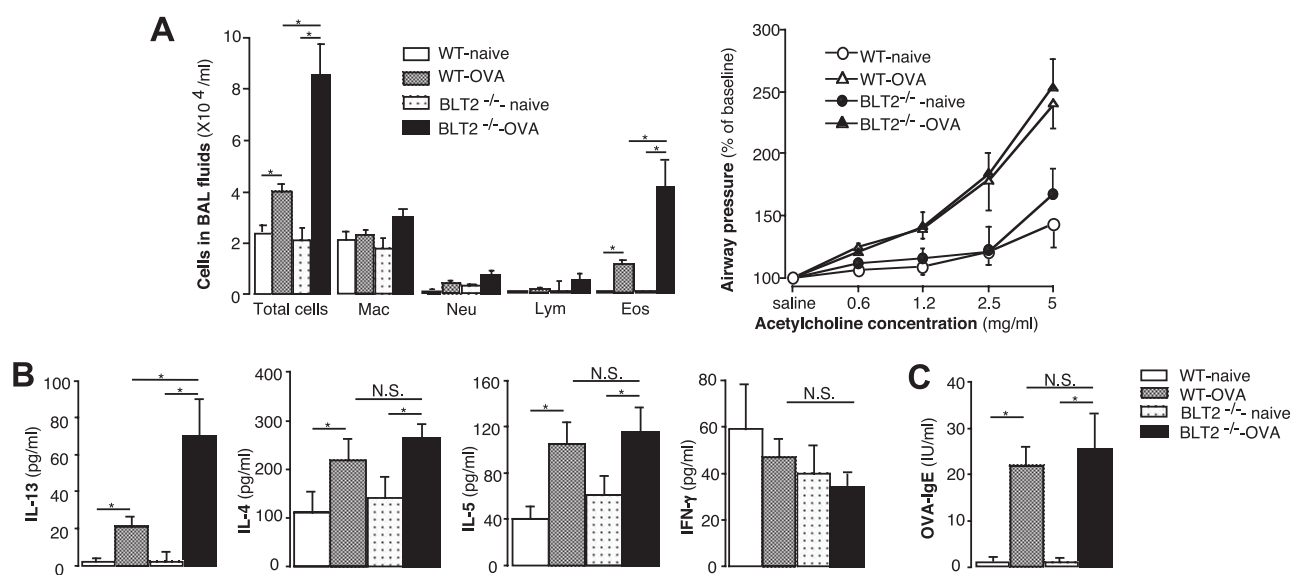


Figure 2. Effects of $BLT2$ deficiency on an OVA-induced asthma model. WT and $BLT2^{-/-}$ mice were sensitized with 20 μ g OVA on d 1 and 14 and exposed to aerosolized 1% OVA on d 26–28. Asthmatic responses were assessed 36 h after the last OVA challenge. A) Differential cell counts in BAL fluid and airway hyperresponsiveness to acetylcholine. * $P < 0.05$. B) Cytokine profiles in the supernatant of BAL fluid, determined using ELISA. C) OVA-specific IgE titers in the sera of WT-OVA and $BLT2^{-/-}$ -OVA mice, determined using ELISA. $n = 5$ for WT-naive, WT-OVA, and $BLT2^{-/-}$ -naive mice; $n = 6$ for $BLT2^{-/-}$ -OVA mice. N.S., not significant. * $P < 0.05$.

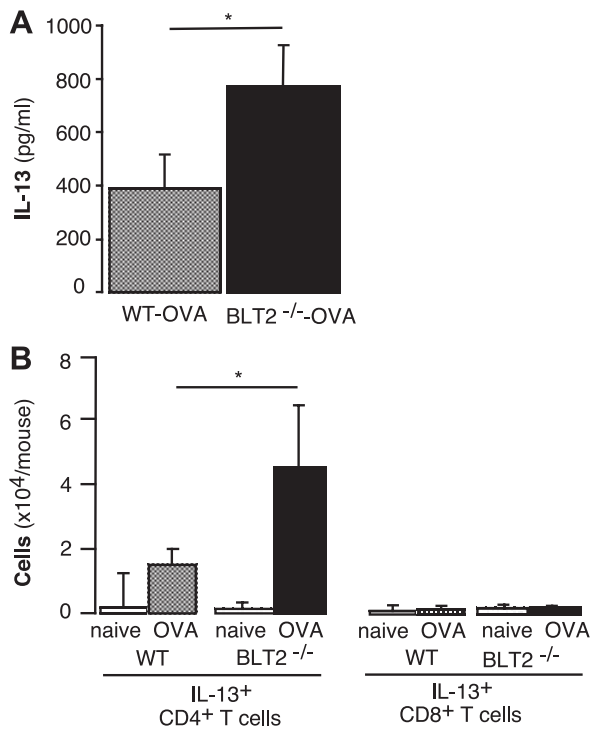


Figure 3. Analysis of IL-13-producing cells in OVA-sensitized/challenged WT and *BLT2*^{-/-} mice. **A)** Following the OVA challenge, PBLN cells were collected and then stimulated *ex vivo* with OVA (10 µg/ml). IL-13 in the cultured supernatant was measured using ELISA. *n* = 8 for WT-OVA mice; *n* = 6 for *BLT2*^{-/-}-OVA mice. Sum of the data collected twice on separate days are shown. **B)** Flow cytometric analysis of IL-13-producing CD4⁺ and CD8⁺ T cells in the lungs. Lungs were collected 36 h after the last OVA challenge. Lung cells were stimulated for 48 h with anti-CD3 plus anti-CD28 mAbs. An electronic gate was set on the CD4⁺ or CD8⁺ T-cell subset, and the percentage of cells positive for IL-13 was multiplied by the total count of lung cells per animal. *n* = 3 for WT-naive and *BLT2*^{-/-}-naive mice; *n* = 8 for WT-OVA mice; *n* = 6 for *BLT2*^{-/-}-OVA mice. N.S., not significant. **P* < 0.05.

deficiency on mast cell numbers in the airways. Toluidine blue-positive mast cells in the trachea and main bronchi were comparable between *BLT2*^{-/-} and WT mice with or without OVA sensitization and challenge (Fig. 4A). BMMCs of WT and *BLT2*^{-/-} mice were stimulated with DNP-BSA, and the secretion of IL-13 was measured. In contrast to the marked increase in IL-13 levels in BAL fluids of *BLT2*^{-/-} mice, IL-13 production from BMMCs of *BLT2*^{-/-} mice was lower than that of WT mice (Fig. 4B).

Functional BLT2 is expressed in effector CD4⁺ T cells

Because controversy exists whether mouse T cells express BLT2, we quantified BLT2 mRNA expression in CD4⁺ T cells using real-time PCR. CD4⁺ T cells were isolated from spleens of naive WT mice and OVA-sensitized/challenged WT mice and from PBLNs of OVA-sensitized/challenged WT mice. The expression of BLT2 mRNA in CD4⁺ T cells from PBLNs of OVA-challenged mice was significantly higher than that

in CD4⁺ T cells from spleens of naive or OVA-treated mice (Fig. 5A). These data suggest that the up-regulation of BLT2 expression in effector CD4⁺ T cells but not in naive/memory CD4⁺ T cells.

To determine the BLT2 function on T cells, CD4⁺ T cells isolated from draining lymph nodes of OVA-sensitized/challenged mice were stimulated with BLT2-specific agonist, 12-HHT at 1 µM (14). The BLT2 receptor is activated by 12-HHT in the 10–100 nM range; thus, the use of µM concentrations ensured a maximal receptor response. CD4⁺ T cells from WT mice showed increased Ca²⁺ transients in response to 12-HHT, whereas 12-HHT did not induce Ca²⁺ transients in *BLT2*^{-/-} CD4⁺ T cells (Fig. 5B). These findings indicate the presence of functional BLT2 on effector CD4⁺ T cells after OVA challenge.

Knockdown of BLT2 by siRNA enhances IL-13 production in CD4⁺ T cells

We next studied the effects of BLT2 knockdown on IL-13 expression in T cells. CD4⁺ T cells isolated from DO11.10 mice were transfected with either BLT2-specific siRNA or control siRNA and cultured under the Th2-skewing condition. The levels of IL-13 were significantly higher in T cells transfected with BLT2-siRNA than in control siRNA (Fig. 6). No significant differences were found in the concentrations of IL-4 and IFN-γ between the two groups. These data indicate that the down-regulation of BLT2 enhanced IL-13 production under Th2-skewing conditions.

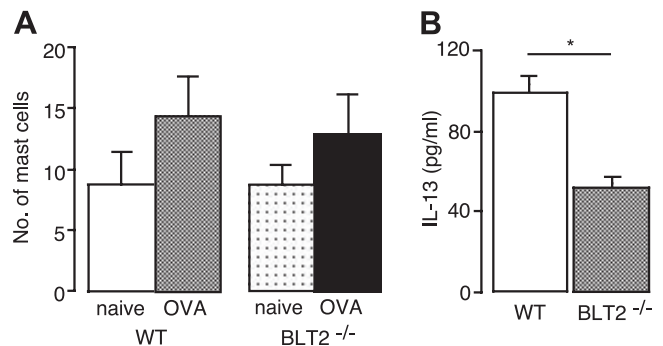


Figure 4. Mast cell numbers in airways of *BLT2*^{-/-} mice and IL-13 production from bone marrow-derived mast cells of *BLT2*^{-/-} mice. **A)** Lung sections of WT or *BLT2*^{-/-} mice with or without OVA treatment were stained with toluidine blue, and mast cell numbers were quantified 3 fields from carina to either side of the main bronchus (×400). No significant difference in numbers of mast cells between WT and *BLT2*^{-/-} mice. *n* = 4 for WT-naive, *BLT2*^{-/-}-naive, and WT-OVA mice; *n* = 6 for *BLT2*^{-/-}-OVA mice. Results are representative of an experiment repeated 3 times on separate days. **P* < 0.05; Student's *t* test. **B)** BMMCs of WT and *BLT2*^{-/-} mice were stimulated with 100 ng/ml DNP-BSA, and the secretion of IL-13 was measured. In contrast to the marked increase in IL-13 levels in BAL fluids of *BLT2*^{-/-} mice, IL-13 production from mast cells of *BLT2*^{-/-} mice was lower than that of WT mice. *n* = 5/group.

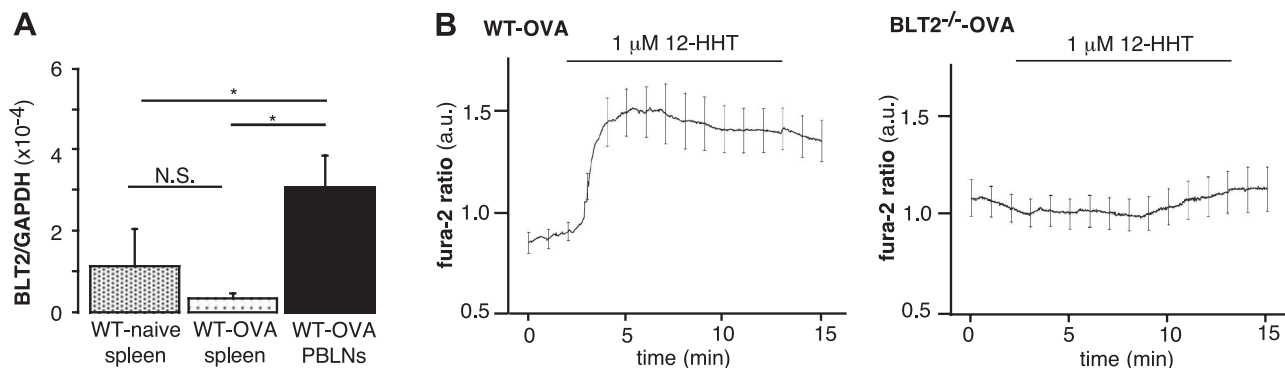


Figure 5. BLT2 mRNA expression in CD4⁺ T cells and [Ca²⁺]_i in CD4⁺ T cells in response to 12-HHT. Mice were sensitized and challenged by OVA, and spleens and PBLNs were collected. A) Relative BLT2 mRNA expression in CD4⁺ T cells from spleens and PBLNs obtained from WT mice was quantified using real-time PCR. BLT2 mRNA expression was standardized using GAPDH. *n* = 3/group. **P* < 0.05. B) [Ca²⁺]_i induced by 12-HHT (1 μM) in CD4⁺ T cells isolated from PBLNs of WT-OVA mice (left panel) and BLT2^{-/-}-OVA mice (right panel). Averaged Ca²⁺ traces of 6 cells in a microscopic field are shown.

12-HHT abundantly exists in BAL fluids

We measured the 12-HHT and LTB₄ levels in BAL fluid to clarify the amount of fluid produced in the locus of allergic airway inflammation. 12-HHT was detected in 3.3 nM levels in BAL fluid of OVA-sensitized/challenged WT mice, whereas LTB₄ was under a detectable limit (<5 pg/ml; **Fig. 7**). Considering the local 12-HHT dilution in saline to obtain BAL fluid, this result suggests that there is enough 12-HHT (10–100 nM) in the lung tissue to activate BLT2.

BLT2 antagonist LY255283 inhibits LTB₄-induced Ca²⁺ influx in BLT1-expressing cells

LY255283 is reported to be a BLT2-selective antagonist (28, 29). In contradiction to the present findings, LY255283 has been shown to attenuate allergic airway inflammation (29). As LY255283 is reported to inhibit the function of eosinophils and neutrophils (30, 31) and reduce the lung parenchymal contractile response to LTB₄ (32), we speculated that LY255283 also has an inhibitory role for BLT1. BLT1- and BLT2-expressing CHO cells were pretreated with 10 μM of LY255283 and then stimulated with 12-HHT or LTB₄. LY255283 completely inhibited the 12-HHT- and LTB₄-induced Ca²⁺ influx in CHO-BLT2 cells (**Fig. 8**, right panel). Similarly, LY255283 inhibited the 1–3 nM LTB₄-induced Ca²⁺ influx in CHO-BLT1 cells almost completely (**Fig. 8**, left panel). Given that LTB₄ exists at <5 pM in BAL fluid of OVA-induced allergic mouse models, these results suggest that LY255283 blockades BLT1 function *in vivo*.

BLT2 mRNA expression in CD4⁺ T cells from individuals with asthma

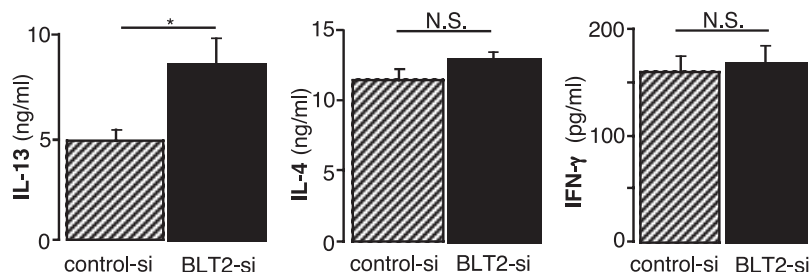
Expression of BLT2 mRNA in CD4⁺ T cells isolated from peripheral blood of the patients with asthma and the healthy volunteers was analyzed using real-time PCR. Regardless of the use of inhaled corticosteroids, the levels of BLT2 expression in CD4⁺ T cells of patients with asthma were lower than those of healthy control subjects (**Fig. 9**).

DISCUSSION

The present study demonstrated that BLT2-deficient mice exhibit enhanced production of IL-13 and augmented eosinophilia in the airways without influencing IL-4 production and IgE levels in a model of allergic airway inflammation. IL-13-producing CD4⁺ T cells were increased in BLT2-deficient mice after allergen challenges. BLT2 down-regulation using BLT2-siRNA also enhanced IL-13 production from Th2 lines generated from OVA-specific T cell receptor transgenic mice. We further demonstrated that BLT2 expression in CD4⁺ T cells in the peripheral blood of patients with asthma is lower than that of healthy control subjects. These data suggest that BLT2 plays a protective role in allergic airway inflammation and that impaired BLT2 expression in CD4⁺ T cells may contribute to the pathophysiology of asthma.

The expression of BLT2 in mouse cells or tissues has been investigated primarily by detecting BLT2 mRNA

Figure 6. Cytokine production from *in vitro*-differentiated CD4⁺ T cells. CD4⁺ T cells from DO11.10 mice were transfected with control siRNA or BLT2-specific siRNA. Cells were differentiated into Th2 cells and stimulated with pOVA. After 48 h, cell culture supernatants were harvested and analyzed using ELISA. *n* = 4/group. N.S., not significant. **P* < 0.05.



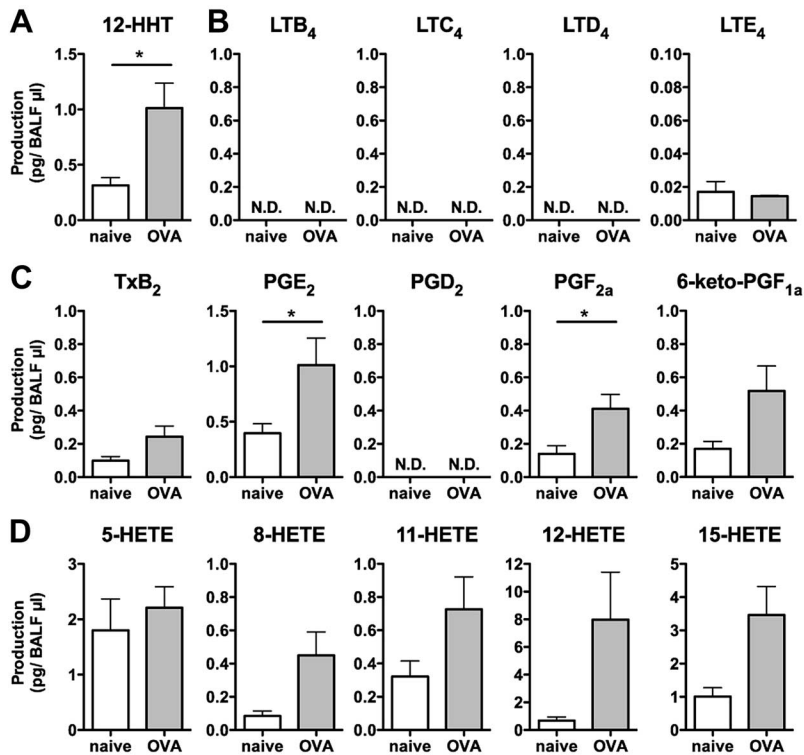


Figure 7. Measurement of eicosanoid levels in BAL fluid. BAL was performed with saline from WT-naive and OVA-sensitized/challenged WT-mice, and BAL fluids were analyzed using mass spectrometry. A, B) 12-HHT was increased in BAL fluid of OVA-sensitized/challenged WT mice (A), whereas LTB₄ was under a detectable level (B). C) Thromboxane and prostaglandin levels in BAL fluid. D) HETE levels in BAL fluid. While leukotriens were not detectable or showed no difference between naive and WT mice, 5-, 8-, 11-, 12-, or 15-HETEs tended to increase in OVA-treated mice. $n = 6$ for WT-naive mice; $n = 5$ for WT-OVA mice. N.D., not detected. * $P < 0.05$; Student's t test.

because no antibodies were available for mouse BLT2. We reported previously on the expression of mouse BLT2 predominantly in the small intestine and skin and in small amounts in the spleen and colon using Northern blotting (10). Other studies suggested the expression of BLT2 in mast cells (26, 27). Although one report using RT-PCR analysis showed that mouse T cells express BLT2 mRNA (26), others using Northern blotting failed to detect BLT2 in T cells (5). In the present study, we show that BLT2 mRNA was up-regulated in CD4⁺ T cells isolated from draining lymph nodes after allergen challenge. Furthermore, 12-HHT induced Ca²⁺ mobilization in CD4⁺ T cells from draining lymph nodes of WT mice but not in those of *BLT2*^{-/-} mice. These findings indicate that functional BLT2 is expressed on antigen-specific effector memory CD4⁺ T cells.

The IL-13 levels in the airways of *BLT2*-deficient mice were higher than those of WT mice after allergen

challenges, while the IL-4, IL-5, and IFN- γ levels were comparable between *BLT2*-deficient and WT mice. These results indicate that *BLT2* does not affect the

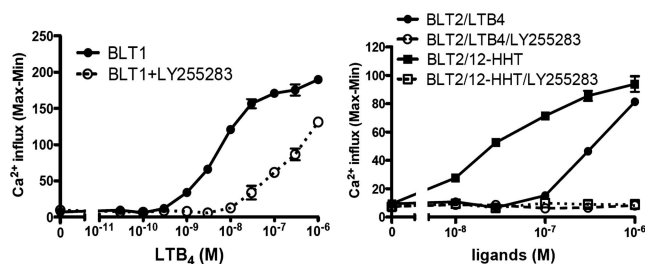


Figure 8. Dose responses of calcium mobilization on CHO cells expressing BLT1 and BLT2. Cells were pretreated or not with 10 mM of LY255283 for 30 min, followed by stimulation with LTB₄ or 12-HHT at indicated concentrations. Left panel: Ca²⁺ influx of CHO-BLT1 cells stimulated with LTB₄. Right panel: Ca²⁺ influx of CHO-BLT2 cells stimulated with LTB₄ or 12-HHT. $n = 3$ /group, average $\pm$ SE.

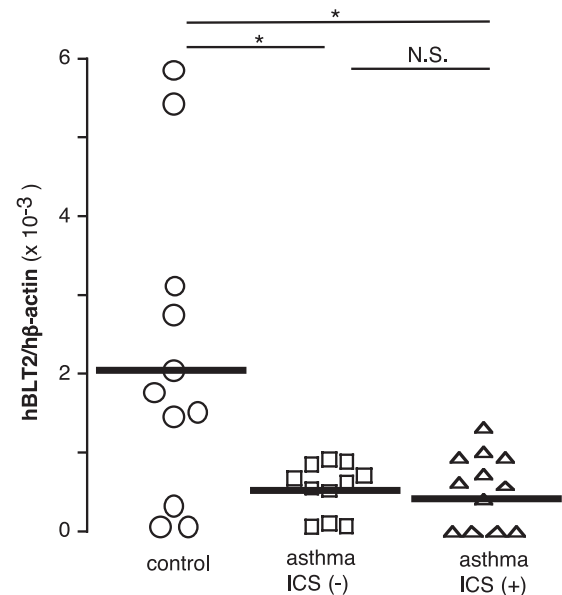


Figure 9. BLT2 mRNA expression in CD4⁺ T cells in the peripheral blood of healthy volunteers and patients with asthma treated or not with therapy *via* inhaled corticosteroid (ICS). All healthy volunteers were negative for antigen-specific IgE (RAST). CD4⁺ T cells were separated using the density-gradient method and the subsequent positive selection method using magnetic beads. hBLT2 mRNA was quantified using real-time PCR. hBLT2 mRNA expressions were standardized using β -actin. Dot plots with lines showing the average of relative hBLT2 expression in each group. $n = 12$ for control group; $n = 16$ for patients without ICS, $n = 11$ for patients with ICS. N.S., not significant. * $P < 0.05$.

process of Th2 differentiation but has an inhibitory role in the effector phase of Th2 responses. The OVA-specific IgE levels were not affected by *BLT2* deficiency either, suggesting that BLT2 is not relevant to B cell activation. IL-13 is produced from a variety of cells, including T cells, NK cells, mast cells, and eosinophils (25), and previous studies have reported that BLT2 is expressed in mast cells (26, 27). In the present study, however, IL-13 production from BMDCs was rather attenuated in *BLT2*-deficient mice, and *BLT2* deficiency did not affect the number of airway mast cells. Increases were found in the numbers of IL-13-producing CD4⁺ T cells in the lungs of allergen-challenged *BLT2*-deficient mice, and BLT2-siRNA treatment enhanced IL-13 production from Th2 cells. This finding suggests that CD4⁺ T cells may be one of the main producers of excessive IL-13. The mechanism for the differential effects of *BLT2* disruption on IL-13 production in T cells and mast cells is not clear at present. It has been demonstrated that IL-4 production is affected differentially by proteasome inhibition; a proteasome inhibitor suppressed IL-4 in activated splenocytes but induced IL-4 production in Jurkat T cells (33).

IL-13 is critical for the development of eosinophilic inflammation and airway hyperresponsiveness (34, 35). *BLT2*-deficient mice exhibited increased eosinophilic inflammation and overproduction of IL-13 in BAL fluid, but airway hyperresponsiveness was not affected by *BLT2* deficiency. The reason for this discrepancy is not clear, but airway hyperresponsiveness can be affected by factors other than airway inflammation, including airway smooth muscle responses and neuronal regulation (36, 37). Although a recent study demonstrated that mast cells do not contribute to the development of airway hyperresponsiveness using newly generated mast cell-deficient mice on a BALB/c background (38), it has been reported that mast cells are required to elicit airway hyperresponsiveness in WBB6F1-*Kit*^W/*Kit*^{W-v} and C57BL/6-*Kit*^{W-sh}/*Kit*^{W-sh} mice (39). We speculate that the decreased release of IL-13 from activated mast cells in *BLT2*-deficient mice might explain the discrepancy; however, further investigations are needed.

The protective role of BLT2 in inflammation is consistent with our previous study showing that dextran sodium sulfate-induced colitis was aggravated in *BLT2*-deficient mice with overproduction of IFN- γ , IL-1 β , IL-6, CXCL9, and CCL19 (16). BLT2 expressed in intestinal epithelial cells is required to maintain the mucosal barrier function, and BLT2 deficiency resulted in the invasion of intestinal flora into the colon tissues, causing severe colitis. Thus, we speculate that BLT2 exhibits protective roles in acute Th1 and asthmatic Th2 response through different mechanisms.

In contrast to our present study, one report showed that a BLT2 antagonist, LY255283, suppressed allergen-induced airway inflammation in mice *in vivo* (29). However, we confirmed that LY255283 works as a potent allosteric antagonist for BLT1. Given that BLT1 plays an important role in the early recruitment of neutrophils and eosinophils (8, 9) and of CD4⁺ and CD8⁺ T cells into the airways in allergic asthma (8, 13), our results suggest that the inhibitory effect of

LY255283 on BLT1 leads to the anti-inflammatory effects of LY255283 in asthma experiments.

In summary, our findings suggest that BLT2 works as an anti-inflammatory receptor for allergic asthma. LTB₄ levels in sputum and exhaled breath condensate are elevated in patients with asthma and COPD (12, 40–42). Although the absence of clinical effects of LTB₄ receptor antagonists in these diseases has brought into question the functional role of LTB₄ in the airways (43, 44), our findings suggest that more selective BLT1 antagonists may have a potential therapeutic application in patients with neutrophilic asthma and COPD. Furthermore, BLT2 might also be a novel therapeutic target for asthma. FJ

The authors thank Prof. Junichi Miyazaki (Osaka University, Osaka, Japan) for providing an expression vector pCXN2 and Ms. Ayako Hashizume, Ms. Makiko Kogo, and Ms. Asami Harumatsu for their technical assistance. This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), a Grant-in-Aid for Challenging Exploratory Research from JSPS, and a Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan, and by the National Institute of Biomedical Innovation, Japan.

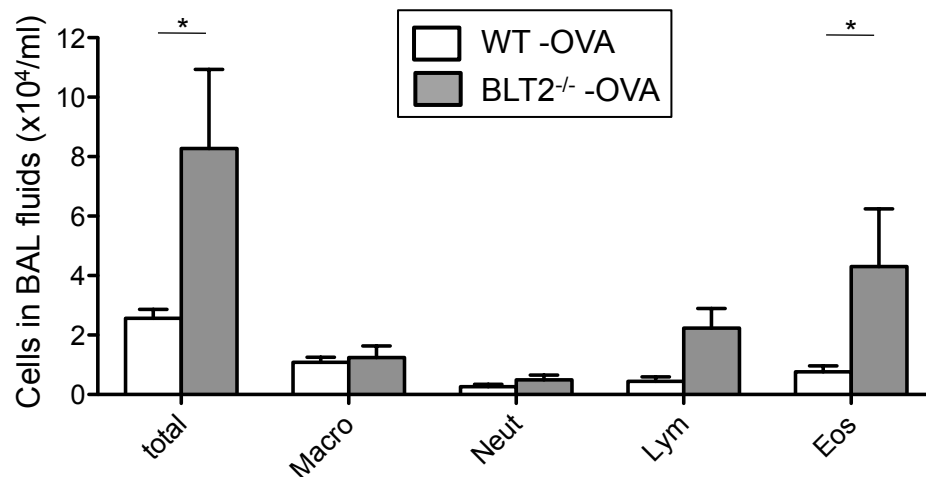
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Received for publication October 17, 2012.

Accepted for publication April 1, 2013.



Supplemental Fig. S1

Supplemental Fig. S1. Effects of BLT2 deficiency on an OVA-induced asthma model. Female BLT2^{-/-} mice were sensitized with 20 microg OVA on days 1 and 14 and exposed to aerosolized 1% OVA on days 26-28. Bronchoalveolar lavage (BAL) was performed 36 h after the last OVA challenge. Differential cell counts in BAL fluid. n= 6. *, $P < 0.05$