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Leukotriene B₄ Receptor 2 Is Critical for the Synthesis of Vascular Endothelial Growth Factor in Allergen-Stimulated Mast Cells

A-Jin Lee, MyungJa Ro, and Jae-Hong Kim

Mast cells are among the principal effector cells in the pathogenesis of allergic asthma. In allergic reactions, allergen (Ag)-induced cross-linking of IgE bound to FcεRI on mast cells results in the production of vascular endothelial growth factor (VEGF), which is essential for the initiation and development of the allergic response. Despite the central role of VEGF in allergic asthma, the signaling events responsible for the production of VEGF remain unclear, particularly in Ag-stimulated mast cells. In the present study, we observed that blocking leukotriene B₄ receptor 2 (BLT2) completely abrogated the production of VEGF in Ag-stimulated bone marrow–derived mast cells (BMMCs). The synthesis of BLT2 ligands (leukotriene B₄ and 12(S)-hydroxyeicosatetraenoic acid) was also required for VEGF production, suggesting a mediating role of an autocrine BLT2 ligands–BLT2 axis in the production of VEGF in mast cells. The NADPH oxidase 1–reactive oxygen species–NF-κB cascade is downstream of BLT2 during Ag signaling to VEGF synthesis in mast cells. Furthermore, the level of VEGF synthesis in genetically mast cell–deficient Kit(W/W^v) mice was significantly lower than that in wild-type mice in the OVA-induced asthma model, suggesting that mast cells play a critical role in the synthesis of VEGF in OVA-induced allergic asthma. Importantly, VEGF production was restored to the levels observed in wild-type mice after adoptive transfer of normal BMMCs into Kit(W/W^v) mice but was not restored in BLT2^{−/−} BMMC-reconstituted Kit(W/W^v) mice in the OVA-induced asthma model. Taken together, our results suggest that BLT2 expression in mast cells is essential for the production of VEGF in OVA-induced allergic asthma. *The Journal of Immunology*, 2016, 197: 2069–2078.

Mast cells are major effector cells in allergic diseases such as asthma, food allergies, and anaphylaxis. Allergen-induced cross-linking of IgE bound to FcεRI leads to the rapid release of allergic mediators. IgE-dependent activation of mast cells releases the following three main classes of mediators: 1) preformed mediators in cytoplasmic granules (e.g., histamine, serotonin, and proteases); 2) newly generated lipid-derived mediators (e.g., PGD₂, leukotriene B₄ [LTB₄] and leukotriene C₄); and 3) a wide variety of chemokines, cytokines including Th2 cytokines (e.g., IL-4 and IL-13), and vascular endothelial growth factor (VEGF) (1–3). VEGF is a widely recognized inflammatory medi-

ator involved in the initiation and development of allergic asthma (4, 5). Mast cells regulate airway inflammation and remodeling by orchestrating VEGF expression in OVA-induced allergic asthma (4). However, although the central role of VEGF in allergic asthma has been established, the signaling events responsible for the production of VEGF remain unclear, particularly in Ag-stimulated mast cells.

LTB₄ and 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE] are inflammatory lipid mediators produced by the metabolism of arachidonic acid by 5-lipoxygenase (5-LO) and 12-lipoxygenase (12-LO), respectively, and can interact with the G protein–coupled receptor LTB₄ receptor 2 (BLT2). Upregulation of BLT2 and its ligands has been associated with the pathogenesis of several inflammatory diseases, including allergic asthma (6–8). Additionally, BLT2 is required for the synthesis of Th2 cytokines during allergen responses in mast cells (9). However, it remains to be determined whether BLT2 is associated with the production of VEGF in the allergic response.

In the present study, we found that VEGF production in Ag-stimulated bone marrow–derived mast cells (BMMCs) is dependent on BLT2. BLT2 blockade clearly abolished VEGF synthesis in BMMCs. We also determined that the NADPH oxidase 1 (Nox1)-reactive oxygen species (ROS)–NF-κB cascade is downstream of BLT2 and mediates VEGF production in BMMCs. Furthermore, we used mast cell–deficient Kit(W/W^v) and BLT2^{−/−} mice to study the roles of mast cells and BLT2 in VEGF production, which revealed that BLT2 expression in mast cells is critical for the production of VEGF in OVA-induced allergic asthma. Taken together, our results demonstrate for the first time, to our knowledge, that the BLT2–Nox1–NF-κB cascade mediates the production of VEGF in allergen-activated mast cells, and thus our results contribute to a better understanding of the signaling mechanism for the production of VEGF in mast cells.

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Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; BLT1, leukotriene B₄ receptor 1; BLT2, leukotriene B₄ receptor 2; BMMC, bone marrow–derived mast cell; cPLA₂, cytosolic phospholipase A₂; DPI, diphenyleneiodonium; 5-LO, 5-lipoxygenase; 12-LO, 12-lipoxygenase; LTB₄, leukotriene B₄; NAC, N-acetylcysteine; Nox1, NADPH oxidase 1; ROS, reactive oxygen species; 12(S)-HETE, 12(S)-hydroxyeicosatetraenoic acid; siRNA, small interfering RNA; SU1498, (E)-3-(3,5-diisopropyl-4-hydroxyphenyl)-2-[(3-phenyl-*n*-propyl)aminocarbonyl]acrylonitrile; Tg, transgenic; VEGF, vascular endothelial growth factor; WT, wild-type.

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Materials and Methods

Reagents

Monoclonal DNP-specific IgE (clone SPE-7), DNP-conjugated BSA (DNP-BSA), DMSO, diphenyliodonium (DPI), and *N*-acetylcysteine (NAC) were obtained from Sigma-Aldrich (St. Louis, MO). AACOCF₃, AA861, baicalein, and Bay11-7082 were purchased from Calbiochem (La Jolla, CA) (10). LTB₄ receptor 1 (BLT1) antagonist U75302 (11, 12), BLT2 antagonist LY255283 (11, 12), LTB₄, and 12(*S*)-HETE were obtained from Cayman Chemical (Ann Arbor, MI). (*E*)-3-(3,5-diisopropyl-4-hydroxyphenyl)-2-[(3-phenyl-*n*-propyl)aminocarbonyl]acrylonitrile (SU1498) was purchased from Calbiochem (San Diego, CA). RPMI 1640, antibiotic and antimycotic compounds, and nonessential amino acids were obtained from Invitrogen (Grand Island, NY) (9).

BMMC culture and stimulation

BMMCs were isolated from the femurs of mice (8–12 wk old) as previously described (9). The isolated BMMCs were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, nonessential amino acids, 50 µM 2-ME, sodium pyruvate, and HEPES buffer containing 20 ng/ml stem cell factor and 10 ng/ml IL-3 (R&D systems, Minneapolis, MN) for 4–8 wk at 37°C and 5% CO₂ (9). The purity of the cells was >97% as determined at 4 wk by flow cytometry analysis (FACSCalibur; BD Biosciences, Franklin Lakes, NJ) using an FITC-conjugated rat anti-mouse c-Kit mAb (BD Biosciences, San Diego, CA). The BMMCs were sensitized overnight with an optimal concentration (1 µg/ml) of monoclonal anti-DNP-specific IgE in complete medium and then stimulated with DNP-BSA (100 ng/ml) in cytokine-free complete medium for the indicated time periods.

Semiquantitative and quantitative RT-PCR analysis

Total cellular RNA was extracted from BMMCs or homogenized lung tissue using easy-BLUE (iNtRON Biotechnology, Seongnam, Korea), and 2 µg of the extracted RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (iNtRON Biotechnology). BLT1, BLT2, VEGF, Nox1, IL-13, and GAPDH were then amplified using a PCR pre-mix kit (iNtRON Biotechnology). For semiquantitative analysis of transcripts, we first identified optimal PCR conditions for the linear amplification of GAPDH. The primers for BLT1, BLT2, and VEGF were described previously (9, 10, 13). The primers (forward and reverse) for Nox1 were 5'-TGGTCATGCAG-CATTAAACTTT-3' and 5'-AAACTCATGTGCCACATTGG-3' and for IL-13 were 5'-ATGAGTCTGCAGTATCCCCG-3' and 5'-CCGTGGCAG-ACAGGAGTGT-3'. The specificities of all primers were confirmed by sequencing the PCR products. Real-time PCR analysis was performed using LightCycler 480 SYBR Green I (Roche Diagnostics, Mannheim, Germany). All data were normalized by the abundance of GAPDH.

Measurement of VEGF and IL-13

The sensitized BMMCs were stimulated with DNP-BSA (100 ng/ml) in cytokine-free complete medium for the indicated time periods. VEGF and IL-13 were quantified in cell-free collected medium or in supernatants of bronchoalveolar lavage fluid (BALF) using an ELISA kit (R&D Systems) according to the manufacturer's instructions. BALF was collected as previously described (8) with slight modifications. In brief, BALF was obtained from mouse lungs using 0.9 ml of PBS after trachea cannulation. The collected BALF was centrifuged at 1000 × *g* for 5 min. The supernatant was collected and stored at -80°C for ELISA assay. The sensitivities of the assays for VEGF and IL-13 were <3.0 and 1.5 pg/ml, respectively.

RNA interference of BLT1, BLT2 and Nox1

To knockdown BLT1, BLT2, and Nox1, BMMCs were transfected with BLT1 small interfering RNAs (siRNAs; Bioneer, Daejeon, Korea), BLT2 siRNAs (Bioneer), or Nox1 siRNAs (Invitrogen) using the MP-100 Micro-Porator (Digital Bio, Seoul, Korea) following the manufacturer's instructions. In brief, 1 × 10⁶ cells in 100 µl of resuspension buffer (Digital Bio) containing control siRNAs (scrambled) (50 nM), BLT1 siRNAs (50 nM), BLT2 siRNAs (50 nM), or Nox1 siRNAs (50 nM) were electroporated by applying two pulses of 1400 V for 30 ms. The cells were cultured in complete medium without antibiotics for 24 h, and the mRNA level of BLT1, BLT2, or Nox1 was then analyzed by RT-PCR to evaluate the level of interference.

Immunoblot analysis

Lysate protein samples were heated at 95°C for 5 min and separated by SDS-PAGE. The separated proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane for 1 h at 100 V. The

membrane was exposed for 1 h to TBS containing 0.05% Tween 20 and 5% dried nonfat milk, followed by incubation for 2 h with primary Abs at a dilution of 1:2000 (or 1:4000 for Abs against β-actin) in TBS containing 0.05% Tween 20. The membrane was then incubated for 2 h at room temperature with HRP-conjugated secondary Abs prior to the detection of immune complexes using an ECL kit (Amersham Biosciences, Little Chalfont, U.K.). Abs to phospho-IκBα and β-actin (loading control) were obtained from Cell Signaling Technology (Danvers, MA). Size estimates for proteins were obtained using molecular mass standards from Thermo Scientific (Rockford, IL).

Mice

The BLT2 gene (*Ltb4r2*) was targeted in the VGB6 embryonic stem cell line in the C57BL/6N genetic background as part of the International Knockout Mouse Consortium (Oakland, CA) (14). A mouse strain harboring the *Ltb4r2*^{tm1(KOMP)Vlcg} allele was generated via blastocyst injection of embryonic stem cell clone 10608A-H4. Insertion of VelociGene cassette ZEN-UB1 induced the deletion of 1164 bp between positions 56,380,746 and 56,381,909 of chromosome 14 (Supplemental Fig. 1A). Mice were routinely genotyped by PCR analysis (Supplemental Fig. 1B). Heterozygous mice were backcrossed with C57BL/6 mice for seven generations under specific pathogen-free conditions. The BLT2 transgenic (Tg) mice and corresponding wild-type (WT) mice (strain FVB) were described previously (9, 15). Genetically mast cell-deficient Kit(W/W^v) mice and congenic WT mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Kit(W/W^v) mice typically contain <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 anatomical sites (4). All animals were housed under 12:12 h light/dark conditions at a density of four to five mice per static polycarbonate microisolator cage on disposable bedding. Wire-lid food hoppers within cages were filled to capacity with rodent chow, and mice were maintained on water supplied by bottle. The study was carried out in strict accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals* of Korea University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Korea University.

Mast cell reconstitution and OVA immunization

Mast cells were selectively reconstituted in W/W^v mice using the method described previously (4) with slight modifications. Bone marrow cells were cultured from WT (BLT2^{+/+}) or BLT2-null (BLT2^{-/-}) mice (strain C57BL/6), and BLT2 levels were determined (Supplemental Fig. 1C). Then, twice weekly, BMMCs (5 × 10⁶) were infused into each W/W^v mouse via the tail vein (6 wk old). Fourteen weeks later, BMMCs were infused once, and the reconstituted mice were then immunized with OVA after 3 d as described previously (8) with slight modifications. In brief, the reconstituted mice were immunized via i.p. injection of 200 mg of OVA emulsified in 2.5 mg of adjuvant aluminum hydroperoxide gel (Pierce, Rockford, IL) on day 1. A second i.p. injection of 20 mg OVA adsorbed onto aluminum hydroperoxide gel (2.5 mg) was administered 14 d later. At 21, 22 (1% OVA), and 23 d (10% OVA) after the initial immunization, the mice were challenged for 30 min with an aerosol of OVA using an ultrasonic nebulizer. The mice were sacrificed on day 25 to assess levels of asthmatic airway inflammation.

Administration of SU1498

BLT2 Tg mice (7–10 wk old) (9) were administered 10 mg/kg SU1498, an inhibitor of VEGF receptor tyrosine kinase. SU1498 was dissolved in DMSO, diluted in saline, and administered i.p. twice at 24-h intervals. The control mice received vehicle (saline containing DMSO) i.p. on the same schedule. These mice were sacrificed 1 h after final administration. BALF was collected from mouse lungs using 0.9 ml of PBS after trachea cannulation for analysis.

Histologic lung analysis

Lungs were fixed (10% formaldehyde) and paraffin embedded. Sections of 6 µm were mounted on Superfrost Plus glass slides (Fisher Scientific) and stained with H&E. The degree of lung inflammation was evaluated on a subjective scale from 0 to 3, as described elsewhere (4, 8). Grade 0 indicated that no inflammation was detectable, grade 1 indicated the occurrence of occasional cuffing with inflammatory cells, grade 2 indicated that most bronchi or vessels were surrounded by a thin layer (one to five cells thick) of inflammatory cells, and grade 3 indicated that most bronchi or vessels were surrounded by a thick layer (more than five cells thick) of inflammatory cells. Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. Images were acquired using a BX51 microscope (Olympus, Tokyo, Japan) equipped with a DP71 digital camera (Olympus).

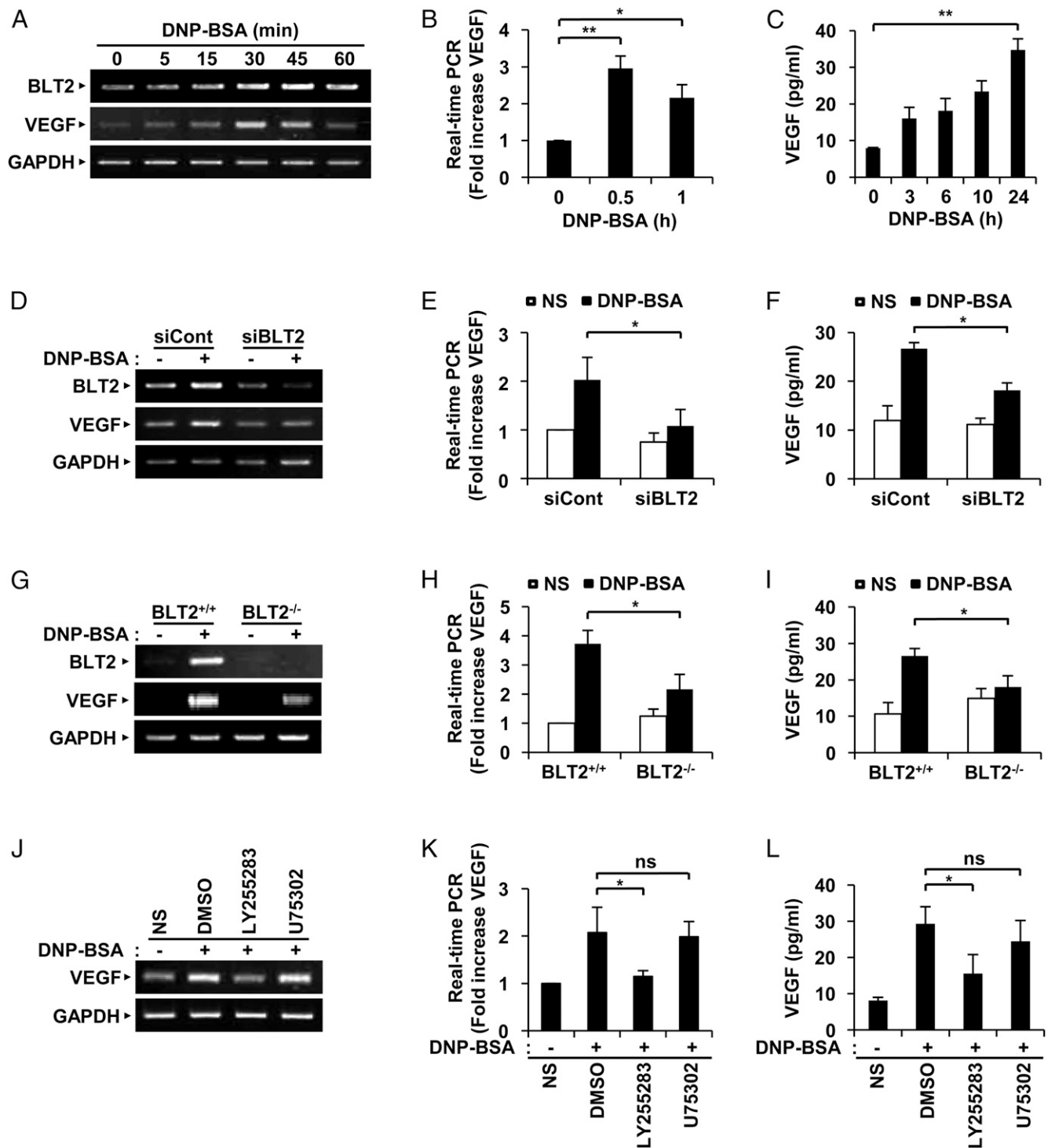


FIGURE 1. Ag-induced VEGF production is dependent on BLT2 in BMMCs. (**A–C**) BMMCs were sensitized with DNP-specific IgE and were stimulated with DNP-BSA (100 ng/ml) for the indicated time periods, after which total cellular RNA was isolated and transcript levels were assessed by semi-quantitative RT-PCR (**A**) and real-time PCR (**B**). VEGF secreted in the culture medium was detected using a specific ELISA (**C**). (**D–F**) BMMCs were transfected with 50 nM control (siCont) or BLT2 siRNA (siBLT2). After 24 h, cells were sensitized with DNP-specific IgE and were then stimulated with DNP-BSA for 30 min (**D** and **E**) or 24 h (**F**). Thereafter, total cellular RNA was isolated, and transcript levels of BLT2 and VEGF were assessed by semi-quantitative RT-PCR (**D**) and real-time PCR (**E**). Additionally, cell-free supernatants were collected and subjected to ELISA analysis for VEGF (**F**). (**G–I**) WT (BLT2^{+/+}) and BLT2-null (BLT2^{-/-}) BMMCs were sensitized with DNP-specific IgE and were stimulated with DNP-BSA for 30 min. Total cellular RNA was then isolated, and transcript levels were assessed by semi-quantitative RT-PCR (**G**) and real-time PCR (**H**). Cell-free supernatants were collected and subjected to ELISA to measure secreted VEGF after 24 h (**I**). (**J** and **K**) BMMCs were first incubated with LY255283 (10 μ M), U75302 (1 μ M), or DMSO (vehicle of inhibitors) for 30 min and then stimulated with DNP-BSA (100 ng/ml) for 30 min, after which total RNA was isolated and subjected to semi-quantitative RT-PCR (**J**) and real-time PCR (**K**). (**L**) Culture supernatants of BMMCs treated as shown in (**J**) were stimulated with DNP-BSA (100 ng/ml) for 24 h to induce VEGF secretion. All quantitative data are the means $\pm$ SD of three independent experiments. * p < 0.05, ** p < 0.01. ns, not significant.

Statistical analysis

Data are presented as the means $\pm$ SD and were analyzed by Student *t* tests and one-way ANOVA for comparisons between two or more groups, respectively. A *p* value <0.05 was considered statistically significant.

Results

BLT2 plays a critical role in VEGF production in allergen-stimulated BMMCs

In accordance with previous reports (16, 17), we observed significant upregulation of VEGF production in Ag-stimulated BMMCs, as indicated by mRNA and protein levels (Fig. 1A–C). Additionally, consistent with our previous observations (9), BLT2 expression was markedly increased in Ag-stimulated BMMCs (Fig. 1A). We then examined whether upregulated BLT2 plays a mediating role in the production of VEGF in Ag-induced BMMCs. For these experiments, we transiently transfected BMMCs with a specific BLT2 siRNA, which resulted in a considerable reduction of Ag-induced VEGF production at both the transcript and protein levels (Fig. 1D–F). However, BLT1 siRNA had no effect on Ag-induced VEGF production (Supplemental Fig. 2). Additionally, we generated BLT2-null mice and isolated BLT2^{-/-} BMMCs from these mice. Ag-induced VEGF production was again considerably attenuated in the BLT2^{-/-} BMMCs compared with cells isolated from WT mice (Fig. 1G–I). Similarly, the BLT2 antagonist LY255283 (11, 12) greatly attenuated Ag-induced VEGF production at both the transcript and protein levels, whereas the BLT1 antagonist U75302 (11, 12) had no effect (Fig. 1J–L). Taken together, these data suggest that BLT2, but not BLT1, is implicated in VEGF production in allergen-stimulated BMMCs.

The synthesis of BLT2 ligands is required for VEGF production in allergen-stimulated BMMCs

Next, we examined the potential roles of BLT2 ligands [LTB₄ and 12(S)-HETE] in VEGF production in Ag-activated BMMCs. Pretreatment of cells with the 5-LO inhibitor AA861 or the 12-LO inhibitor baicalein clearly suppressed VEGF production (Fig. 2A–C). The cytosolic phospholipase A₂ (cPLA₂) inhibitor AACOCF₃ also attenuated Ag-induced VEGF synthesis (Fig. 2A–C). These results suggest that cPLA₂, 5-LO, and 12-LO, which are necessary for the synthesis of BLT2 ligands, are involved in VEGF production in BMMCs. We next examined the direct effect of BLT2 ligands on VEGF production. We isolated BMMCs from WT or BLT2-overexpressing Tg mice and analyzed VEGF levels after incubation with the BLT2 ligands LTB₄ and 12(S)-HETE. VEGF levels were clearly enhanced by treatment with BLT2 ligands in BMMCs derived from BLT2 Tg mice compared with cells derived from WT mice (Fig. 2D–F). Taken together, these results suggest that BLT2 ligands [LTB₄ and 12(S)-HETE], which are produced via the consecutive actions of cPLA₂ and either 5-LO or 12-LO, are required for VEGF production in allergen-stimulated BMMCs.

The Nox1-ROS cascade is downstream of BLT2 in signaling to VEGF production in allergen-stimulated BMMCs

ROS have been reported to be involved in the production of VEGF in vitro and in vivo (18). Additionally, VEGF levels are decreased by antioxidants in murine asthma models (19). Furthermore, we recently determined that BLT2 upregulates ROS generation via Nox1 activation in Ag-stimulated BMMCs (9). Thus, we hypothesized that the mechanism of BLT2-mediated VEGF production

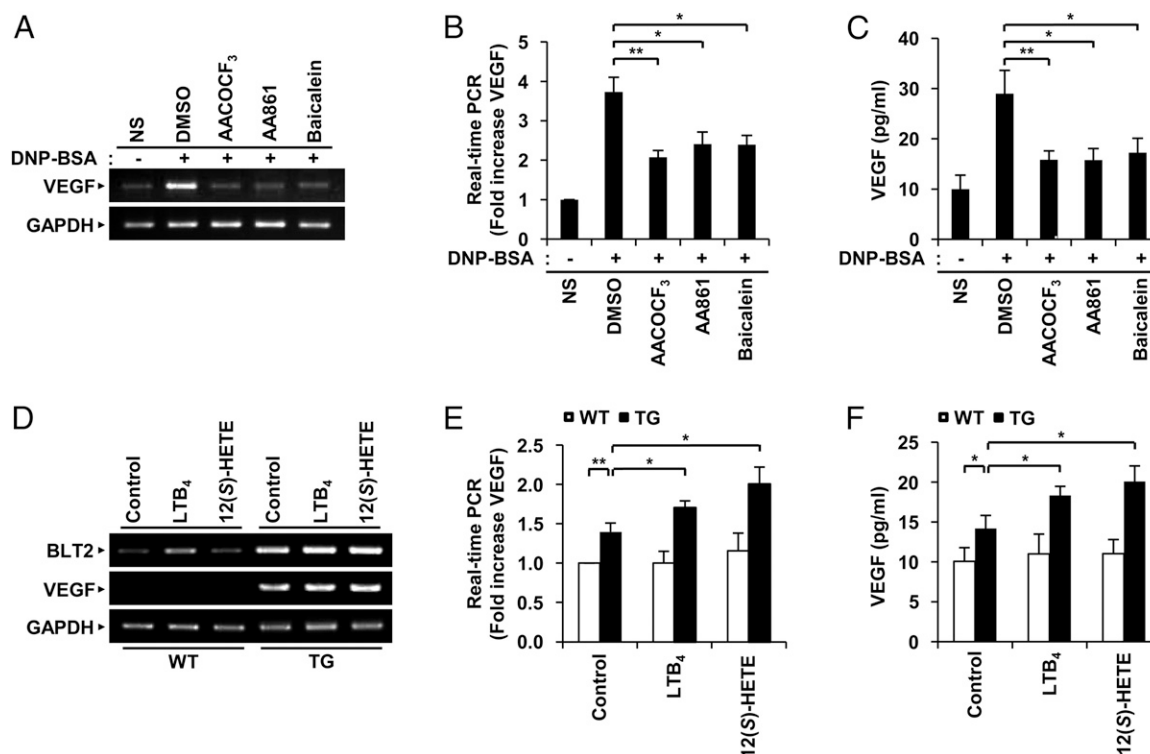


FIGURE 2. Synthesis of BLT2 ligands is necessary for VEGF production in allergen-stimulated BMMCs. (A and B) BMMCs were first incubated with AACOCF₃ (20 μ M), AA861 (10 μ M), baicalein (10 μ M), or DMSO for 30 min and then stimulated with DNP-BSA (100 ng/ml) for 30 min, after which total RNA was isolated and subjected to semiquantitative RT-PCR (A) and real-time PCR (B). (C) Culture supernatants of BMMCs treated as shown in (A) were stimulated for 24 h to induce VEGF secretion. (D and E) WT BMMCs or BLT2 Tg BMMCs were incubated with 300 nM each LTB₄ and 12(S)-HETE for 30 min. These cells were harvested to detect VEGF transcripts by semiquantitative RT-PCR (D) and real-time PCR (E) with specific primers. (F) WT BMMCs or Tg BMMCs were incubated for 24 h with BLT2 ligands as shown in (D), after which released VEGF was assayed. All quantitative data are means $\pm$ SD of three independent experiments. **p* < 0.05 , ***p* < 0.01 .

involves a Nox1–ROS cascade in Ag-stimulated BMMCs. We therefore examined the effects of DPI, an inhibitor of Nox-like flavoproteins, and NAC, a free radical scavenger, on Ag-induced VEGF synthesis. As shown in Fig. 3A–C, DPI or NAC significantly inhibited the production of VEGF in Ag-stimulated BMMCs, suggesting that ROS-generating flavoproteins such as Nox may be involved in signaling events for VEGF production. Consistent with this conclusion, Ag-induced VEGF production was dramatically inhibited by Nox1 siRNA (Fig. 3D–F). To further examine the role of Nox1 downstream of the BLT2-linked cascade, we transiently transfected BMMCs with Nox1 siRNA in BMMCs isolated from BLT2 Tg mice and analyzed VEGF levels. When Nox1 was knocked down by siRNA, VEGF production was significantly reduced in BMMCs isolated from BLT2 Tg mice (Fig. 3G–I), suggesting that the Nox1–ROS cascade lies downstream of BLT2 in signaling to VEGF synthesis in BMMCs.

NF- κ B activation is downstream of BLT2 in signaling to VEGF production in allergen-stimulated BMMCs

ROS has been reported to regulate the activity of NF- κ B (20), a principal transcription factor involved in VEGF expression (21–23). Additionally, we recently reported that BLT2 regulates NF- κ B activation in human mast cells (11). We therefore examined the involvement of NF- κ B in VEGF production downstream of the

BLT2–Nox1–ROS cascade in BMMCs. First, we examined the effects of the NF- κ B inhibitor Bay11-7082 on Ag-induced VEGF synthesis. As shown in Fig. 4A–C, VEGF production was greatly inhibited by pretreatment with Bay11-7082 in Ag-stimulated BMMCs. Similarly, the enhanced production of VEGF in BMMCs isolated from BLT2 Tg mice was also greatly inhibited by Bay11-7082 (Fig. 4D–F). Additionally, BLT2 knockdown resulted in marked attenuation of Ag-induced NF- κ B activation, as indicated by reduced levels of I κ B α phosphorylation (Fig. 4G). Similar results were obtained for BMMCs derived from BLT2-null mice (Fig. 4H). Pretreatment with LY255283 also resulted in marked attenuation of Ag-induced NF- κ B activation in BMMCs (Fig. 4I). Finally, Nox1 knockdown also markedly inhibited NF- κ B activation in BLT2 Tg BMMCs (Fig. 4J). Taken together, these results indicate that VEGF production in BMMCs mainly occurs through a BLT2–Nox1–ROS–NF- κ B-dependent signaling pathway.

BLT2 in mast cells is required for the expression of VEGF and airway inflammation in the OVA-induced asthma model

To determine whether BLT2 expression in mast cells plays a role in VEGF production in vivo, we used genetically mast cell-deficient Kit(W/W^v) and WT OVA-induced asthma model mice. First, we observed that BLT2 levels were markedly elevated in the lungs of OVA-sensitized and challenged mice compared with normal controls

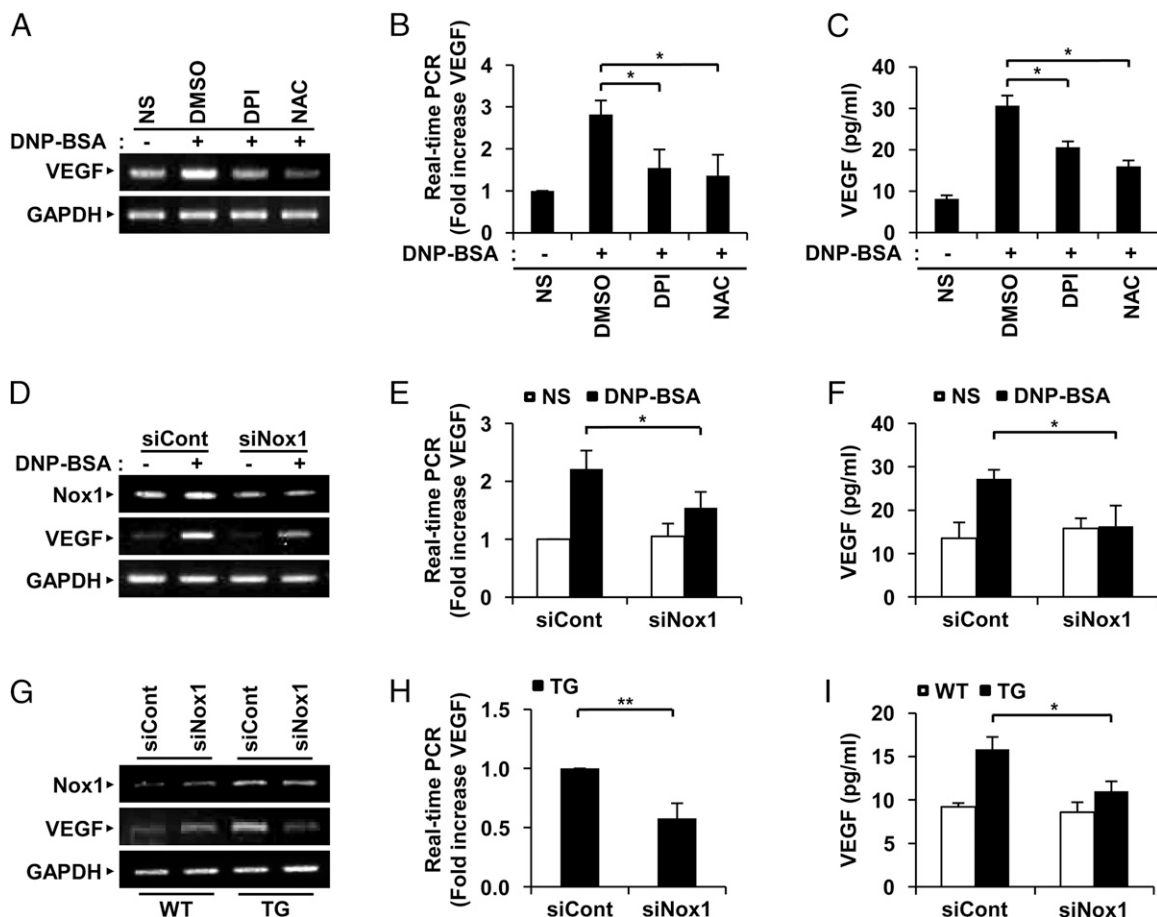


FIGURE 3. The Nox1–ROS cascade lies downstream of BLT2 in signaling to VEGF production in allergen-stimulated BMMCs. (**A** and **B**) Cells were pretreated for 30 min with DPI (1 μ M), NAC (1 mM), or DMSO and were then stimulated for 30 min with DNP-BSA (100 ng/ml), followed by analysis of total RNA. (**C**) Cells were exposed as shown in (**A**) and incubated with DNP-BSA for 24 h. VEGF release into the culture medium was then assayed. (**D** and **E**) Cells were transfected with 50 nM Nox1 or control siRNAs, incubated for 24 h, and then incubated with DNP-BSA for 30 min. Total RNA was then analyzed. (**F**) Cells were transfected and incubated as shown in (**D**) and stimulated with DNP-BSA for 24 h, followed by assays of VEGF release. (**G** and **H**) WT BMMCs or Tg BMMCs were transfected with Nox1 or control siRNAs and then incubated for 24 h. The cells were then harvested for the detection of VEGF transcripts. (**I**) Cells were transfected as shown in (**G**) and incubated for 24 h before assaying VEGF release into the culture medium. All quantitative data are mean $\pm$ SD values of three independent experiments. * p < 0.05, ** p < 0.01.

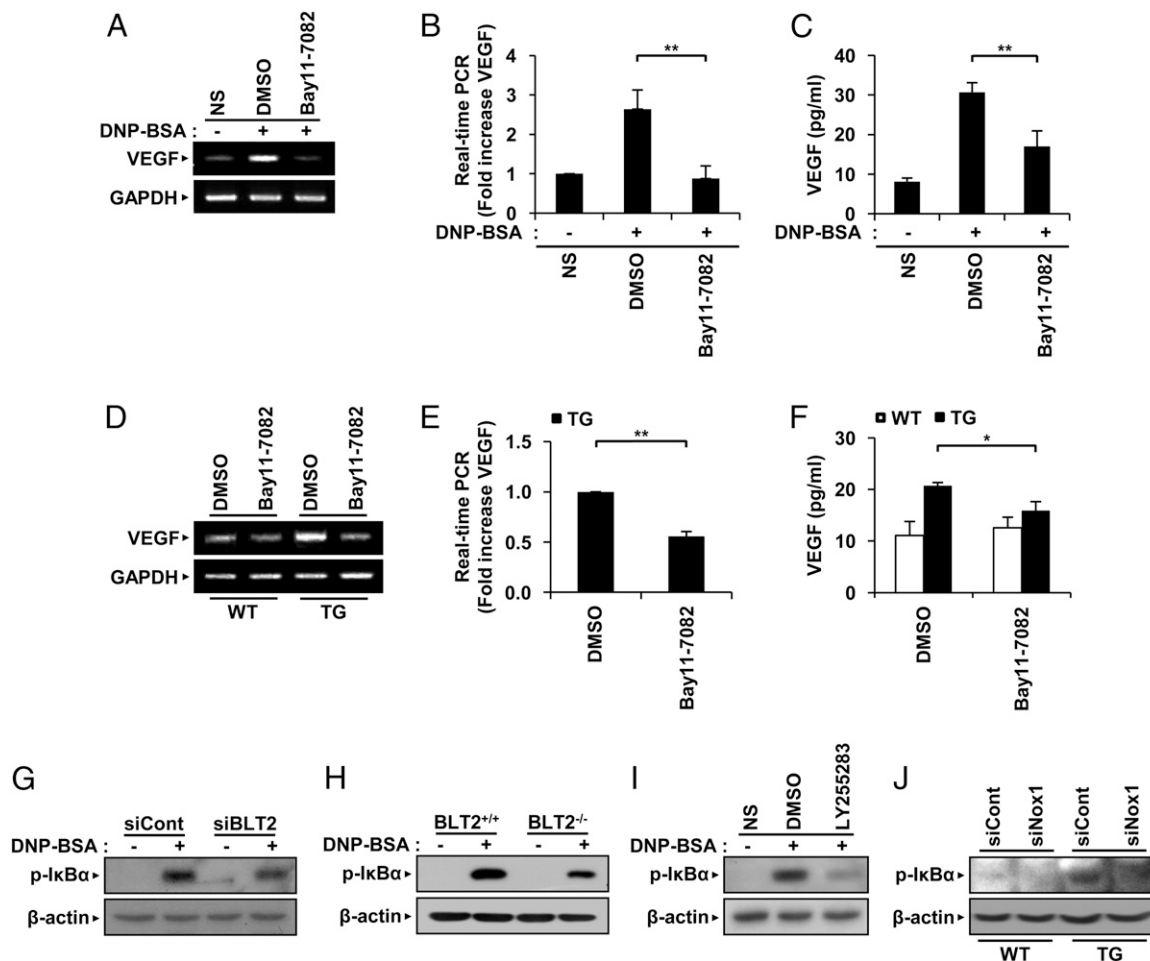


FIGURE 4. NF- κ B mediates VEGF production downstream of the BLT2-Nox1-ROS cascade in allergen-stimulated BMMCs. (A–C) Cells were first incubated with Bay11-7082 (10 μ M) or DMSO for 30 min and then stimulated for 30 min (A and B) or 24 h (C) with DNP-BSA (100 ng/ml). Total RNA was then isolated for VEGF transcript analysis, or VEGF released into the culture medium was assayed. (D) WT BMMCs or Tg BMMCs were incubated for 30 min with Bay11-7082 or DMSO, after which total RNA was isolated. The transcript levels of VEGF were then assessed with specific primers. (E) Tg BMMCs were incubated with Bay11-7082 or DMSO for 30 min. Thereafter, total cellular RNA was isolated and converted into cDNA, and real-time PCR was performed. (F) WT BMMCs or Tg BMMCs were incubated for 24 h with inhibitors as shown in (D), after which cultured supernatants were collected and subjected to ELISA to measure secreted VEGF. All quantitative data are the mean $\pm$ SD of three independent experiments. * p < 0.05, ** p < 0.01. (G) BMMCs were transfected with BLT2 or control siRNAs, incubated for 24 h, and stimulated with DNP-BSA for 5 min, after which the cell lysates were subjected to immunoblot analysis with Abs to the indicated proteins. (H) WT (BLT2^{+/+}) and BLT2-null (BLT2^{-/-}) BMMCs were incubated with DNP-BSA for 5 min, after which the cell lysates were subjected to immunoblot analysis as shown in (G). (I) BMMCs were first incubated with LY255283 (10 μ M) or DMSO for 30 min and then stimulated with DNP-BSA for 5 min, after which the cell lysates were subjected to immunoblot analysis as shown in (G). (J) WT BMMCs or Tg BMMCs were transfected with Nox1 or control siRNAs and incubated for 24 h; cell lysates were then prepared and subjected to immunoblot analysis. The results shown are representative of three independent experiments with similar results.

(Fig. 5A). We also compared BLT2 levels in WT mice, Kit(W/Wv) mice, WT mast cell-reconstituted Kit(W/Wv) mice (referred to hereafter as WT BMMC $\rightarrow$ W/Wv mice), and BLT2^{-/-} mast cell-reconstituted Kit(W/Wv) mice (referred to hereafter as BLT2^{-/-} BMMC $\rightarrow$ W/Wv mice) in the OVA-induced asthma group (Fig. 5A). BLT2 levels were significantly lower in the lung tissues of the OVA-sensitized and challenged W/Wv or W/Wv mice reconstituted with BLT2^{-/-} BMMCs than in the OVA-sensitized and challenged WT or W/Wv mice reconstituted with WT BMMCs (Fig. 5A).

Moreover, VEGF levels in the lung tissues and BALF were much higher in the OVA-sensitized and challenged mice than in the normal control group (Fig. 5B, 5C). VEGF levels were significantly lower in the genetically mast cell-deficient Kit(W/Wv) mice than in the WT mice in the OVA-induced asthma model (Fig. 5B, 5C), suggesting a role of mast cells in the synthesis of VEGF in OVA-induced allergic asthma. Interestingly, VEGF production levels were restored to those observed in WT mice

after adoptive transfer of WT BMMCs into Kit(W/Wv) mice, whereas VEGF production was not restored in BLT2^{-/-} BMMC-reconstituted Kit(W/Wv) mice in the OVA-induced asthma model (Fig. 5B, 5C).

Additionally, histologic analysis by H&E staining after formalin fixation revealed typical pathologic features of allergic airway inflammation as well as thickening and narrowing of the airway lumen in the OVA-sensitized and challenged WT or W/Wv mice reconstituted with WT BMMCs (Fig. 5D). In contrast, no significant changes in airway wall and lumen thickness were observed among the saline-treated control mice and the OVA-sensitized and challenged W/Wv mice or W/Wv mice reconstituted with BLT2^{-/-} BMMCs (Fig. 5D). Additionally, the degree of lung inflammation was evaluated on a subject scale from 0 to 3, and we found that the inflammation scores were significantly increased in OVA-sensitized and challenged WT or W/Wv mice reconstituted with WT BMMCs (Fig. 5E). In contrast, the inflammation scores of OVA-sensitized

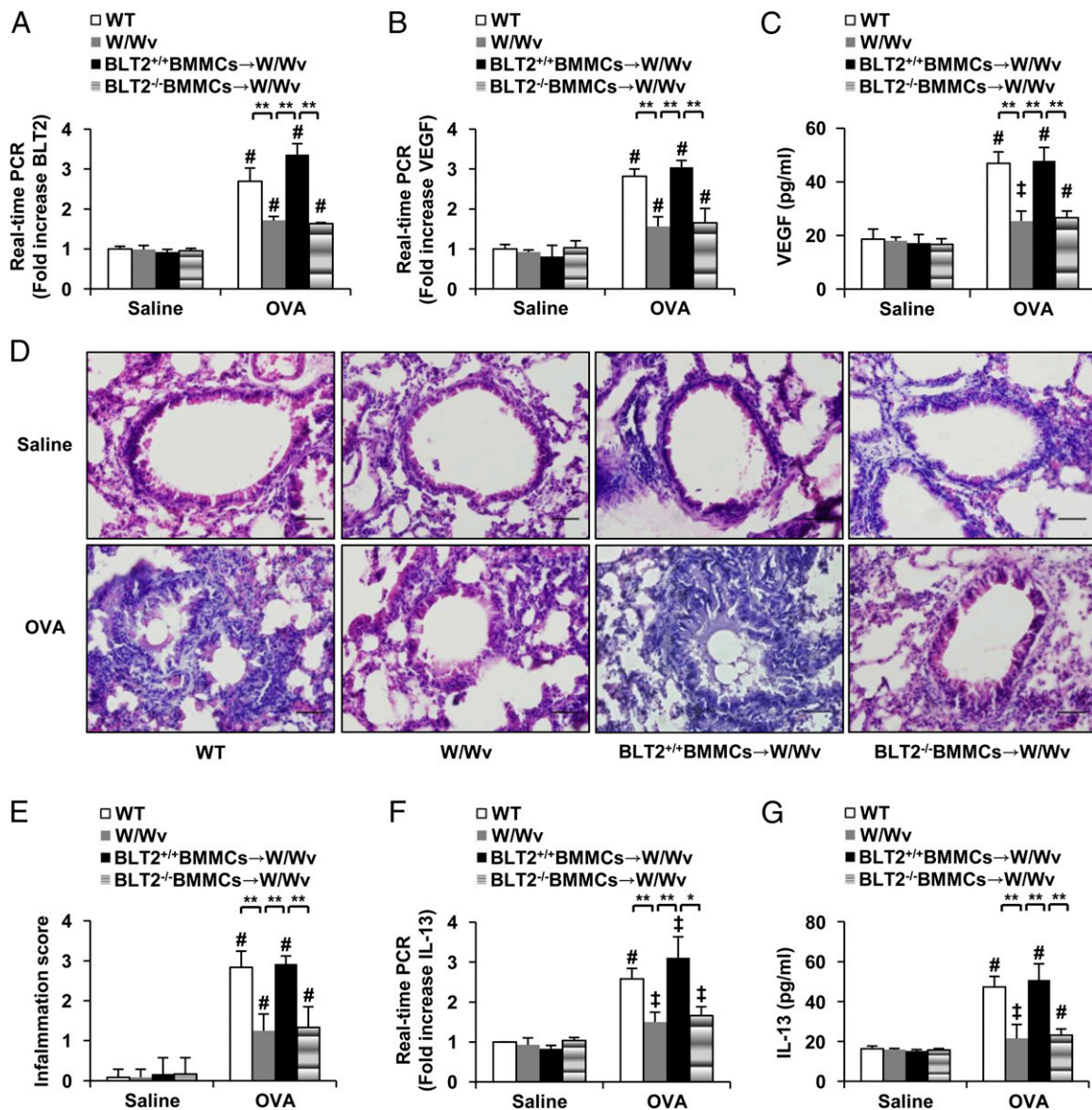


FIGURE 5. BLT2 expression in mast cells is crucial for VEGF production and airway inflammation in the OVA-induced asthma model. OVA-induced mice were sacrificed on day 25 to assess asthmatic phenotypes. (**A** and **B**) The lungs of the mice were homogenized, and RNA was isolated and converted to cDNA. Real-time PCR was performed using BLT2- or VEGF-specific primers. (**C**) VEGF levels in BALF were analyzed using a specific ELISA. Data are the mean $\pm$ SD ($n = 4-5$ per group). ** $p < 0.01$ versus the group indicated. † $p < 0.05$, # $p < 0.01$ versus the corresponding normal saline group. (**D**) Airway inflammation in lung tissues was analyzed by histologic analysis. Lungs were excised, fixed, and stained with H&E. ($n = 4-5$ per group). Scale bars, 50 μ m. (**E**) Peribronchial and perivascular lung inflammation was measured, and total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. ** $p < 0.01$ versus the group indicated; # $p < 0.01$ versus the corresponding normal saline group. (**F**) The transcript levels of IL-13 in lung tissue were analyzed by real-time PCR. (**G**) IL-13 levels in BALF were analyzed using a specific ELISA ($n = 4-5$ per group). * $p < 0.05$, ** $p < 0.01$ versus group indicated. † $p < 0.05$, # $p < 0.01$ versus corresponding normal saline group.

and challenged W/Wv or W/Wv mice reconstituted with BLT2^{-/-} BMMCs were significantly decreased (Fig. 5E).

Interestingly, we observed that IL-13 levels were much lower in W/Wv mice than in the normal controls but were restored to levels similar to those in WT mice after adoptive transfer of WT BMMCs into W/Wv mice. In contrast, IL-13 production was not restored in OVA-sensitized and challenged W/Wv mice reconstituted with BLT2^{-/-} BMMCs (Fig. 5F, 5G). These results suggest that the production of IL-13 in lung tissues and BALF may occur through a BLT2-dependent cascade in BMMCs in OVA-induced allergic asthma. Indeed, we recently reported that BLT2 plays a role in IL-13 synthesis in allergen-activated mast cells (9). Taken together, these results suggest that BLT2 expression in mast cells plays a

pivotal role in the production of VEGF and potentially IL-13 in OVA-induced asthma, thus causing airway inflammation.

IL-13 production may occur downstream of the BLT2-VEGF cascade

Next, we investigated whether IL-13 production occurs downstream of VEGF. Previous studies have reported that VEGF enhances Th2 cytokine-mediated inflammation in a murine model (24, 25). Thus, we hypothesized that the BLT2-mediated increase in VEGF triggers the synthesis of IL-13. We first compared VEGF levels in BALF of WT and Tg mice. As shown in Fig. 6A, VEGF levels were higher in BALF of Tg mice compared with WT mice. Elevated IL-13 production was completely lost following administration of

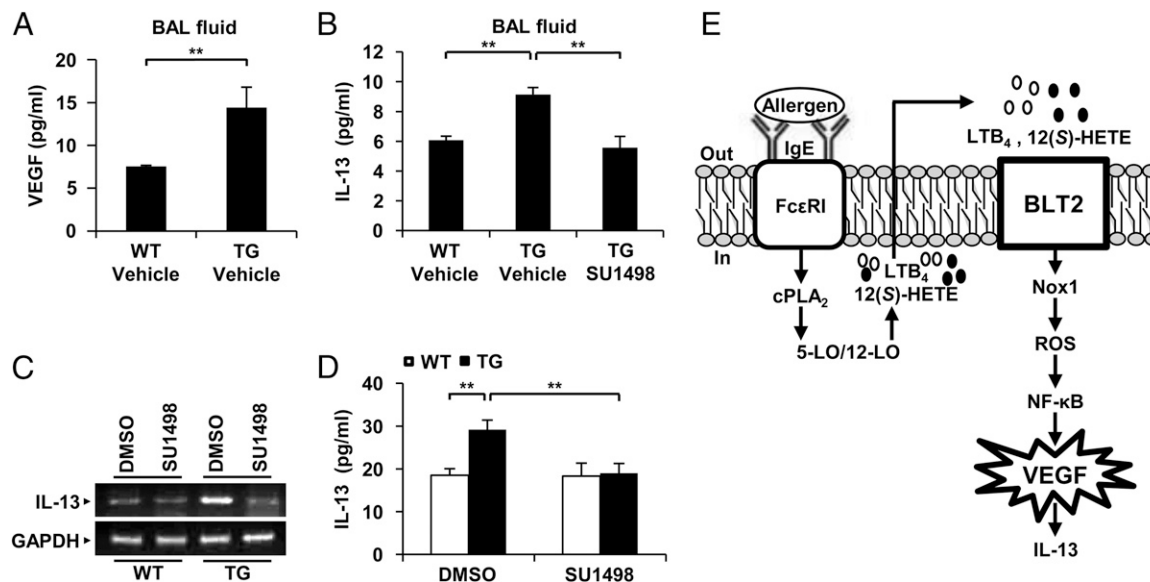


FIGURE 6. The BLT2-VEGF cascade may mediate IL-13 production. (A and B) WT mice or BLT2 Tg mice received vehicle (saline containing DMSO) or SU1498 (10 mg/kg) i.p. twice at 24-h intervals. The mice were sacrificed 1 h after the second administration. BALF was collected to measure VEGF content in the WT/vehicle- and Tg/vehicle-administered mice (A) and to analyze IL-13 content in the WT/vehicle-, Tg/vehicle-, and Tg/SU1498-administered mice (B). Data are the mean $\pm$ SD ($n = 4$ per group). $**p < 0.01$. (C) WT BMMCs and Tg BMMCs were incubated with DMSO or SU1498 (20 μ M) for 30 min, followed by isolation of total RNA and measurement of IL-13 transcript levels. The results shown are representative of three independent experiments with similar results. (D) WT BMMCs and Tg BMMCs were incubated with inhibitors as shown in (C) for 24 h, followed by collection of the culture supernatants and ELISA analysis of secreted IL-13 levels. All quantitative data are the mean $\pm$ SD of three independent experiments. $**p < 0.01$. (E) Scheme of intracellular signaling responsible for Ag-induced VEGF synthesis in mast cells.

SU1498, an inhibitor of VEGF receptor tyrosine kinase, in Tg mice (Fig. 6B). Next, to determine whether enhanced VEGF generated via a BLT2-linked signaling pathway upregulates IL-13 synthesis in mast cells, we compared BMMCs drawn from WT and Tg mice. As shown in Fig. 6C and 6D, the enhanced levels of IL-13 were significantly attenuated by SU1498 in BMMCs from BLT2 Tg mice. These results suggest that the BLT2-VEGF cascade may be associated with IL-13 synthesis in mast cells.

Discussion

In the present study, we demonstrated that Ag-stimulated VEGF production occurs through a BLT2-dependent pathway in BMMCs. We also determined that a Nox1-ROS-NF- κ B-linked cascade is downstream of BLT2 in Ag signaling to VEGF production in BMMCs. Moreover, BMMCs derived from BLT2 Tg mice produced VEGF even in the absence of Ag stimulation, and adoptive transfer of BMMCs derived from normal mice into genetically mast cell-deficient W/Wv mice restored the production of VEGF in OVA-induced animal models, whereas adoptive transfer of BMMCs from BLT2^{-/-} mice did not. Taken together, our results suggest that BLT2 expression in mast cells plays a critical role in the production of VEGF in allergen-induced allergic responses. Additionally, our results suggest that the VEGF produced may induce the synthesis of IL-13 in BMMCs, thus potentially contributing to allergic airway inflammation.

VEGF mediates the initiation and development of allergic asthma (4, 5), and VEGF levels are significantly increased in the airway mucosa and sputum of asthmatic patients (26, 27). Despite the critical roles of VEGF in asthma, the signaling mechanism of VEGF synthesis in Ag-stimulated allergic asthma has not been explored. The results of the present study clearly suggest that VEGF synthesis is largely mediated in a BLT2-dependent manner in allergen-activated mast cells (Fig. 1D-L). Additionally, we demonstrated that BLT2 in mast cells affects VEGF synthesis processes in OVA-induced asthma models using genetically mast

cell-deficient mice (W/Wv) (Fig. 5B, 5C). For example, VEGF levels were significantly lower in OVA-induced W/Wv mice than in WT mice with OVA inhalation (Fig. 5B, 5C). However, the introduction of WT BMMCs in W/Wv mice restored VEGF levels to levels similar to those in WT mice, resulting in airway inflammation (Fig. 5B, 5C). In contrast to WT BMMC $\rightarrow$ W/Wv mice, BLT2^{-/-} BMMC $\rightarrow$ W/Wv mice did not exhibit a significant increase in VEGF production following OVA inhalation (Fig. 5B, 5C). Taken together, these findings suggest that BLT2 in mast cells is critical for VEGF synthesis and may orchestrate airway inflammation.

We also demonstrated that the synthesis of the BLT2 ligands LTB₄ and 12(S)-HETE is essential for VEGF production in Ag-stimulated mast cells, indicating that BLT2 ligands may act in an autocrine or paracrine manner through BLT2 as shown in the hypothetical model (Fig. 6E). Consistent with the proposed models of BLT2 activities during VEGF production, BLT2 overexpression alone significantly increased VEGF production, and the addition of BLT2 ligands [LTB₄ and 12(S)-HETE] further enhanced VEGF production even in the absence of Ag stimulation (Fig. 2D-F). In accordance with the suggested role of BLT2 ligands in VEGF synthesis, a number of recent studies have suggested that eicosanoids, lipid metabolites derived from cyclooxygenase and LO metabolism of cPLA₂-released arachidonic acid, are closely associated with VEGF synthesis in various cells (28-30). For instance, cysteinyl leukotrienes induce VEGF production in human monocytes and bronchial smooth muscle cells (28). Additionally, 5-LO, which catalyzes LTB₄ synthesis, mediates the synthesis of VEGF in malignant mesothelial cells (30), and 12-LO, which catalyzes 12(S)-HETE synthesis, contributes to VEGF production in prostate cancer cells (29) in accordance with the role of BLT2 in VEGF production proposed in the present study.

Earlier studies have shown that ROS mediates the production of VEGF in vitro and in vivo (18). We recently demonstrated that BLT2 upregulates ROS generation via Nox1 activation in Ag-stimulated

mast cells (9). Similarly, we found that this Nox1–ROS cascade plays a role in BLT2-mediated VEGF production in BMMCs (Fig. 3G–I) and that NF- κ B is downstream of the Nox1–ROS cascade in BLT2 signaling for VEGF production (Fig. 4J). Consistent with our findings, a recent study demonstrated that ROS generation in asthma models leads to the activation of the redox-sensitive transcription factor NF- κ B (20), one of the principal transcription factors of VEGF expression (21–23). We also previously reported that BLT2 regulates NF- κ B activation in human mast cells (11). Recently, it was reported that VEGF production is regulated by the PI3K–AKT pathway in OVA-induced asthma models (4). Thus, we investigated whether the BLT2-dependent cascade is associated with the PI3K–AKT pathway in VEGF synthesis in Ag-stimulated BMMCs. We found that BLT2 blockade had no effect on PI3K–AKT activation (Supplemental Fig. 3), thus implying that the BLT2-linked cascade is independent of the PI3K–AKT cascade during VEGF synthesis. Further studies are necessary to elucidate how the PI3K–AKT pathway is related to our BLT2-linked pathway with respect to VEGF production in BMMCs.

Most studies have suggested that VEGF regulates angiogenesis and vascular permeability, thus contributing to asthmatic pathogenesis (4, 31–33). Interestingly, recent studies have shown that VEGF also induces Th2 cytokine-mediated lung inflammation in vivo (24, 25). Similarly, we observed that VEGF-stimulated BMMCs upregulate IL-13 production (data not shown). Our results clearly demonstrate that IL-13 levels are elevated in BLT2 Tg mice compared with control mice and that this elevated level of IL-13 production is completely attenuated by the blockade of VEGF receptors via SU1498 (Fig. 6B). Additionally, we observed that IL-13 levels were significantly lower in W/W^v mice than in OVA-induced WT mice after OVA inhalation (Fig. 5F, 5G). Moreover, the reconstitution of WT BMMCs in W/W^v mice resulted in an increase in IL-13 similar to that observed in WT mice (Fig. 5F, 5G), thus potentially mediating allergic asthma. In contrast, no significant change in IL-13 production was observed in BLT2^{-/-} BMMC→W/W^v mice after OVA inhalation (Fig. 5F, 5G). Additionally, we evaluated BMMCs isolated from WT and Tg mice. We observed that the enhanced IL-13 levels in BMMCs from BLT2 Tg mice were significantly attenuated by SU1498 (Fig. 6C, 6D), supporting the regulation of IL-13 production by VEGF in mast cells. Clearly, further studies are needed to elucidate the detailed mechanism by which BLT2–VEGF contributes to IL-13 production in allergen-induced asthma.

Our results differ from those of Matsunaga et al. (34), who found that BLT2 negatively regulates allergen-induced pulmonary responses. The discrepancy between the Matsunaga et al. study and the findings of the present study may result from the different cell types focused on in each study; we focused on mast cells to study the role of BLT2 in the production of VEGF and Th2 cytokine, whereas the Matsunaga group focused on CD4⁺ T cells to study the role of BLT2 in allergic eosinophilia. Additionally, the disparate results may be due to the different experimental conditions used in the two studies. Because of these discrepancies, further studies are clearly necessary to reveal the precise role of BLT2 in asthmatic diseases.

In summary, our study shows that BLT2 plays a critical role in Ag-induced VEGF production in mast cells via a Nox1–ROS–NF- κ B-linked cascade, thus potentially contributing to allergic asthma. Our results contribute to a better understanding of the signaling mechanism for the production of VEGF in mast cells.

Disclosures

The authors have no financial conflicts of interest.

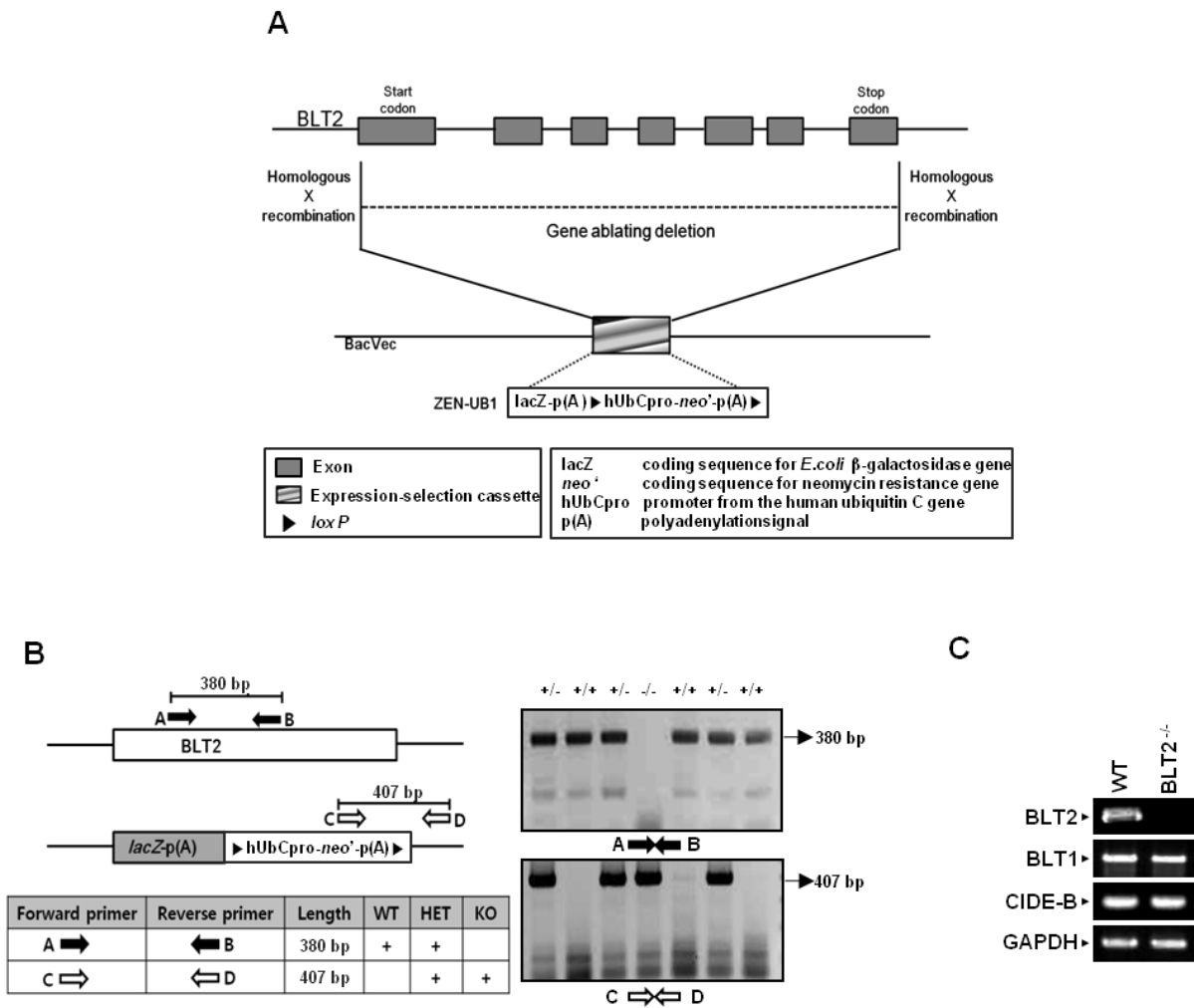
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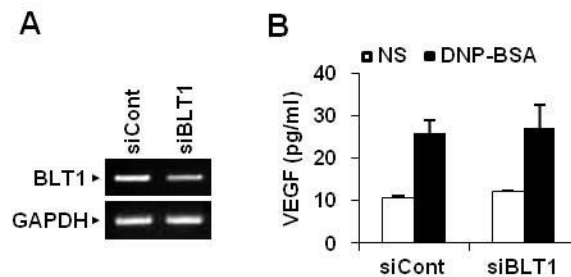
Supplemental Data

Supplemental Figure S1



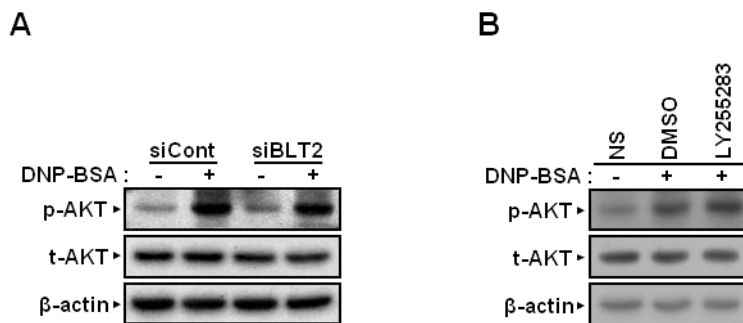
Supplemental Figure S1. Targeted disruption of the BLT2 gene in mice. (A) Schematic representation of the mouse BLT2 gene and the bacterial artificial chromosome (BAC)-based targeting vector. Homologous recombination results in replacement of the entire BLT2 gene with the ZEN-UB1 cassette, which contains the coding sequences for the LacZ and neomycin resistance genes, of the vector. (B) The PCR primer pairs A-B and C-D amplify 380- and 407-bp fragments from the WT and targeted BLT2 alleles, respectively. Genomic DNA extracted from mouse toe was analyzed by PCR with the two pairs of primers for genotyping of WT (+/+), heterozygous (HET, +/-), and homozygous mutant (KO, -/-) mice. (C) Disruption of the BLT2 gene did not affect expression of the BLT1 or CIDE-B genes. Semiquantitative RT-PCR analysis of BLT2, BLT1, CIDE-B, and GAPDH mRNAs in BMMCs-derived from BLT2^{+/+} (WT) mice and BLT2^{-/-} mice.

Supplemental Figure S2



Supplemental Figure S2. BLT1 has no effect on VEGF production in Ag-stimulated BMMCs. (A-B) BMMCs were transfected with 50 nM control (siCont) or BLT1 siRNA (siBLT1). After 24 h, total cellular RNA was isolated, and transcript levels of BLT1 was assessed by semiquantitative RT-PCR (A). Control siRNA- or BLT1 siRNA-introduced cells were sensitized with DNP-specific IgE and were then stimulated with DNP-BSA for 24 h. Thereafter, cell-free supernatants were collected and subjected to ELISA analysis for VEGF (B).

Supplemental Figure S3



Supplemental Figure S3. BLT2 has no effect on PI3K-AKT activation in Ag-stimulated BMMCs. (A) BMMCs were transfected with 50 nM control (siCont) or BLT2 siRNA (siBLT2). After 24 h, cells were sensitized with DNP-specific IgE and were then stimulated with DNP-BSA for 5 min, after which the cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins. (B) BMMCs were first incubated with LY255283 (10 μM) or DMSO for 30 min and then stimulated with DNP-BSA for 5 min, after which the cell lysates were subjected to immunoblot analysis as shown in panel (A).