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5-/12-Lipoxygenase-linked cascade contributes to the IL-33-induced synthesis of IL-13 in mast cells, thus promoting asthma development

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Abstract

Background: As asthma progresses, the levels of IL-33 in serum are markedly increased and contribute to asthmatic development and exacerbation. Mast cells, one of the principal effector cells in the pathogenesis of asthma, express high levels of the IL-33 receptor ST2 and have been shown to be activated by IL-33. Thus, IL-33 stimulates mast cells to produce Th2-type cytokines such as IL-13, thus contributing to asthmatic development. However, the signaling mechanism for IL-33-induced synthesis of Th2 cytokines, particularly IL-13, has not been fully elucidated in mast cells.

Methods: The role of 5- or 12-LO in the IL-33-induced synthesis of IL-13 was investigated using knockdown or pharmacological inhibitors in bone marrow-derived mast cells (BMMCs) and animal model.

Results: Blockade of 5- or 12-LO significantly suppressed IL-33-induced synthesis of IL-13 in BMMCs. The subsequent action of 5- and 12-LO metabolites through their specific receptor, BLT2, was also critical for IL-33-induced synthesis of IL-13. We also demonstrated that the MyD88-p38 kinase cascade lies upstream of 5-/12-LO and that NF- κ B lies downstream of 5-/12-LO to mediate the IL-33-induced synthesis of IL-13 in mast cells. Consistent with these findings, we observed that in an IL-33-administered asthmatic airway inflammation model, IL-13 levels were markedly increased in bronchoalveolar lavage fluid, but its levels were markedly suppressed by treatment with inhibitors of 5-LO, 12-LO or BLT2, further suggesting roles of 5-/12-LO in IL-33-induced IL-13 production.

Conclusion: Our results suggest that “MyD88-5-/12-LO-BLT2-NF- κ B” cascade significantly contributes to the IL-33-induced synthesis of IL-13 in mast cells, thus potentially contributing to asthmatic development and exacerbation.

KEYWORDS

BLT2, IL-13, IL-33, lipoxygenase, mast cells

Abbreviations: 12(S)-HETE, 12(S)-hydroxyeicosatetraenoic acid; 12-LO, 12-lipoxygenase; 5-LO, 5-lipoxygenase; BAL fluid, bronchoalveolar lavage fluid; BLT2, leukotriene B₄ receptor-2; cPLA₂, cytosolic phospholipase A₂; LTB₄, leukotriene B₄.

1 | INTRODUCTION

IL-33, a member of the IL-1 family of cytokines, is mainly secreted by damaged epithelial cells and acts as an alarmin that is immediately released in response to cell damage.¹ Recently, IL-33 was shown to be closely associated with asthmatic development and exacerbation, and its levels were increased with the severity of the disease.^{2,3} The relationship between asthma and IL-33 was also demonstrated using an IL-33-overexpressing transgenic mouse, which spontaneously showed asthmatic airway inflammation with infiltration of inflammatory cells and enhanced Th2-type cytokines.⁴ Consistent with the suggested role of IL-33 in asthma, earlier studies demonstrated that blockade of IL-33 clearly reduced disease severity, as evident from the suppression of Th2 cytokine production, airway inflammation, hyper-responsiveness (AHR), and mucus production.^{5,6}

Although mast cells have been shown to respond to exogenous danger signals,⁷ recent studies have suggested that mast cells also play an important role in the recognition of endogenous danger signals such as IL-33.⁸ Indeed, mast cells are one of the major ST2-expressing cell types and have been demonstrated to be critically associated with the IL-33-associated development of asthma.^{9,10} For instance, activation of mast cells by IL-33 was shown to enhance AHR and asthmatic inflammation via the production of Th2 cytokines such as IL-13.⁹⁻¹¹ In addition, IL-13 secreted from IL-33-stimulated mast cells heightened airway smooth muscle contraction and contributed to the development of airway inflammation.^{9,10} However, the detailed signaling mechanism for the production of Th2 cytokines, particularly IL-13, in activated mast cells by IL-33 remains unclear.

Many previous studies have shown that 5-LO or 12-LO is closely associated with allergic asthmatic development.¹²⁻¹⁶ For instance, leukotriene B₄ (LTB₄), a 5-LO product, has been demonstrated to perform important functions during the development of asthma.^{13,14} Similarly, 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), a product of 12-LO, was detected at higher levels in the sputum and bronchoalveolar lavage fluid (BALF) in asthmatics than in healthy subjects.¹⁵ Furthermore, 12-LO-overexpressing mice showed spontaneous asthmatic symptoms such as elevated AHR and airway inflammation, and inhibition of 12-LO markedly reduced airway resistance and inflammation as well as goblet cell metaplasia in an OVA-induced asthma model.¹⁶ Recently, mast cells were suggested to be one of the principal cell types for the action of 5-/12-LO during the progression of asthmatic inflammation.¹⁷⁻¹⁹ In particular, 5-/12-LO was suggested to contribute to mast cell-derived IL-13 production.^{14,19} LTB₄ and 12(S)-HETE, products of 5-/12-LO, were shown to act through their specific cell surface G-protein-coupled receptor BLT2.²⁰ In addition, many previous reports have suggested that BLT2 in mast cells plays a key mediatory role in the production of Th2 cytokines, such as IL-13.^{14,19} Despite these suggested roles of 5-/12-LO in IL-13 production, the relationship between IL-33 and 5-/12-LO has not yet been elucidated.

In this study, we found that 5-/12-LO plays critical roles in IL-33 signaling to induce IL-13 production in mast cells, and we further

characterized the involved pathway. IL-33 stimulates the production of IL-13 via the upregulation of 5-LO and 12-LO in mast cells. We found that the "MyD88-p38 kinase-cPLA₂" cascade lies upstream of 5-/12-LO, and the "BLT2-NF- κ B" cascade lies downstream of 5-/12-LO in IL-33 signaling to induce IL-13 production in mast cells. Taken together, our findings demonstrated that "MyD88-p38 kinase-cPLA₂-5-/12-LO-BLT2-NF- κ B" signaling contributes to IL-33-induced IL-13 synthesis in mast cells, thereby potentially exacerbating airway inflammation. This finding may provide a new perspective for the development of new therapies for asthma, including severe asthma.

2 | MATERIALS AND METHODS

2.1 | Reagents

Recombinant mouse IL-33 was purchased from R&D systems (Minneapolis, MN, USA). RPMI 1640, antibiotics, antimycotic compounds, and nonessential amino acids were purchased from Invitrogen (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (St. Louis, MO, USA). MK886, Bay11-7082, PD98059, and SB203580 were purchased from Calbiochem (La Jolla, CA, USA). Baicalein was purchased from Enzo Life Sciences (Farmingdale, NY, USA). U75302 and LY255283 were obtained from Cayman Chemical (Ann Arbor, MI, USA).

2.2 | BMMC culture and stimulation

Bone marrow-derived mast cells (BMMCs) were obtained by in vitro differentiation of bone marrow cells obtained from mouse (8-12 weeks old) femurs and cultured as described previously.^{19,21} The purity of the BMMCs was typically over 97%, as determined by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) using an FITC-conjugated rat anti-mouse c-Kit mAb (eBioscience, San Diego, CA, USA). BMMCs were stimulated with IL-33 (10 ng/mL) in cytokine-free complete medium for the indicated time periods.

2.3 | Semi-quantitative and quantitative reverse transcriptase PCR analysis

Total cellular RNA was extracted using easy-BLUE (iNtRON Biotechnology, Seongnam, Korea). Then, 2 μ g of the extracted RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (iNtRON Biotechnology), and BLT1, BLT2, IL-13, ST2, MyD88, and GAPDH were amplified using an RT-PCR PreMix kit (iNtRON Biotechnology). For semi-quantitative analysis of the transcripts, we first determined the optimal PCR conditions for linear amplification of GAPDH. The primers for ST2,²² MyD88,²⁰ IL-13,²¹ BLT1, BLT2, and GAPDH¹⁹ were described previously, respectively. For real-time PCR reactions, a LightCycler 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's instructions.

2.4 | RNA interference

To knockdown ST2, MyD88, 5-LO, 12-LO, BLT1, or BLT2, BMMCs were transfected with small interfering RNAs (siRNA; Bioneer, Daejeon, Korea) using the MP-100 MicroPorator (Digital Bio, Seoul, Korea) according to the manufacturer's instructions. Then, cells were cultured in complete medium without antibiotics for 24 hours, and the mRNA levels of ST2, MyD88, 5-LO, 12-LO, BLT1, and BLT2 were analyzed by RT-PCR to evaluate the level of interference.

2.5 | Measurement of IL-13, LTB₄, and 12(S)-HETE

Bone marrow-derived mast cells were stimulated with IL-33 (10 ng/mL) in cytokine-free complete medium for the indicated time periods. The amount of IL-13, IL-5, LTB₄, or 12(S)-HETE was quantified in cell-free collected medium or in the supernatants of BALF using an ELISA kit (R&D systems for IL-13 or IL-5; Enzo life sciences for LTB₄ and 12(S)-HETE) according to the manufacturer's instructions. BALF was obtained from mouse lungs using 0.7 mL of PBS after trachea cannulation, and the collected BALF was centrifuged at 1000 g for 3 minutes. Then, the supernatant was collected for an ELISA.

2.6 | Immunoblotting analysis

Immunoblotting analysis was carried out as described previously.¹⁹ Antibodies against cPLA₂, p-cPLA₂, 5-LO, and 12-LO were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies against ERK, p-ERK, p38, phospho-p38, p-IkB α , and β -actin (loading control) were obtained from Cell Signaling Technology (Danvers, MA, USA). Size estimates for proteins were obtained using molecular weight standards from Thermoscientific (Rockford, IL, USA).

2.7 | IL-33 administration-induced Airway inflammation

Female BALB/c mice (8 weeks old) were obtained from Orient Bio (Seongnam, Korea). Mice were intranasally administered IL-33 daily (1 μ g/day) for 3 days. For the inhibition experiments, mice were orally injected with MK886 (5 mg/kg) or baicalein (75 mg/kg) 1 h before each IL-33 administration. In addition, mice were intraperitoneally injected with LY255283 (10 mg/kg), U75302 (10 μ g/mouse) or vehicle (DMSO) 1 hour before each IL-33 administration. Mice were killed on the next day after the final IL-33 administration. Genetically mast cell-deficient Kit (W/Wv) mice and congenic wild-type (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Typically, W/Wv mice exhibit less than 1.0% of the number of dermal mast cells present in the skin of WT mice and have no detectable mature mast cells in the respiratory system or other sites.²³ In addition, we performed the mast cell reconstitution experiments using the methodology described previously.^{21,24,25} Bone marrow cells were cultured from WT mice. Then, twice weekly,

BMMCs were infused into each W/Wv mice via the tail vein. Eight weeks later, BMMCs were infused once, and the reconstituted mice were then administered with IL-33 daily (1 μ g/day) for 3 days. All experimental animals used in this study were treated according to the guidelines approved by the Institutional Animal Care and Use Committee of Korea University.

2.8 | BALF cells and analysis of lung histology

Inflammatory cells were collected by BALF by centrifugation (1000 g for 3 minutes) and washed with PBS. Next, BAL cells were fixed on glass slides and then stained with hematoxylin and eosin (H&E). Lung sections (6 μ m thickness) were mounted onto Superfrost Plus glass slides (Fisher Scientific Pittsburgh, PA, USA), deparaffinized, and stained with H&E or periodic acid-Schiff (PAS). A quantitative histological analysis was performed to measure the degree of inflammation by five independent, blinded investigators. The degree of peribronchial and perivascular lung inflammation was evaluated on a subjective scale from 0 to 3, as previously described.¹⁴ Images were acquired using a BX51 microscope (Olympus, Tokyo, Japan) equipped with a DP71 digital camera (Olympus).

2.9 | Statistical analysis

Student's *t* test was performed to assess most of the data, which were expressed as the means $\pm$ SD. In all cases, the experiments were repeated three times with similar results, and representative data are shown. *P*-values < .05 were considered statistically significant.

3 | RESULTS

3.1 | IL-33 stimulates the synthesis of IL-13 in mast cells via a 5-/12-LO-dependent pathway

To determine whether mast cells play a role in the IL-33-driven production of IL-13 *in vivo*, we used genetically mast cell-deficient Kit (W/Wv) mice. The IL-13 levels in BALF were significantly lower in W/Wv mice than in WT mice when IL-33 was administered intranasally (Figure 1A), suggesting a role of mast cells in the synthesis of IL-13 by IL-33. Similarly, histological analysis revealed that W/Wv mice showed diminished airway inflammation (Figure 1A). To further demonstrate the role of mast cells, we performed the mast cell reconstitution experiments using the methodology described previously.^{21,24,25} Clearly, the production of IL-13 and associated airway inflammation as well as mucus production was restored by the transfer of BMMCs derived from WT mice into W/Wv mice and subsequent administration of IL-33 (Figure 1A). Taken together, mast cells appear to be critical for the production of IL-13 and the airway inflammation in IL-33-administered mice.

Next, we isolated BMMCs to test whether IL-33 stimulates the synthesis of IL-13 and found that the synthesis of IL-13 was significantly elevated following IL-33 stimulation (Figure 1B). To determine

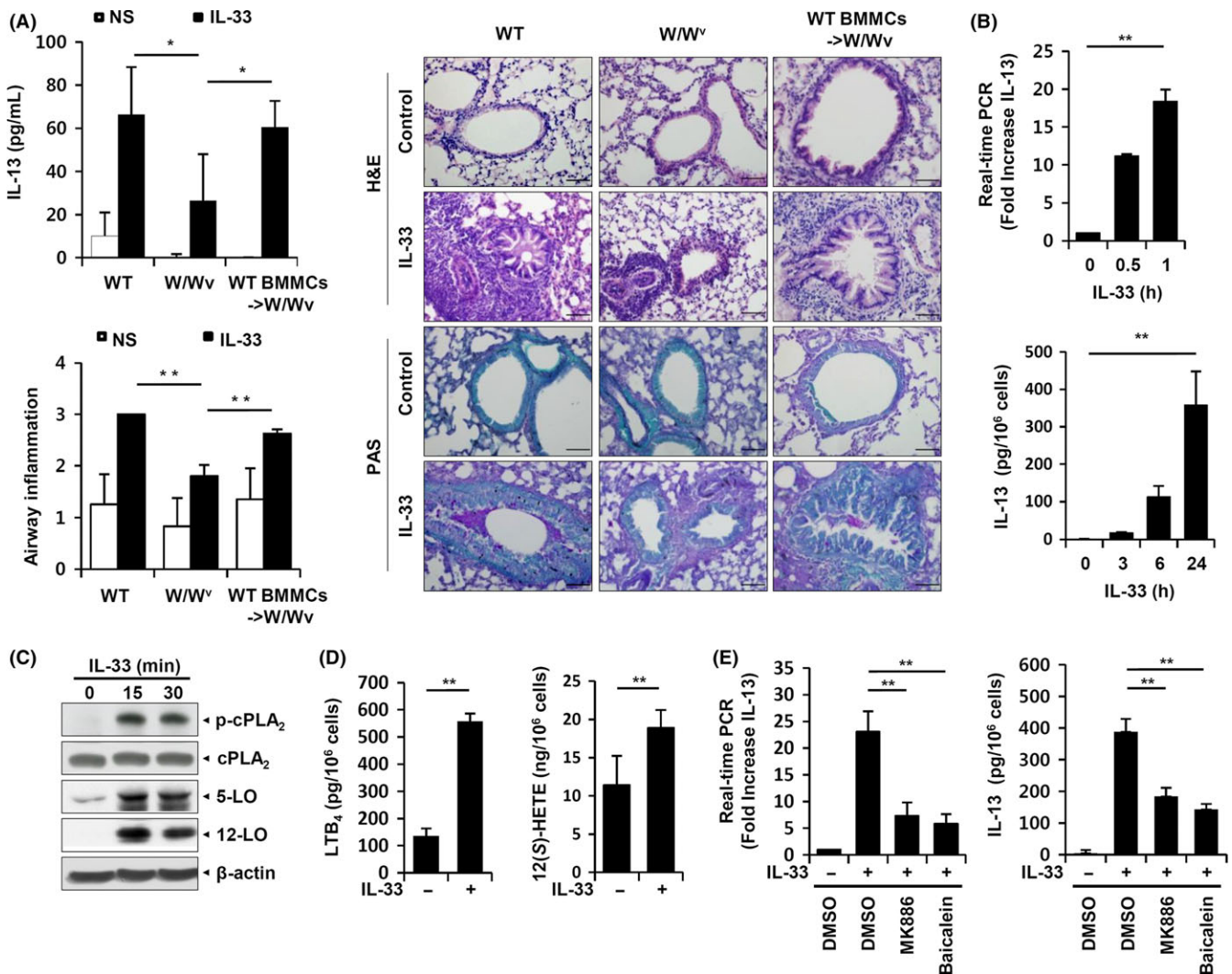


FIGURE 1 5-/12-LO contributes to the synthesis of IL-13 in IL-33-stimulated bone marrow-derived mast cells (BMMCs). A, Airway inflammation was induced by IL-33 (1 μ g per animal, intranasal) for three consecutive days in W/Wv or W/Wv reconstituted with WT BMMCs as well as control mice (WT). The levels of IL-13 in BALF were analyzed using an ELISA. Airway inflammation in lung tissues was assessed by histological analysis. The lungs were excised, fixed, and stained with H&E or PAS ($n = 3$ –5 per group). Scale bars, 50 μ m. Peribronchial and perivascular lung inflammation were measured and scored. B, BMMCs were stimulated with IL-33 (10 ng/mL) for the indicated time periods. Then, transcript levels were assessed by real-time PCR. The levels of IL-13 in the culture medium were detected using an ELISA. C, BMMCs were stimulated with IL-33 (10 ng/mL) for the indicated times. Then, cell lysates were subjected to immunoblotting analysis. D, BMMCs were stimulated with IL-33 for 24 h. Then, the levels of LTB₄ and 12(S)-HETE released into the culture medium were assayed using ELISA. E, BMMCs were first incubated with MK886 (5 μ mol/L), baicalein (20 μ mol/L), or DMSO for 30 min and then stimulated with IL-33 for 1 h. Next, total RNA was isolated and subjected to real-time PCR. Culture supernatants of BMMCs treated as shown in (B) were assayed to measure the level of IL-13. All quantitative data are expressed as the mean $\pm$ SD from three independent experiments. * $P < .05$, ** $P < .01$.

whether 5-/12-LO is associated with IL-33-stimulated signaling to induce IL-13 synthesis in mast cells, we examined the levels of 5-/12-LO and cPLA₂ phosphorylation. We found that the levels of cPLA₂ phosphorylation and 5-/12-LO were highly increased following IL-33 stimulation (Figure 1C). Also, we found that LTB₄ and 12(S)-HETE were elevated by IL-33 (Figure 1D). Furthermore, prior exposure of the cells to the 5-LO inhibitor MK886 or the 12-LO inhibitor baicalein significantly suppressed the levels of IL-13 (Figure 1E). Together, these results suggested that 5-/12-LO contributes to IL-13 production by IL-33 in mast cells.

3.2 | 5-/12-LO lies downstream of MyD88 in IL-33-induced signaling to promote IL-13 production in BMMCs

IL-33 activates mast cells by binding to its receptor, ST2, and transmits a signal via a cytoplasmic adaptor protein, MyD88. The ST2/MyD88 cascade has been shown to produce IL-13 in mast cells.²⁶ Consistent with this report, depletion of ST2 or MyD88 by siRNA resulted in a marked attenuation of IL-33-induced IL-13 production (Figure 2A,B). Next, we tested whether upregulation of 5-/12-LO in

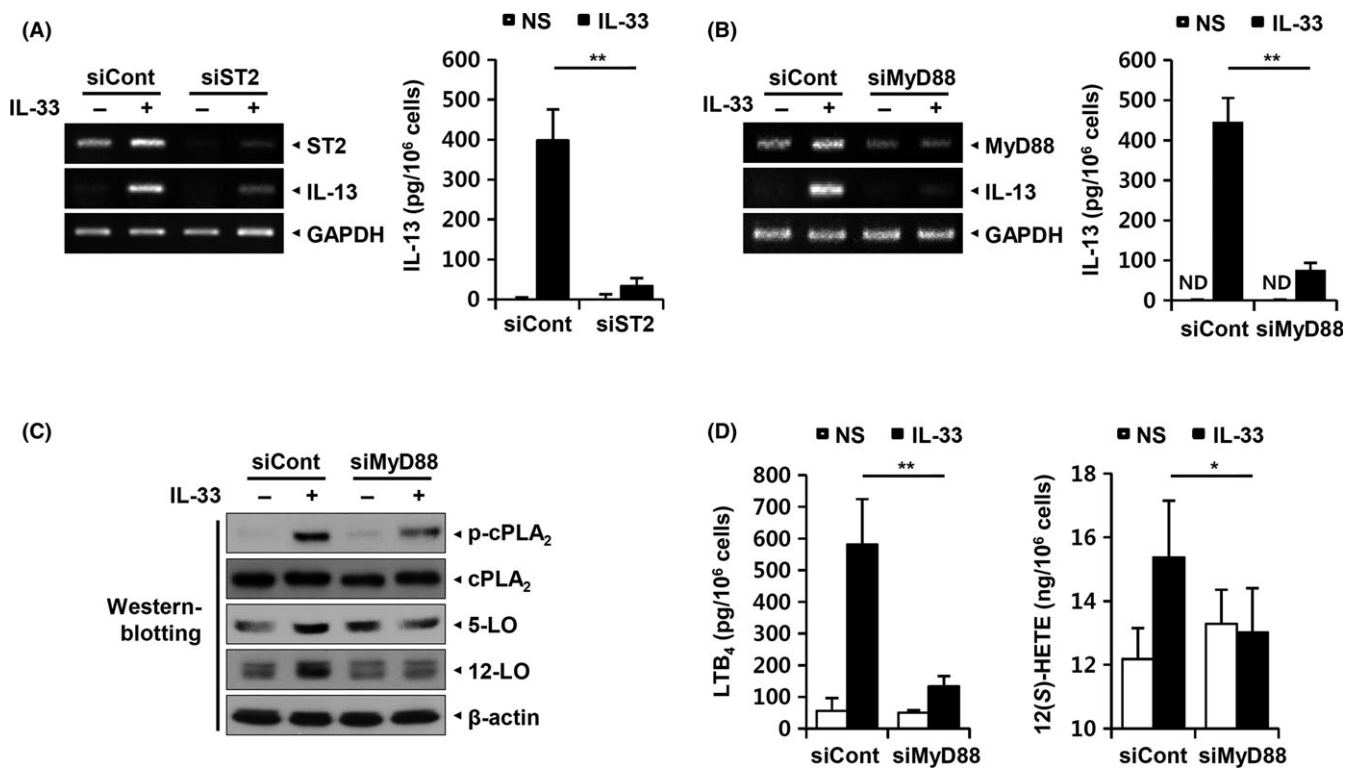


FIGURE 2 5-/12-LO functions downstream of MyD88 in IL-33-induced signaling to promote IL-13 production in bone marrow-derived mast cells (BMMCs). A, BMMCs were transfected with control or ST2 siRNAs, incubated for 24 h and then stimulated with IL-33 (10 ng/mL) for 1 h. Transcript levels were assessed by semi-quantitative RT-PCR. Independently, BMMCs were transfected, incubated as described above, and stimulated with IL-33 (10 ng/mL) for 24 h, followed by assays of IL-13 release. B, BMMCs were transfected with control or MyD88 siRNAs, incubated for 24 h, and stimulated with IL-33 (10 ng/mL) for 1 h. Then, transcript levels were assessed by semi-quantitative RT-PCR. Cells were transfected and incubated as described above and stimulated with IL-33 for 24 h, followed by assays of IL-13 release. C, BMMCs were transfected as described in (B) and stimulated with IL-33 for 15 min. Then, cell lysates were subjected to immunoblot analysis. D, BMMCs were transfected as in (B) and stimulated with IL-33 for 24 h. Next, the levels of LTB₄ and 12(S)-HETE were determined using an ELISA assay. All quantitative data are expressed as the mean $\pm$ SD from three independent experiments. * P < .05, ** P < .01

BMMCs is dependent on MyD88. Depletion of MyD88 clearly attenuated the IL-33-induced increase in the levels of cPLA₂ phosphorylation and 5-/12-LO in BMMCs (Figure 2C). The IL-33-induced generation of LTB₄ and 12(S)-HETE was also significantly inhibited by MyD88 depletion (Figure 2D). Collectively, these results suggested that a cPLA₂-5-/12-LO-linked cascade is activated via MyD88 and predominantly contributes to IL-33-induced IL-13 synthesis in BMMCs.

3.3 | p38 Kinase lies upstream of 5-/12-LO in IL-33 signaling to promote IL-13 synthesis in BMMCs

Previous studies have suggested that MAPKs play a role in IL-33 signaling in mast cells, thereby facilitating the synthesis of IL-13 in these cells.^{27,28} Consistent with these reports, we found that pretreatment with the ERK inhibitor PD98059 or the p38 kinase inhibitor SB203580 markedly inhibited IL-33-induced IL-13 production in BMMCs (Figure 3A,B). Next, we found that PD98059 and SB203580 clearly inhibited the levels of cPLA₂ phosphorylation and 5-/12-LO (Figure 3C). In addition, IL-33-induced generation of LTB₄ and 12(S)-HETE in BMMCs was significantly inhibited by prior

exposure of the cells to PD98059 or SB203580 (Figure 3D). Together, these results suggest that MAPKs, specifically p38 kinase, are associated with the phosphorylation of cPLA₂ and upregulation of 5-LO and 12-LO, thereby contributing to the production of IL-13 in IL-33 activated BMMCs.

3.4 | The action of 5-/12-LO-derived lipid products through their receptor, BLT2, is necessary for the production of IL-13 in response to IL-33 in BMMCs

LTB₄ and 12(S)-HETE are produced through the metabolism of arachidonic acid by 5-LO and 12-LO, respectively, and can interact with their specific receptor, BLT2.^{29,30} Next, we examined whether BLT2 plays any role in IL-13 production by IL-33 in BMMCs. Pretreatment with a BLT2-specific inhibitor, LY255283, or knockdown of BLT2 by siRNA decreased the production of IL-13 by IL-33 (Figure 4A,B), suggesting that the 5-/12-LO-BLT2 cascade is involved in IL-33-induced IL-13 synthesis. In contrast to BLT2, BLT1 inhibition by U75302 or knockdown of BLT1 had no effect on IL-13 production in IL-33-stimulated BMMCs (Figure 4C,D). Next, we investigated whether upregulation of BLT2 is dependent on 5-/12-LO.

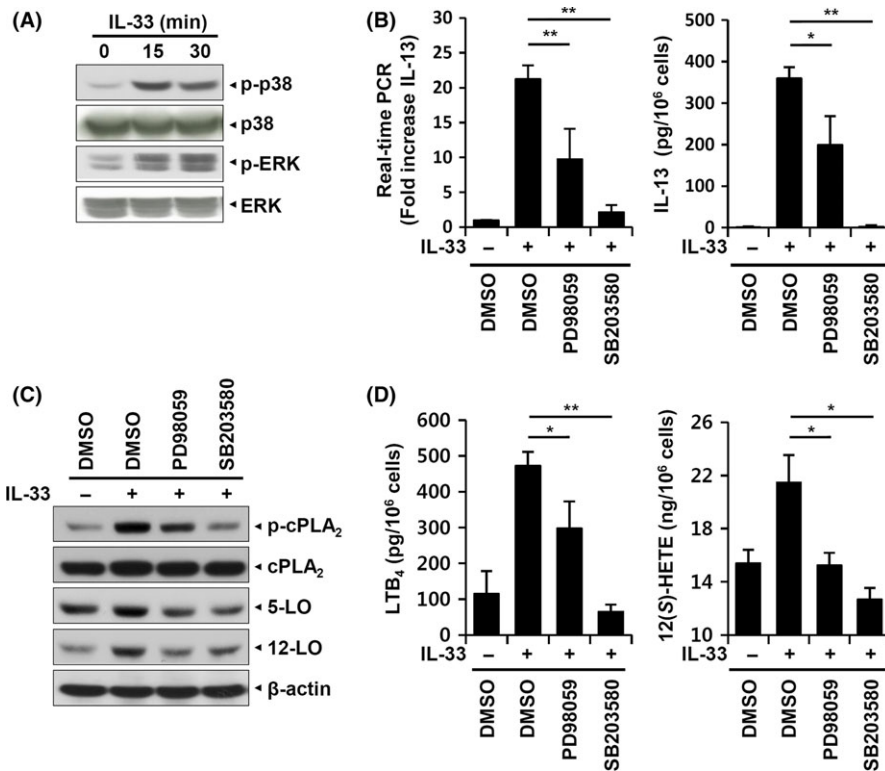


FIGURE 3 p38 Kinase functions upstream of 5-/12-LO in IL-33 signaling to promote IL-13 synthesis in bone marrow-derived mast cells (BMMCs). A, BMMCs were stimulated with IL-33 (10 ng/mL) for the indicated times. Then, cell lysates were subjected to immunoblot analysis. B, Cells were pretreated for 30 min with PD98059 (20 μ mol/L), SB203580 (20 μ mol/L) or DMSO and then stimulated for 1 h with IL-33 (10 ng/mL), followed by analysis of total RNA using real-time PCR. Cells were exposed as described above and incubated with IL-33 (10 ng/mL) for 24 h. The levels of IL-13 released into the culture medium were then assayed. C, Cells were treated as in (B) and incubated with IL-33 (10 ng/mL) for 15 min. Next, cell lysates were subjected to immunoblot analysis. D, Cells were treated as in (B), and the levels of LTB₄ and 12(S)-HETE were determined in the culture medium using an ELISA assay. All quantitative data are expressed as the mean $\pm$ SD from three independent experiments. * P < .05, ** P < .01

Pretreatment of cells with the 5-LO inhibitor MK886 or the 12-LO inhibitor baicalein clearly suppressed the upregulation of BLT2 (Figure 4E). In addition, knockdown of MyD88 by siRNA resulted in a clear attenuation of BLT2 upregulation in IL-33-treated BMMCs (Figure 4F). Together, these results suggested that BLT2 is potentially downstream of 5-/12-LO in mediating IL-13 production in IL-33 activated BMMCs.

3.5 | NF- κ B lies downstream of 5-/12-LO in IL-33 signaling to IL-13 production in BMMCs

IL-33 stimulates NF- κ B activity, which contributes to the transcription of IL-13 in BMMCs.²⁸ Consistent with this report, we found that NF- κ B is activated in response to IL-33 by measuring the levels of I κ B α phosphorylation (Figure 5A), and pretreatment of the cells with the NF- κ B inhibitor Bay11-7082 markedly suppressed the production of IL-13 by IL-33 in BMMCs (Figure 5B). Next, we investigated the involvement of 5-/12-LO in NF- κ B activation by IL-33 and found that inhibition of 5-LO or 12-LO with MK886 or baicalein, respectively, clearly inhibited IL-33-induced NF- κ B activation in BMMCs (Figure 5C and 5D), suggesting that NF- κ B activation lies downstream of 5-/12-LO in IL-33 signaling to induce IL-13 production in BMMCs. In addition, transfection of siMyD88 resulted in a marked attenuation of IL-33-induced NF- κ B activation (Figure 5E).

3.6 | 5-/12-LO contributes to the production of IL-13 in the IL-33-induced airway inflammation model

Next, we examined whether 5-/12-LO contributes to the production of IL-13 in response to IL-33 in vivo. To achieve this, BALB/c mice

were intranasally administered IL-33 daily (1 μ g/day) for 3 days. To inhibit 5-/12-LO, mice were orally injected with MK886 (5 mg/kg) or baicalein (75 mg/kg) 1 hour before IL-33 administration (Figure 6A). We observed that the levels of IL-13 as well as IL-5 were highly increased in BALF in the IL-33-administered asthmatic airway inflammation model (Figure 6B), but the levels were dramatically suppressed by treatment with inhibitors of 5-LO or 12-LO. In addition, the levels of LTB₄ and 12(S)-HETE were greatly elevated in serum after IL-33 treatment, and injection of MK886 or baicalein significantly reduced LTB₄ and 12(S)-HETE levels (Figure 6C). Accumulation of total immune cells and eosinophils was also highly enhanced in BALF by IL-33, and this effect was also greatly suppressed by MK886 or baicalein (Figure 6D). In addition, histology analysis showed that MK886 or baicalein clearly reduced airway inflammation compared to those of controls (Figure 6E). Together, these results suggest that IL-33-induced production of IL-13 and airway inflammation are predominantly dependent on the 5-/12-LO cascade, supporting the critical mediatory roles of 5-/12-LO in mast cells in IL-33-induced IL-13 production.

Similarly, we examined the effect of BLT2 blockade on IL-13 synthesis in an IL-33-induced murine model. To achieve this, LY255283 (10 mg/kg) was intraperitoneally injected at 1 hour before IL-33 administration (Figure 7A). We found that LY255283 significantly reduced the levels of IL-13 as well as IL-5 (Figure 7B), and also the infiltration of inflammatory cells (Figure 7C). In addition, airway inflammation and mucus production were markedly reduced by LY255283 as determined by histological analysis and quantitative analysis of inflammation scores (Figure 7D). Together, these results suggested that BLT2, which is downstream of the 5-/12-LO cascade, contributes to the production of IL-13 and airway inflammation induced by IL-33,

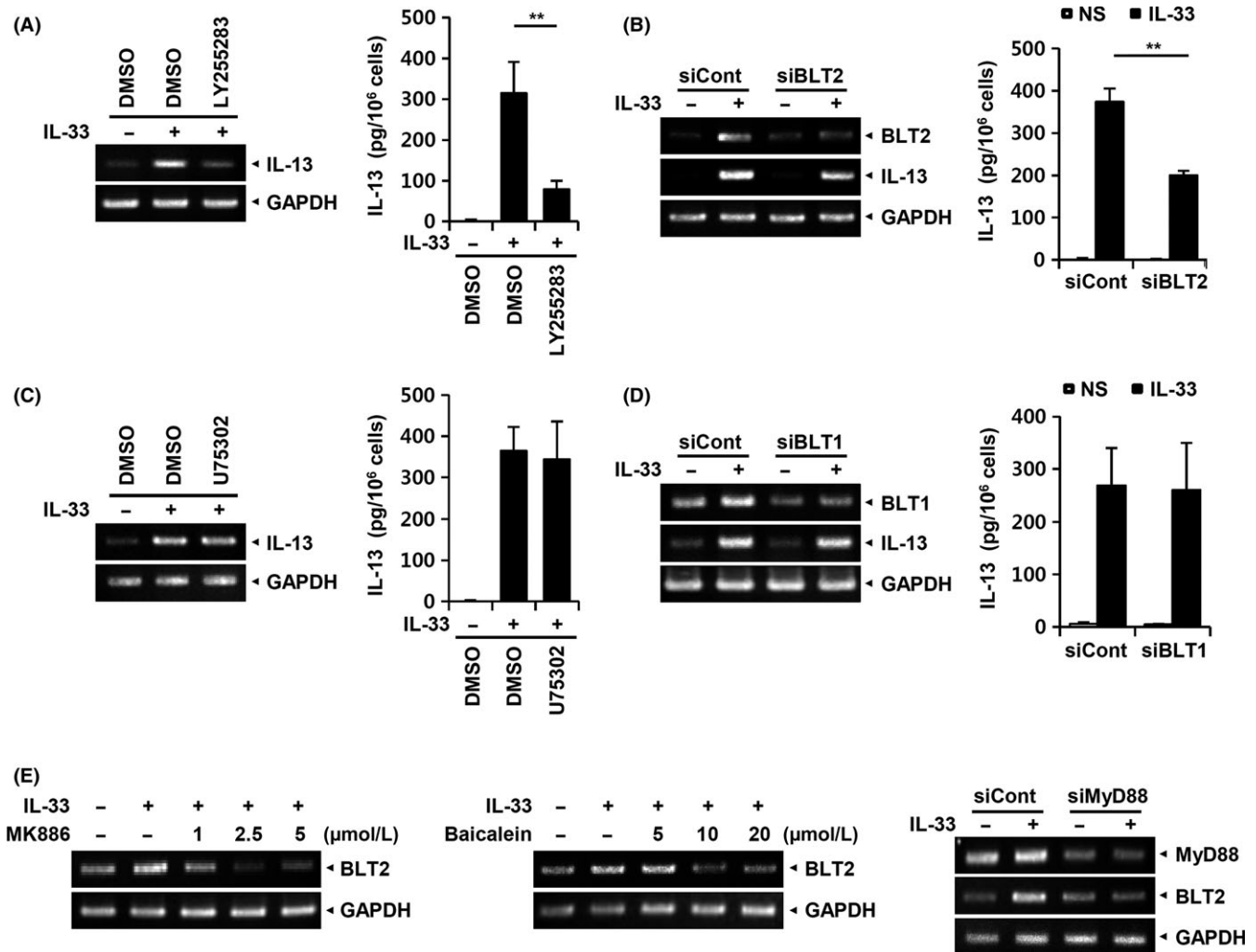


FIGURE 4 BLT2 functions downstream of the 5-/12-LO cascade in IL-33 signaling to induce IL-13 synthesis in bone marrow-derived mast cells (BMMCs). A and C, BMMCs were first incubated with LY255283 (10 μmol/L), U75302 (1 μmol/L), or DMSO for 30 min and then stimulated with IL-33 (10 ng/mL) for 1 h. Then, transcript levels were assessed by semi-quantitative RT-PCR. Cells were treated as described above (A) and incubated with IL-33 (10 ng/mL) for 24 h. Next, the levels of IL-13 released into the culture medium were assayed. B and D, BMMCs were transfected with 50 nmol/L of control, BLT1 or BLT2 siRNAs, incubated for 24 h and then stimulated with IL-33 (10 ng/mL) for 1 h. Then, transcript levels were assessed by semi-quantitative RT-PCR. Cells were transfected and incubated as described above and stimulated with IL-33 (10 ng/mL) for 24 h, followed by assays of IL-13 levels. E, BMMCs were incubated for 30 min with the indicated dose of MK886 or baicalein and then stimulated with IL-33 (10 ng/mL) for 1 h. Then, total RNA was isolated and subjected to semi-quantitative RT-PCR analysis. F, siMyD88 was transfected as in (B), and cells were treated with IL-33 (10 ng/mL) for 1 h. Then, semi-quantitative RT-PCR analysis was performed. The results shown are representative of three independent experiments with similar results. All quantitative data are expressed as the mean ± SD. **P* < .05, ***P* < .01

further supporting the role of 5-/12-LO in IL-33-induced IL-13 production and airway inflammation response (Figure 7E).

4 | DISCUSSION

In the present study, we demonstrated that 5-/12-LO plays a critical role in IL-33 signaling to induce IL-13 production in mast cells. In addition, we demonstrated that the "MyD88-p38 kinase-cPLA₂" cascade functions upstream of 5-/12-LO, and the "BLT2-NF-κB" cascade functions downstream of 5-/12-LO in the IL-33 signaling

pathway to induce IL-13 production in mast cells. Our findings may provide insight into the mechanisms underlying how IL-33 contributes to asthmatic development and exacerbation.

IL-33, a member of the IL-1 family, functions as an alarmin to alert the immune system after endothelial or epithelial cell damage.³¹ In addition, recent studies have suggested that IL-33 is also implicated in the promotion and exacerbation of asthmatic pathogenesis by inducing the production of Th2 cytokines, such as IL-13.³² And, mast cells have been demonstrated to be closely associated with IL-33-induced production of IL-13.^{9,27} Consistent with these reports, we found that IL-13 levels were significantly lower

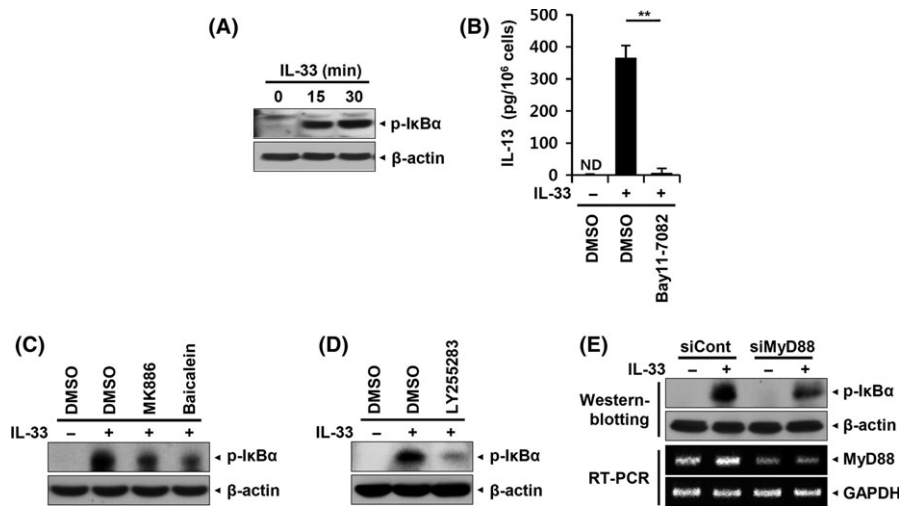


FIGURE 5 NF-κB activation functions downstream of 5-/12-LO in IL-33 signaling to induce IL-13 production in bone marrow-derived mast cells (BMMCs). A, BMMCs were stimulated with IL-33 (10 ng/mL) for the indicated times. Then, cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated IκBα. B, BMMCs were incubated for 30 min with Bay11-7082 (20 μmol/L) and then stimulated for 24 h with IL-33 (10 ng/mL). The level of IL-13 was determined with an ELISA. C and D, BMMCs were incubated for 30 min with MK886 (5 μmol/L), baicalein (20 μmol/L), or LY255283 (10 μmol/L) and then stimulated with IL-33 for 15 min. Cell lysates were subjected to immunoblot analysis. E, BMMCs were transfected with control or MyD88 siRNAs and incubated for 24 h. Next, the BMMCs were stimulated with IL-33 for 15 min. Cell lysates were subjected to immunoblot analysis. Cells were treated as described above and stimulated with IL-33 for 1 h. Total RNA was isolated and subjected to semi-quantitative RT-PCR analysis. All quantitative data are expressed as the mean ± SD from three independent experiments. *P < .05, **P < .01

in W/Wv mice than in WT mice when IL-33 was administered intranasally (Figure 1A), suggesting a pivotal role of mast cells in the synthesis of IL-13 by IL-33.

In the present study, we found that the 5-/12-LO is associated with the synthesis of IL-13 by IL-33 in BMMCs (Figure 1). In addition, the levels of 5-/12-LO metabolites, LTB₄ and 12(S)-HETE, were shown to be highly elevated following IL-33 stimulation (Figure 1). It was shown that IL-33 activates mast cells by binding to ST2 and transmits its signal via an adaptor protein, MyD88. Indeed, the ST2/MyD88 cascade was shown to produce IL-13 in mast cells.²⁶ Consistent with this report, depletion of ST2 or MyD88 resulted in a marked attenuation of IL-33-induced IL-13 production (Figure 2A,B). Moreover, we found that depletion of MyD88 clearly attenuated the IL-33-induced increase in the levels of cPLA₂ phosphorylation and 5-/12-LO as well as the production of LTB₄ and 12(S)-HETE in BMMCs (Figure 2C,D), which together further support the role of 5-/12-LO in the IL-33-induced synthesis of IL-13. Recently, IL-13 released from mast cells exposed to IL-33 was shown to indirectly induce airway smooth muscle contraction and play a critical role in the development of airway inflammation.⁹ Thus, we suspect that 5-/12-LO inhibition might attenuate airway smooth muscle contraction and inflammation. Consistent with this idea, it was shown that 5-LO inhibition was effective in alleviating asthmatic symptoms induced by allergic responses.³³ Similarly, 12-LO inhibition reduced airway resistance, recruitment of inflammatory cells to the lung, fibrosis, and goblet cell metaplasia in an OVA-induced asthma model.¹⁶ In addition, overexpression of 12-LO caused spontaneous asthmatic symptoms,

such as increased AHR and airway inflammation. Moreover, atopic asthmatics show higher levels of 12(S)-HETE in the sputum and BAL fluid than healthy subjects.¹⁵

The 5-/12-LO metabolites LTB₄ and 12(S)-HETE interact with the same cell surface receptor, BLT2, and it is likely that an autocrine- or paracrine-acting "LTB₄/12(S)-HETE-BLT2" cascade is implicated in the production of IL-13 in response to IL-33. Consistent with this idea, we observed that the levels of BLT2 were markedly upregulated by IL-33 (Figure 4B) and that blockade of BLT2 significantly reduced the IL-33-induced IL-13 synthesis in BMMCs (Figure 4A,B). We found that blockade of BLT1 had no effect on the levels of IL-13 in response to IL-33 (Figure 4C,D), suggesting that the BLT1-linked cascade is not associated with IL-33 signaling to induce IL-13 production in mast cells. Consistent with the suggested role of BLT2 in the production of IL-13, it was previously shown that BLT2 overexpression alone significantly increased IL-13 production in BMMCs, and the addition of BLT2 ligands [LTB₄ and 12(S)-HETE] further enhanced IL-13 production.¹⁹ Moreover, we recently demonstrated that the synthesis of IL-13 synthesis are strictly dependent on BLT2 in mast cells,^{21,34} which is consistent with the role of BLT2 in IL-33 signaling to induce IL-13 production. Many nonepithelial cell types including macrophages express 5-/12-LO and can secrete lipid mediators such as LTB₄ and 12(S)-HETE during airway inflammation in mice.^{35,36} In addition, 5-LO and its product LTB₄ were shown to play critical roles in IL-13 production in innate immune cells including macrophages and as group 2 innate lymphoid cells (ILC2) in allergic responses.³⁷ Thus, we speculate that a similar BLT2-

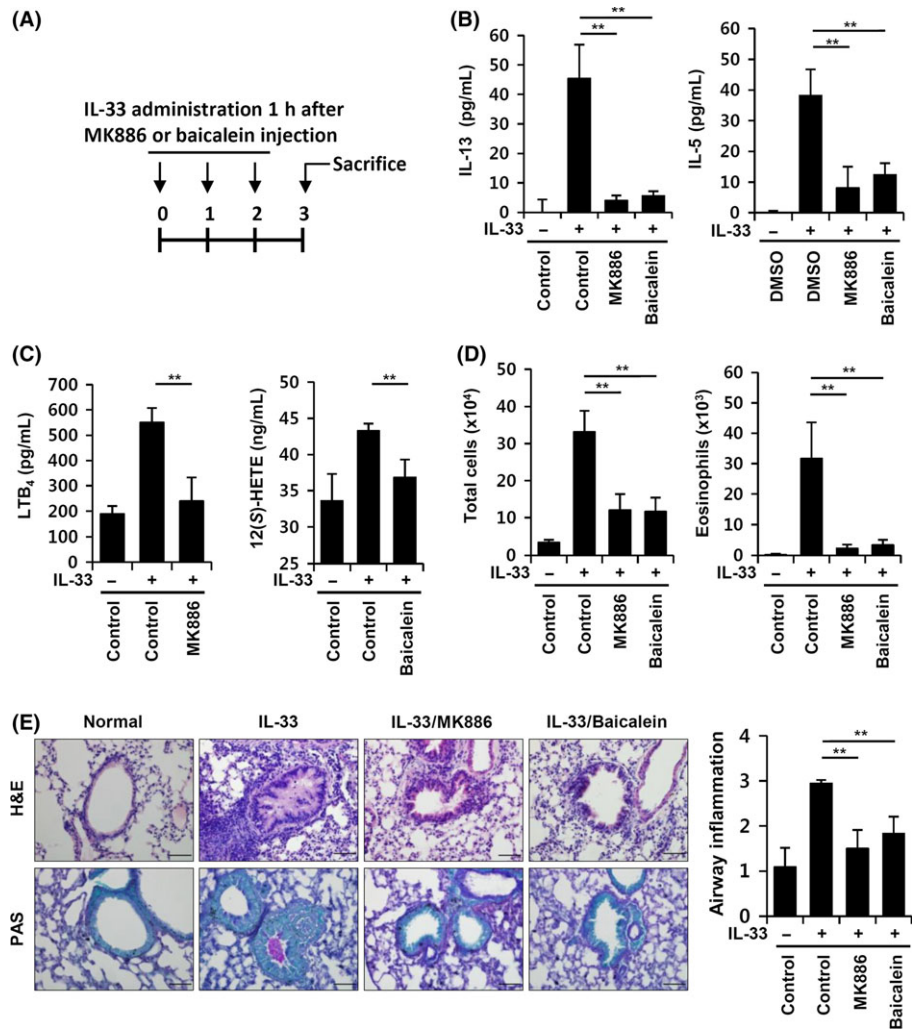


FIGURE 6 Blockade of 5-/12-LO suppresses the production of IL-13 in an IL-33-induced airway inflammation model. A, Airway inflammation was induced by intranasal administration of 1 μ g of IL-33 per day for 3 consecutive days in BALB/c mice. MK886 (5 mg/kg) or baicalein (75 mg/kg) was injected orally 1 h before each IL-33 administration in some mice, and the mice were then killed on the day after the final IL-33 administration ($n = 4$ –6 per group). B, The levels of IL-13 and IL-5 in BALF were analyzed using ELISA. C, The LTB₄ and 12(S)-HETE levels in serum were analyzed using ELISAs. D, Eosinophils and total immune cells in BALF were obtained using cytospin and stained with H&E. E, The lungs were excised, fixed, and stained with H&E or PAS. Peribronchial and perivascular lung inflammation were measured and scored. All quantitative data are expressed as the mean $\pm$ SD from three independent experiments. * $P < .05$, ** $P < .01$.

linked signaling cascade may exist in other cell types such as macrophages or ILC2. Nonetheless, we think that the observations (Figures 1, 6, and 7), especially from the mast cell reconstitution experiment, are mainly due to the contribution by mast cells. Clearly, further studies are necessary to understand the roles of other cell types in IL-33-IL-13 axis in vivo.

Similar to our findings in BMMCs, the levels of IL-13 in BALF were also markedly suppressed in vivo by blockade of 5-LO or 12-LO in IL-33-induced murine models (Figure 6). The levels of LTB₄ and 12(S)-HETE were also highly elevated in serum after IL-33 treatment (Figure 6C). In addition, blockade of 5- or 12-LO resulted in a dramatic suppression of the accumulation of eosinophils in lung airways (Figure 6D). Moreover, blockade of BLT2 significantly reduced the production of IL-13 and airway inflammation in IL-33-induced asthmatic models (Figure 7). Interestingly, blockade of BLT1 with U75302 has no effect (Fig. S1), thus excluding the contribution of BLT1 in IL-33-IL-13 axis in vivo. Taken together, these results suggested that IL-33-induced production of IL-13 and eosinophilic airway inflammation is predominantly due to the 5-/12-LO cascade. In summary, our results suggested that the 5-/12-LO-linked cascade significantly contribute to IL-33 signaling, resulting in the production

of IL-13 in mast cells. Considering the critical role of IL-33 in asthmatic development and exacerbation, our results may provide a basis for the development of new treatments for allergic diseases, including severe asthma.

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CONFLICTS OF INTEREST

The authors have no financial conflict of interests.

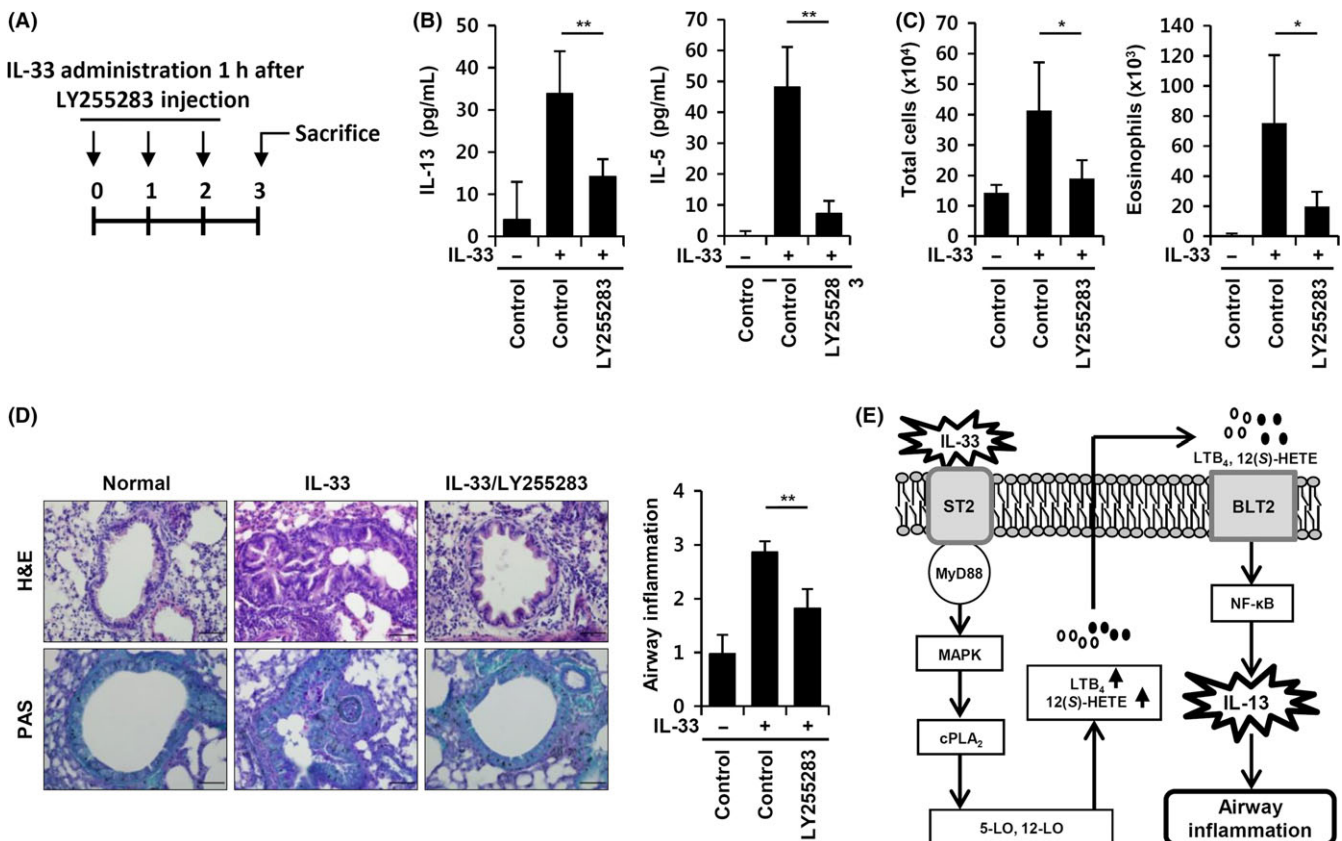


FIGURE 7 BLT2 contributes to the production of IL-13 in the IL-33-induced airway inflammation model. A, Airway inflammation was induced as described in Figure 6. Mice were intranasally administered IL-33 (1 μ g/d) daily for 3 d. LY255283 (10 mg/kg) or DMSO was injected intraperitoneally 1 h before each IL-33 administration, and the mice were then killed on the day after the final IL-33 administration ($n = 4$ –6 per group). B, The levels of IL-13 and IL-5 in BALF were analyzed using an ELISA. C, Eosinophils and total immune cells in BALF were obtained using cytopsin and stained with H&E or PAS. D, The lungs were excised, fixed, and stained with H&E or PAS. Peribronchial and perivascular lung inflammation were measured and scored. E, The scheme for the role of the 5-/12-LO-linked signaling pathway responsible for IL-33-induced IL-13 production in mast cells is shown. All quantitative data are expressed as the mean $\pm$ SD from three independent experiments.

* $P < .05$, ** $P < .01$

AUTHOR CONTRIBUTIONS

MR planned the study, performed the experiments, analyzed data, and wrote the manuscript. AJL performed the experiments and analyzed data. JHK supervised the study and wrote the manuscript.

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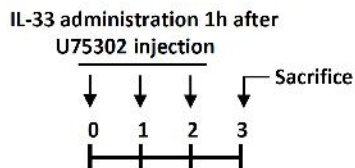
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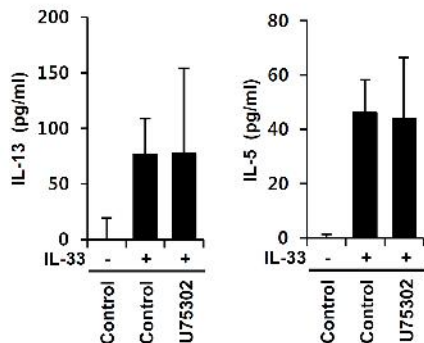
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Supplementay figure 1

A



B



C

