



Leukotriene B₄ receptors play critical roles in house dust mites-induced neutrophilic airway inflammation and IL-17 production

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ABSTRACT

Increased levels of neutrophils in bronchoalveolar lavage fluid (BALF) were associated with asthma severity. As leukotriene B₄ (LTB₄) is a principal chemoattractant molecule for neutrophils, its receptors, BLT1 and BLT2, may contribute to neutrophil-dominant airway inflammation. In the present study, we established a mouse model of steroid-resistant, neutrophil-dominant airway inflammation by house dust mite (HDM)/lipopolysaccharide (LPS) sensitization and HDM challenge, and we investigated whether BLT1/BLT2 signaling was associated with the development of neutrophilic airway inflammation. Blockade of BLT1 or BLT2 significantly suppressed airway inflammation and IL-17 production in this mouse model. The 5-LO and 12-LO enzymes, which catalyze the synthesis of BLT1/BLT2 ligands, were also critically associated with neutrophil-dominant airway inflammation and the synthesis of IL-17. Collectively, our results suggest that the 5-/12-LO-BLT1/BLT2-linked cascade significantly contributes to neutrophil-dominant severe airway inflammation via IL-17 synthesis in HDM-induced neutrophilic asthma.

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1. Introduction

Asthma is a chronic inflammatory disease of the airways. Asthmatic allergies are triggered by multiple stimuli, such as respiratory infection, occupational chemicals, and house dust mites (HDMs) [1,2]. HDMs are the most prevalent indoor allergen, and HDM exposure is a common cause of asthmatic allergies. Previous clinical studies reported that HDM sensitization is present in up to 50% of asthma patients, and continuous exposure to HDM allergens is associated with asthma severity [3,4]. Therefore, HDMs are frequently used for experimental asthma studies. However, an HDM-induced mouse model of neutrophilic airway inflammation has not yet been established.

Neutrophils are a dominant cell type found during severe exacerbations and chronic airway narrowing in asthma [5,6]. In some asthmatic patients, an increase in neutrophil number in the sputum is associated with persistent severe asthma, which is called steroid-resistant asthma. Recently, IL-17 was shown to be associated with severe neutrophilic asthmatic development and exacerbation, and the levels of IL-17 in serum and sputum were increased with the

severity of the disease [7,8].

Leukotriene B₄ (LTB₄), a 5-LO product, has been demonstrated to perform major functions during the development of asthma [9,10]. Similarly, 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), a product of 12-LO, was highly expressed in the sputum and bronchoalveolar lavage fluid (BALF) in asthma patients compared with healthy subjects [11]. LTB₄ is a principal chemoattractant for neutrophils, and there are two receptors for LTB₄, BLT1 and BLT2. These receptors are a family of G protein-coupled receptors (GPCRs) that are highly expressed on the surface of cells. Although BLT1/BLT2 were suggested to contribute to asthmatic inflammation, their roles in HDM-induced neutrophilic airway inflammation have not yet been demonstrated [8,12,13].

In the present study, we established a mouse model of neutrophil-dominant, steroid-resistant airway inflammation using HDMs. In addition, we found that BLT1/2 as well as the synthesis of their ligands (LTB₄ and 12(S)-HETE) by 5-/12-LO play critical roles in the development of HDM-induced neutrophilic airway inflammation and further identified IL-17 as a downstream target of the BLT1/2-linked cascade. Taken together, our findings suggest that the '5-/12-LO-BLT1/2-IL-17' signaling pathway potentially contributes to HDM-induced neutrophilic airway inflammation. This finding may provide a new perspective for the development of new therapies for neutrophil-dominant severe asthma.

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2. Materials & methods

2.1. Reagents

Recombinant mouse IL-17 was purchased from R&D Systems (Minneapolis, MN, USA). LPS (*Escherichia coli* serotype O55:B5), dimethyl sulfoxide (DMSO), and dexamethasone were obtained from Sigma-Aldrich (St. Louis, MO). MK886 was purchased from Calbiochem (La Jolla, CA, USA). U75302 and baicalein were purchased from Enzo Life Sciences (Farmingdale, NY, USA). LY255283 was obtained from Cayman Chemical (Ann Arbor, MI, USA).

2.2. Mouse model of HDM-induced neutrophilic airway inflammation

Female BALB/c mice (8–10 weeks old) were obtained from Young Bio (Seongnam Korea). HDM extract (Greer Laboratories, Lenoir, NC) was resuspended in saline and introduced by intranasal instillation, with the mice lightly anesthetized with 5% isoflurane before the immunization. The mice were intranasally immunized with 10 μ g of LPS and 100 μ g of HDM on days 0, 1, 2, and 7 and then challenged with 50 μ g of HDM on days 14, 15, 18, and 19. The mice were sacrificed on day 20 (Fig. 1A). At 8 weeks of age, the mice were distributed into the following groups: (A) normal group; (B) HDM/LPS group; (C) dexamethasone + HDM/LPS group; (D) U75302 + HDM/LPS group; (E) LY255283 + HDM/LPS group; (F) MK886 +

HDM/LPS group; (G) baicalein + HDM/LPS group; (H) anti-IL-17 + HDM/LPS group. For the inhibition experiments, dexamethasone (1 mg/kg), U75302 (50 μ g/kg), LY255283 (10 mg/kg), MK886 (5 mg/kg), baicalein (75 mg/kg) or vehicle control (DMSO) was administered via intraperitoneal (i.p.) injection 1 h before every challenge. For the IL-17 inhibition experiment, the mice were treated with either 0.5 mg/kg of control IgG1 or anti-IL-17A antibody via i.p. injection 1 h before every challenge. The mouse room was maintained at a temperature of 22 ± 1 °C and a humidity of $50 \pm 10\%$ with a 12 h light/dark cycle. Wire-lidded food hoppers within the cages were filled to capacity with rodent chow, and the mice had access to bottle-supplied water. All animal procedures were conducted in accordance with the guidelines of the Korea University Institutional Animal Care & Use Committee.

2.3. Preparation of tissue lysates and immunoblot analysis

Lung tissues were homogenized and lysed in lysis buffer (50 mM Tris Cl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.02% NaN₃, 100 mM phenylmethyl sulfonyl fluoride, 1 M sodium fluoride, leupeptin (0.5 mg/ml), aprotinin (1 mg/ml), and pepstatin A (5 mg/ml)) at 4 °C. The lysate protein samples were heated at 95 °C for 5 min and subjected to SDS-PAGE. The separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane for 45 min at 110 V. The membrane was incubated for 1 h with TBS-T containing 5% nonfat dried milk at room

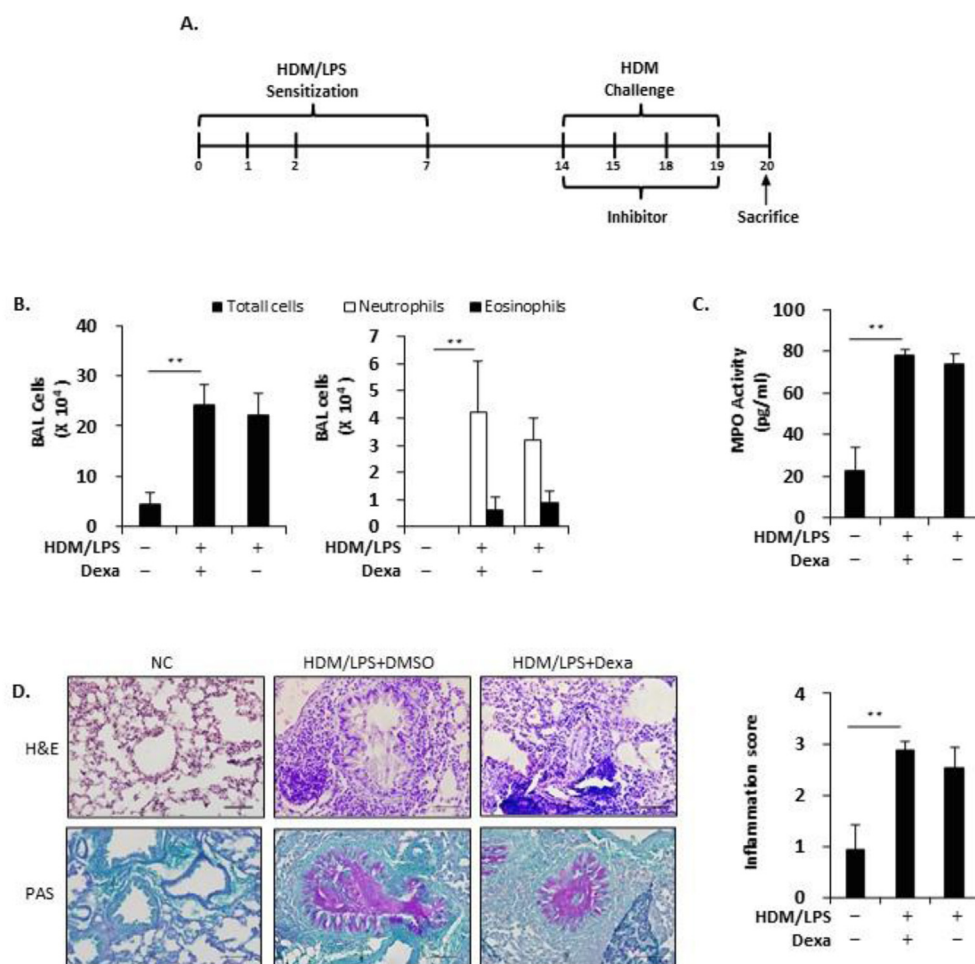


Fig. 1. Induction of steroid-resistant neutrophilic airway inflammation by HDM and LPS administration.

temperature (RT) and for 1 h at RT with primary antibodies at a 1:1000 dilution (1:500 for 5-LO and 12-LO or 1:5000 for β -actin) in TBS-T. The membrane was then incubated for 1 h at RT with HRP-conjugated secondary antibodies before the detection of immune complexes with an enhanced chemiluminescence kit (Amersham Biosciences, Little Chalfont, UK).

2.4. Measurement of IL-17, LTB₄ and myeloperoxidase

The levels of IL-17, LTB₄ and myeloperoxidase were quantified in the protein lysates of the lung tissue or serum using an ELISA kit (R&E system for IL-17; Enzo Life Sciences for LTB₄; Abcam for myeloperoxidase) according to the manufacturer's instructions.

2.5. BAL cell counting

Inflammatory cells were collected from BALF by centrifugation (1000×g for 3 min) and washed with PBS. Next, BAL cells were fixed on glass slides and stained with a Diff-Quik kit.

2.6. Histological analysis of lung tissue

Lung tissues were harvested, fixed in 10% formaldehyde for 10 days and embedded in paraffin. Lung sections (4.5 μ m thickness) were mounted onto Superfrost™ Plus glass slides (Fisher Scientific, Pittsburgh, PA, USA), deparaffinized and stained with H&E and PAS. All images were acquired using a BX51 microscope (Olympus, Tokyo, Japan) equipped with a DP71 digital camera (Olympus).

2.7. Statistical analysis

All statistical analyses were performed with one-way analysis of variance, followed by Tukey's post-hoc test. SPSS software (IBM SPSS Statistics for Windows, version 21.0; IBM Corp., Armonk, NY, USA) was used for statistical analysis. In all experiments, 3 to 6 mice were included in each group. The results are presented as the mean $\pm$ SD. *P*-values <0.05 indicated statistical significance.

3. Results

3.1. Induction of steroid-resistant neutrophil-dominant airway inflammation by HDM administration

For establishment of a mouse model of steroid-resistant, neutrophil-dominant airway inflammation, mice were intranasally immunized with 10 μ g of lipopolysaccharide (LPS) and 100 μ g of HDM on days 0, 1, 2, and 7 and then challenged with 50 μ g of HDM on days 14, 15, 18, and 19. The mice were sacrificed on day 20 (Fig. 1A). To examine whether the HDM-induced murine model is suitable for steroid-resistant and neutrophil-dominant airway inflammation, we tested the efficacy of steroids (dexamethasone; Dexa). Dexa did not significantly suppress the recruitment of immune cells and myeloperoxidase activity (Fig. 1B and C). In addition, highly increased airway inflammation and mucus secretion in the group of HDM-induced mice were not suppressed by Dexa (Fig. 1D). Together, these results suggested that our HDM-induced neutrophil dominant airway inflammation model is suitable for studying steroid-resistant pulmonary inflammation.

3.2. IL-17 plays a critical role in HDM-induced neutrophilic airway inflammation

For analysis of the contribution of IL-17 to HDM-induced neutrophil airway inflammation, an anti-IL-17 neutralizing antibody (0.5 mg/kg) was intraperitoneally administered 1 h before

every challenge. Clearly, anti-IL-17 strongly reduced the levels of IL-17, and Dexa had no effect on the levels of IL-17 (Fig. 2A). Under these experimental conditions, the numbers of neutrophils in the BALF were significantly suppressed by an anti-IL-17 neutralizing antibody (Fig. 2B). In addition, airway inflammation and mucus secretion were substantially suppressed by the anti-IL-17 neutralizing antibody (Fig. 2C). Thus, these results suggested that IL-17 significantly contributes to neutrophil-dominant airway inflammation.

3.3. The LTB₄ receptors BLT1 and BLT2 play important roles in HDM-induced neutrophilic airway inflammation and IL-17 production

To examine the roles of the LTB₄ receptors BLT1 and BLT2 in neutrophilic airway inflammation and IL-17 production in the HDM-induced neutrophilic mouse model, we first tested the effect of BLT1 or BLT2 antagonists in these mice. The BLT1 antagonist U75302 (50 μ g/kg) or the BLT1/2 antagonist LY255283 (10 mg/kg) was intraperitoneally injected 1 h before each challenge. By these antagonists, the numbers of neutrophils in the BALF, the levels of IL-17 and myeloperoxidase activity were substantially reduced by U75302 or LY255283 (Fig. 3A and B). Under these experimental conditions, U75302 or LY255283 had no effect on the differentiation or maturation of leukocytes, including neutrophils (data not shown), suggesting that the suppressive effect on neutrophil infiltration into the airway by the antagonists is solely due to the blockade of the BLT1/2-linked signaling cascade. Next, we found that airway inflammation was significantly reduced by U75302 or LY255283, as determined by histological analysis and quantitative analysis of the inflammation scores (Fig. 3C). Additionally, mucus secretion was clearly suppressed by U75302 or LY255283 (Fig. 3C). Taken together, these results suggest that BLT1 and BLT2 contribute to IL-17 production and airway inflammation in HDM-induced neutrophilic airway inflammation.

3.4. The 5/12-lipoxygenases play roles in HDM-induced neutrophilic airway inflammation and IL-17 production

Next, we examined whether the synthesis of BLT1/2 ligands by 5-/12-LO is also associated with neutrophil-dominant airway inflammation and the synthesis of IL-17. To test the roles of 5-/12-LO, we used inhibitors of each enzyme. For the inhibition of 5/12-LO, the HDM-induced mice received the 5-LO inhibitor MK886 (5 mg/kg) or the 12-LO inhibitor baicalein (75 mg/kg) 1 h before every challenge by intraperitoneal injection. We observed that the 5-/12-LO expression levels and the levels of LTB₄ and 12(S)-HETE in the lung lysates were highly elevated by the HDM-induced inflammation (Fig. 4A and B). And, these elevated levels of the 5-/12-LO expressions were clearly attenuated by these inhibitors (Fig. 4A). Additionally, the numbers of neutrophils in the BALF and the levels of IL-17 and myeloperoxidase activity were significantly reduced by MK886 and baicalein (Fig. 4C and D). Histological analysis showed that MK886 and baicalein alleviated immune cell infiltration of the airway and inflammation compared to those of the control group. Furthermore, the administration of MK886 and baicalein significantly reduced mucus production (Fig. 4E). Thus, together, these results suggest that HDM-induced IL-17 production and neutrophilic airway inflammation are dependent on 5-/12-LO, further supporting the contribution of BLT1/2-linked cascade to the HDM-induced neutrophilic severe airway inflammation.

4. Discussion

In the present study, we demonstrated that 5-LO, 12-LO, BLT1,

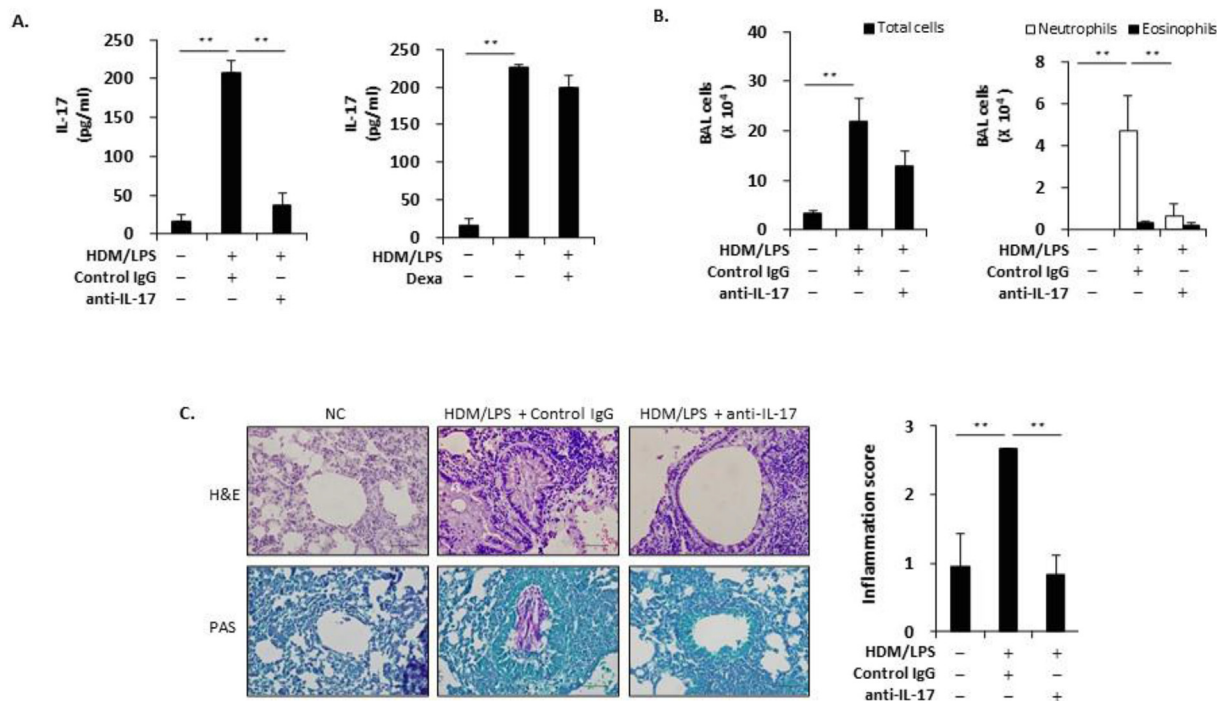


Fig. 2. IL-17 is critical for HDM-induced neutrophilic airway inflammation.

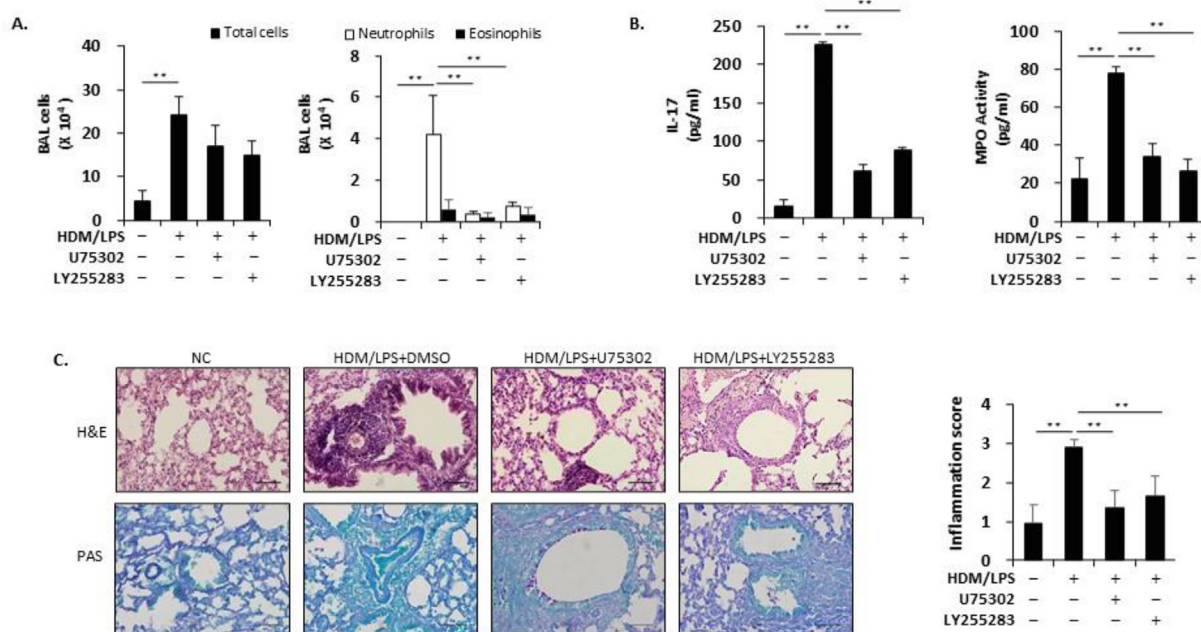


Fig. 3. The LTB₄ receptors BLT1 and BLT2 are critical for HDM-induced neutrophilic airway inflammation.

and BLT2 play critical roles in airway inflammation and IL-17 production in HDM-induced neutrophilic airway inflammation. Our findings may provide insight into the mechanisms underlying HDM-associated neutrophil-dominant airway inflammation and asthmatic exacerbation.

Asthma phenotypes can be classified according to the inflammatory immune cell types infiltrated in the airway, such as eosinophilic, neutrophilic or mixed eosinophilic/neutrophilic inflammation [14]. Generally, eosinophilic inflammation is mostly

observed in mild-to-moderate asthma, while neutrophilic inflammation is observed in a more severe asthma phenotype [15]. Eosinophilic inflammation has been associated with the Th2 response [16]. In contrast, neutrophil-dominant airway inflammation has been associated with Th17- or Th1-related responses [17,18], and the levels of IL-17 in serum and sputum were increased with the severity of the disease. IL-17 plays an important role in neutrophil recruitment and activation, which contribute to the development of severe neutrophilic inflammation in the airways of

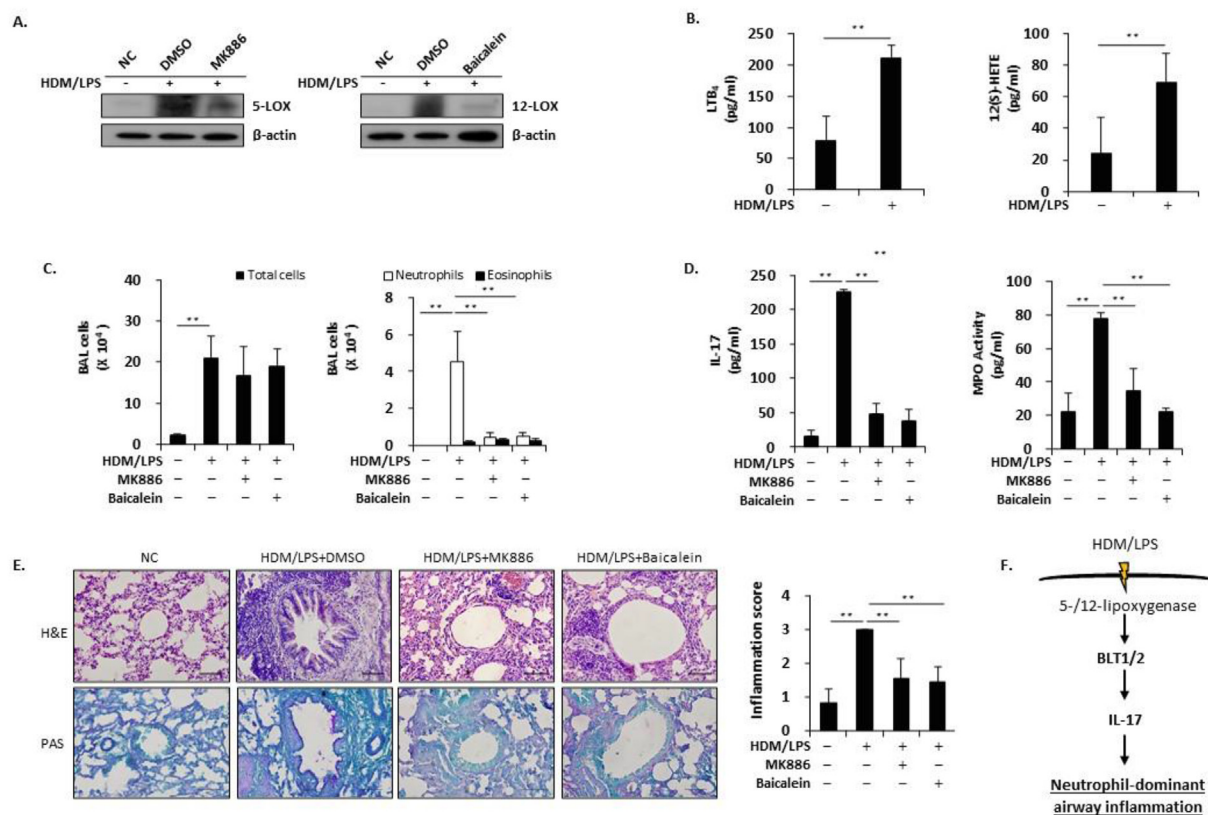


Fig. 4. The synthesis of the BLT1/2 ligands LTB₄ and 12(S)-HETE is necessary for HDM-induced neutrophilic airway inflammation and IL-17 production.

lungs [7,8,19–21]. In addition, enhanced levels of IL-17 mRNA were found in the sputum of asthmatic patients with neutrophilic inflammation [22].

One of the most widely used aeroallergens in experimental animal models is ovalbumin. Ovalbumin is rarely implicated in clinical asthma and is often used in acute challenge protocols [23,24]. Therefore, OVA may not be a disease-relevant allergen. In contrast, exposure to HDMs, the major source of allergens, has been shown to play an important role in asthma and in other allergic diseases, such as dermatitis and rhinitis [25–28]. Therefore, investigating the physiological role of HDMs or the design of an experimental animal model using HDMs is pathologically important to understand asthmatic airway inflammation. Some recent studies have reported that chronic exposure to HDMs can be associated with neutrophil-dominant asthmatic inflammation [29–31]. Nevertheless, many experimental studies of HDM-induced mouse models have not shown steroid-resistant or neutrophil-dominant airway inflammation phenotypes. Thus, we believe our HDM model established in the present study may be useful for studying steroid-resistant and neutrophil-dominant airway inflammation phenotypes.

In the present study, we found that the levels of BLT1 and BLT2 as well as the synthesis of their ligands LTB₄ and 12(S)-HETE were markedly upregulated in our HDM-induced neutrophilic mouse model, and blockade of BLT1/2 or 5-/12-LO significantly reduced the production of IL-17, thus attenuating neutrophilic airway inflammation (Figs. 3 and 4). Consistent with the proposed role of BLT1/2 in neutrophilic inflammation, previous studies reported that BLT1 is associated with neutrophil infiltration in several inflammatory diseases, including arthritis, spinal cord injury and allergic skin inflammation. These researchers reported that BLT1 blockade dramatically reduces neutrophil recruitment in

inflammatory sites [32,33]. In addition, BLT2 has proinflammatory activities by cigarette smoke exposure, which enhances neutrophil adhesion and contributes to airway neutrophilia [34]. Further, our previous results demonstrated that BLT2 mediates the signaling associated with the promotion and exacerbation of severe neutrophilic asthmatic pathogenesis [12]. Thus, along with the results of the present study, BLT2 is likely to serve as a therapeutic target for severe neutrophilic airway inflammatory symptoms.

In conclusions, our results suggested that the “5-/12-LO-BLT1/2” cascade significantly contributes to the development of HDM-induced neutrophilic airway inflammation via IL-17 production. This is the first report on the role of BLT1/2 in HDM-associated neutrophilic airway inflammation, and our results may provide a new perspective for the development of treatments for asthmatic patients that are less sensitive to steroid treatment.

Author contributions

D.W.K planned the study, performed the experiments, analyzed data, and wrote the manuscript. D.P performed the experiments. J.H.K supervised the study and wrote the manuscript.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

(A) Schematic of the HDM/LPS-induced neutrophilic airway inflammation model. Airway inflammation was induced by immunization with LPS (10 μg) plus HDM (100 μg) and challenge with HDM (50 μg) (n = 5–7 per group). Dexamethasone (1 mg/kg) was administered via i.p. injection 1 h before every challenge (n = 5–7

per group). **(B)** Total immune cells, neutrophils, and eosinophils in BALF were obtained using cytopsin and stained with Diff-Quik. **(C)** Levels of myeloperoxidase in lung lysate were analyzed using ELISAs. **(D)** The lungs were excised, fixed and stained with H&E and PAS. Peribronchial and perivascular lung inflammation was measured and scored. All quantitative data are expressed as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$.

For the IL-17 inhibition experiment, mice received 0.5 mg/kg of control IgG1 or anti-IL-17A antibody via i.p. injection 1 h before every challenge ($n = 5$ –7 per group). **(A)** Levels of IL-17 in lung lysates were analyzed using ELISAs. **(B)** Total immune cells, neutrophils, and eosinophils in BALF were obtained using cytopsin and stained with Diff-Quik. **(C)** The lungs were excised, fixed and stained with H&E and PAS. Peribronchial and perivascular lung inflammation was measured and scored. All quantitative data are expressed as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$.

The control DMSO, U75302 (50 μ g/kg) and LY255283 (10 mg/kg) were administered via i.p. injection 1 h before every challenge ($n = 5$ –7 per group). **(A)** Total immune cells, neutrophils, and eosinophils in BALF were obtained using cytopsin and stained with Diff-Quik. **(B)** Levels of IL-17 and myeloperoxidase in lung lysates were analyzed using ELISAs. **(C)** The lungs were excised, fixed and stained with H&E and PAS. Peribronchial and perivascular lung inflammation was measured and scored. All quantitative data are expressed as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$.

The control DMSO, MK886 (5 mg/kg) and baicalein (75 mg/kg) were administered via i.p. injection 1 h before every challenge ($n = 5$ –7 per group). **(A)** The mouse lungs were homogenized, protein was isolated, and the levels of 5-LO and 12-LO were assessed by western blotting. **(B)** The levels of LTB₄ and 12(S)-HETE in lung lysates were analyzed using ELISAs. **(C)** Total immune cells, neutrophils, and eosinophils in BALF were obtained using cytopsin and stained with Diff-Quik. **(D)** The levels of IL-17 and myeloperoxidase activity in lung lysates were analyzed using ELISAs. **(E)** The lungs were excised, fixed and stained with H&E and PAS. Peribronchial and perivascular lung inflammation was measured and scored. All quantitative data are expressed as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$. **(F)** Scheme of the 5/12-LOX-BLT1/2-IL-17 signaling cascade in HDM/LPS-induced neutrophilic airway inflammation.

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