

Leukotriene B₄ Receptor 1 Expression on Dendritic Cells Is Required for the Development of Th2 Responses and Allergen-Induced Airway Hyperresponsiveness^{1,2}

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Dendritic cells (DC) are important APCs that control allergen-induced airway responses by interacting directly with T cells. Leukotriene B₄ (LTB₄), interacting with its high-affinity receptor, LTB₄ receptor 1 (BLT1), is known to attract and activate leukocytes during inflammation. We have previously shown that BLT1 expression on Ag-primed T cells is required for the development of airway hyperresponsiveness (AHR; Miyahara et al. 2005. *Am. J. Respir. Crit. Care Med.* 172: 161–167). However, the role for the LTB₄-BLT1 pathway in DC function in allergen-induced airway responses has not been defined. Bone marrow-derived DCs (BMDC) were generated. Naive BALB/c mice received OVA-pulsed BLT1-deficient (BLT1^{-/-}) BMDCs or wild-type BMDCs intratracheally and were then challenged with OVA for 3 days. Airway responses were monitored 48 h after the last allergen challenge. BLT1^{-/-} BMDCs showed normal maturation judged from surface expression of CD markers. Compared with recipients of wild-type BMDCs, mice that received BLT1^{-/-} BMDCs developed significantly lower AHR to inhaled methacholine, lower goblet cell metaplasia, and eosinophilic infiltration in the airways and decreased levels of Th2 type cytokines in the bronchoalveolar lavage fluid. Migration of BLT1^{-/-} BMDCs into peribronchial lymph nodes was significantly impaired compared with BLT1^{+/+} BMDCs after intratracheal instillation. These data suggest that BLT1 expression on DCs is required for migration of DCs to regional lymph nodes as well as in the development of AHR and airway inflammation. *The Journal of Immunology*, 2008, 181: 1170–1178.

Asthma is characterized by persistent airway inflammation and airway hyperresponsiveness (AHR).⁵ Allergen-specific memory T cells that secrete Th2-type cytokines such as IL-4, IL-5, and IL-13 are thought to play a central role in the development of the response (1, 2). A critical step in the induction of T cell activation is the uptake, processing, and presentation of Ag by APCs. Among the different types of APCs, dendritic cells (DC) play a key role for the initiation

of immune responses in the airways. DCs recognize and take up Ag in the peripheral tissues, and process them. After Ag uptake and processing, DCs mature and migrate to secondary lymphoid organs via the activation of chemotactic receptors (3, 4). DCs then present processed peptides to the surface bound MHC molecules, which are recognized by T cells (5, 6), initiating T cell priming. It has been reported that extracellular stimuli such as chemokines and some lipid mediators such as prostaglandins and cysteinyl leukotrienes, stimulate DC chemotaxis (5–7). In animal models of allergic airway responses, DCs play crucial roles in both the sensitization and challenge phases (8, 9).

Leukotriene B₄ (LTB₄) is a proinflammatory lipid mediator which is derived from membrane phospholipids (10–12). LTB₄ activates leukocytes through a high-affinity G protein-coupled cell surface receptor, LTB₄ receptor 1 (BLT1), and leads to granulocyte and macrophage accumulation at sites of inflammation (13–17). We and others have recently shown that the LTB₄-BLT1 pathway may be important in effector T cell movement to sites of acute inflammation and Th2 cytokine production from T cells, and is critical to the development of AHR and inflammation (18–24). However, the effect of LTB₄ on allergic airway responses mediated through DCs in the airways has not been elucidated.

In this study, we investigated the role of BLT1 expression on DCs in the development of allergen-induced AHR and airway inflammation using mice that do not express this receptor. We found that murine lung DCs express BLT1 and that in an adoptive transfer model of allergen-induced airway responses, BLT1 expression on DCs was required for migration of DCs to regional lymph nodes as well as for the development of AHR and airway inflammation.

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Received for publication July 12, 2007. Accepted for publication May 13, 2008.

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¹ This work was supported by National Institutes of Health Grants HL-36577 and HL-61005 and by Environmental Protection Agency Grant R825702. N.M. was supported by a grant from the Takeda Science Foundation.

² The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute or the National Institutes of Health.

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⁵ Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BLT1, leukotriene B₄ receptor 1; DC, dendritic cell; BMDC, bone marrow-derived DC; Cdyn, dynamic compliance; LTA₄H, leukotriene A₄ hydrolase; LTB₄, leukotriene B₄; MNC, mononuclear cells; PAS, periodic acid-Schiff; PBLN, peribronchial lymph node; RL, lung resistance; WT, wild type.

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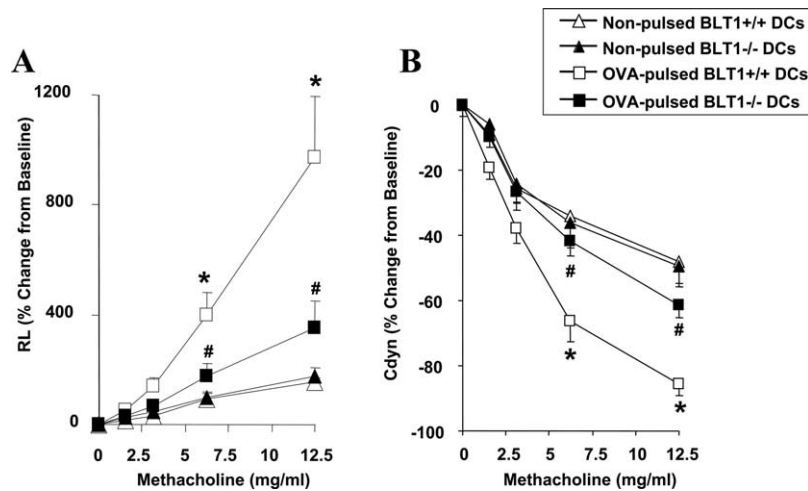


FIGURE 1. Development of altered airway function in mice that received BLT1^{+/+} or BLT1^{-/-} DCs. OVA-pulsed BLT1^{+/+} BMDCs (1×10^6), OVA-pulsed BLT1^{-/-} BMDCs (1×10^6), nonpulsed BLT1^{+/+} BMDCs (1×10^6), or nonpulsed BLT1^{-/-} BMDCs (1×10^6) were administered intratracheally to naive mice. Ten days after DC transfer, animals were challenged with OVA (1% in saline) for 20 min on three consecutive days. Forty-eight hours after the last OVA challenge, AHR was assessed, and BAL and tissues were obtained for further analysis. RL (A) and Cdyn values (B) were obtained in response to increasing concentrations of inhaled methacholine as described in *Materials and Methods*. Data represent mean $\pm$ SEM from three separate experiments, $n = 12$. *, Significant differences ($p < 0.01$) between mice that received OVA-pulsed BLT1^{+/+} BMDCs (BLT1^{+/+} DCs) vs mice that received nonpulsed BLT1^{+/+} BMDCs (nonpulsed BLT1^{+/+}) or nonpulsed BLT1^{-/-} BMDCs (nonpulsed BLT1^{-/-}). #, Significant differences ($p < 0.05$) between mice that received OVA-pulsed BLT1^{-/-} BMDCs (BLT1^{-/-} DCs) or BLT1^{+/+} BMDCs.

Materials and Methods

Animals

Female mice with a targeted disruption of the LTB₄ receptor 1 (BLT1^{-/-} mice) were backcrossed for seven generations onto a BALB/c genetic background (25). Leukotriene A₄ hydrolase-deficient (LTA₄H^{-/-}) mice and LTA₄H^{+/+} mice (129/SvEv mice) were obtained from Dr. B. H. Koller (University of North Carolina, Chapel Hill, NC; Ref. 26). All mice were used at 8–12 wk of age. The mice were housed under specific pathogen-free conditions and maintained on an OVA-free diet in the Biological Resources Center at the National Jewish Medical and Research Center. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

Generation of bone marrow-derived DCs (BMDC)

DCs were generated from bone marrow cells of naive BLT1^{+/+} or BLT1^{-/-} mice according to the procedure previously described (27). In brief, bone marrow cells obtained from femurs and tibias of mice were placed in culture medium with recombinant mouse GM-CSF (10 ng/ml) and recombinant mouse IL-4 (10 ng/ml; R&D Systems). Nonadherent cells were collected by aspirating the medium and transferred into fresh flasks. On day 8, cells were pulsed with OVA (grade V; Sigma-Aldrich; 200 μ g/ml) for 24 h and washed three times with PBS. More than 90% of the cells were determined to be myeloid DCs (CD11c⁺, CD11b⁺, Gr-1⁻). BLT1^{+/+} BMDCs or BLT1^{-/-} BMDCs (2×10^5 cells) were incubated in 96-well culture plates with or without OVA (100 μ g/ml). After 24 h, culture supernatants were harvested.

Transfer of BMDCs

Under anesthesia, 1×10^6 OVA-pulsed BMDCs in 40 μ l of PBS were instilled into naive BLT1^{+/+} mice through the trachea, under fiberoptic illumination (27). Control groups of mice received OVA-non-pulsed BMDCs. Ten days after BMDC transfer, mice were exposed to aerosolized OVA (1% in saline) for 20 min/day for three consecutive days; 48 h after the last challenge, AHR was assessed, and bronchoalveolar lavage (BAL) fluid and lung tissues were obtained.

To study the distribution of injected BMDCs, cells were labeled with the green fluorescent dye CFSE (Molecular Probes), as described previously (28). A total of 1×10^6 BMDCs were instilled intratracheally into naive mice and traced 24, 48, and 120 h later in the BAL fluid, lung tissue, and peribronchial lymph nodes (PBLN). Samples were then analyzed by flow cytometry (FACSCalibur; BD Biosciences). In these experiments, mice were not exposed to aerosolized OVA after transfer of BMDCs.

DCs were also generated from bone marrow cells of naive LTA₄H^{+/+} mice, and CFSE-labeled OVA-pulsed BMDCs were instilled into naive LTA₄H^{+/+} or LTA₄H^{-/-} mice through the trachea. Distribution of the transferred BMDCs was evaluated in the same way.

Assessment of airway function

Airway function was assessed as previously described by measuring changes in lung resistance (RL) and dynamic compliance (Cdyn) in response to increasing doses of inhaled methacholine (29). Data are expressed as percent change from baseline RL and Cdyn values obtained after inhalation of saline. There were no significant differences in baseline values among the different groups.

Bronchoalveolar lavage

Immediately after assessment of AHR, lungs were lavaged via the tracheal tube with HBSS. Total leukocyte numbers were counted by cell counter (Coulter Counter; Coulter). Cytospin slides were stained with Leukostat (Fisher Diagnostics) and differentiated by standard hematological procedures.

Histological studies

After BAL fluid was obtained, the lungs were fixed in 10% formalin and processed into paraffin blocks. Tissue sections 5 μ m thick were stained with H&E and periodic acid-Schiff (PAS) for identification of mucus-containing cells. The number of mucus-containing cells per millimeter of basement membrane was determined as previously described (30).

Eosinophils were identified by immunohistochemistry using rabbit anti-mouse eosinophilic major basic protein Ab (kindly provided by Dr. J. J. Lee, Mayo Clinic, Scottsdale, AZ) as previously described (30). Numbers of peribronchial eosinophils in the tissues were evaluated as previously described (30).

PBLN cell preparations

PBLN were removed, and mononuclear cells (MNC) were purified by Ficoll-Hypaque gradient centrifugation (Organon Teknica) as previously described (31, 32). To evaluate cytokine production from T cells, T cells from PBLN MNC were isolated with a Mouse T Cell Recovery Column Kit (Cedarlane; purity >95%). T cells (2×10^5) were cultured for 24 h in 96-well round-bottom plates in the presence or absence of OVA (100 μ g/ml) with irradiated spleen cells (2×10^5) as APCs.

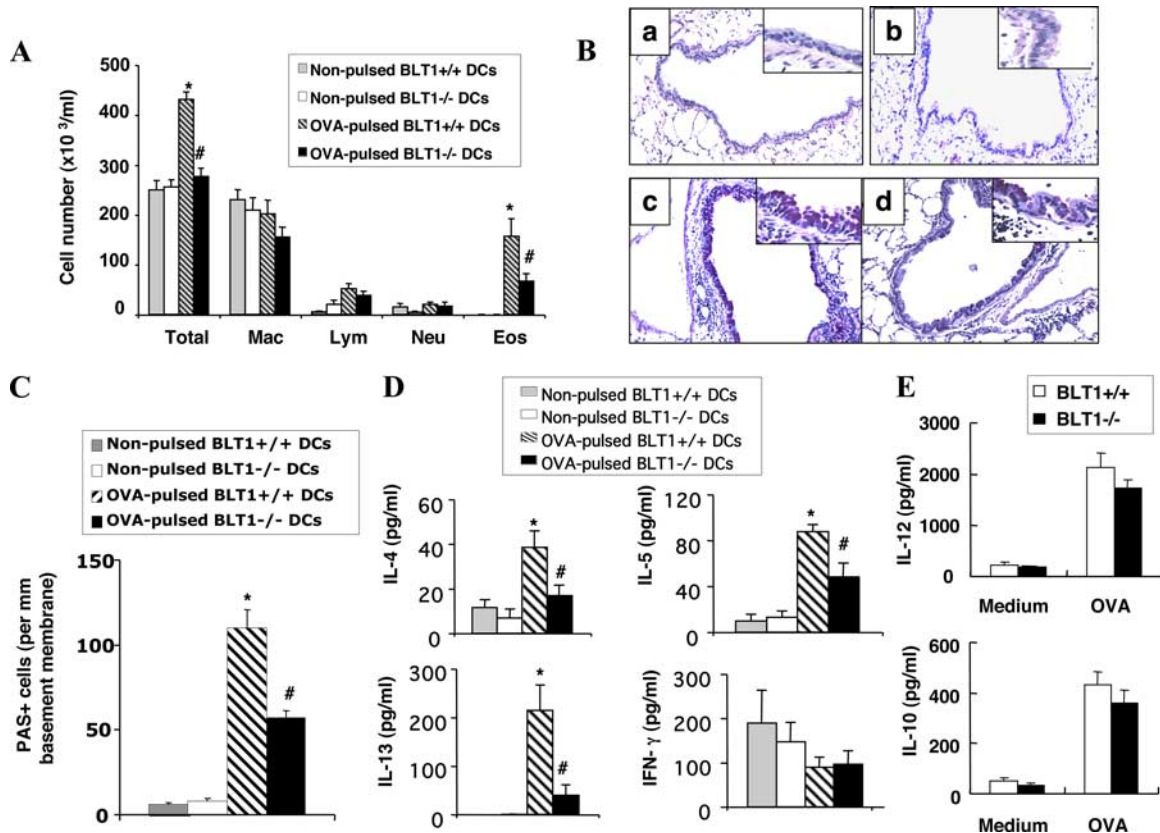


FIGURE 2. Development of airway inflammation in the lung and cytokine levels in BAL fluid. Samples were obtained from the same groups described in the legend to Fig. 1. **A**, Cellular composition in BAL fluid. *, Significant differences ($p < 0.05$) between mice that received OVA-pulsed BLT1^{+/+} BMDCs (BLT1^{+/+} DCs) vs mice that received nonpulsed BLT1^{+/+} BMDCs (nonpulsed BLT1^{+/+}) or non-pulsed BLT1^{-/-} BMDCs (nonpulsed BLT1^{-/-}). #, Significant differences ($p < 0.05$) between mice that received OVA-pulsed BLT1^{-/-} BMDCs (BLT1^{-/-} DCs) or BLT1^{+/+} DCs. **B**, Representative photomicrographs of PAS⁺ cells in the lung tissues. Mice that received nonpulsed BLT1^{+/+} DC (**a**), nonpulsed BLT1^{-/-} DC (**b**), or OVA-pulsed BLT1^{+/+} DCs (**c**), or OVA-pulsed BLT1^{-/-} DCs (**d**). **C**, Quantitative analysis of PAS⁺ cells in lung tissues was performed as described in *Materials and Methods*. *, Significant differences ($p < 0.001$) between mice that received OVA-pulsed BLT1^{+/+} BMDCs (BLT1^{+/+} DCs) vs mice that received nonpulsed BLT1^{+/+} BMDCs (nonpulsed BLT1^{+/+}) or OVA nonpulsed BLT1^{-/-} DCs (nonpulsed BLT1^{-/-}). #, Significant differences ($p < 0.05$) between mice that received OVA-pulsed BLT1^{-/-} BMDCs (BLT1^{-/-} DCs) or BLT1^{+/+} BMDCs. **D**, Cytokine levels in BAL fluid. IL-4, IL-5, IL-13, and IFN-γ levels in BAL fluid from the same groups of mice as in Fig. 1. *, Significant differences ($p < 0.05$) between mice that received OVA-pulsed BLT1^{-/-} BMDCs (BLT1^{-/-} DCs) or OVA-pulsed BLT1^{+/+} BMDCs (BLT1^{+/+} DCs). #, Significant differences ($p < 0.05$) between recipients of OVA-pulsed BLT1^{+/+} BMDCs vs mice that received nonpulsed BLT1^{+/+} DCs or nonpulsed BLT1^{-/-} DCs. **E**, IL-12 and IL-10 levels from BMDCs cultured in the presence of OVA. BMDCs were generated as described in *Materials and Methods*. After three washing in PBS, 2×10^5 OVA-pulsed BLT1^{+/+} BMDCs (BLT1^{+/+}) or BLT1^{-/-} BMDCs (BLT1^{-/-}) were incubated in 96-well culture plates with or without OVA (100 μg/ml) for 24 h. Data represent means $\pm$ SEM from three independent experiments.

In vitro sensitization of naive T cells with OVA-pulsed BLT1^{+/+} or BLT1^{-/-} DCs

Spleens of naive BLT1^{+/+} mice were removed, MNC were first isolated by Ficoll-Hypaque gradient centrifugation, and then T cells were isolated with the Mouse T Cell Recovery Column Kit as described in *PBLN cell preparations* (purity >95%). Bone marrow-derived BLT1^{+/+} or BLT1^{-/-} DCs were generated and pulsed with OVA as described in *PBLN cell preparations*, and isolated T cells (1×10^6 /ml) were cultured with OVA-pulsed BLT1^{+/+} or BLT1^{-/-} BMDCs (0.5×10^6 /ml) for 4 days in 24-well flat-bottom tissue culture plates. Then, T cells were isolated with the Mouse T Cell Recovery Column Kit again, and 2×10^5 cells were cultured for 24 h in 96-well round-bottom plates in the presence or absence of OVA (100 μg/ml) with irradiated spleen cells (2×10^5) as APCs.

In vitro stimulation of OVA-primed T cells with OVA-pulsed BLT1^{+/+} or BLT1^{-/-} DCs

Spleens of OVA-sensitized BLT1^{+/+} mice were removed, and MNC were first isolated by Ficoll-Hypaque gradient centrifugation, and then T cells were isolated with the Mouse T Cell Recovery Column Kit (purity >95%). Bone marrow-derived BLT1^{+/+} or BLT1^{-/-} DCs were generated and pulsed with OVA as described in *In vitro sensitization of naive T cells with OVA-pulsed BLT1^{+/+} or BLT1^{-/-} DCs*, and isolated (spleen) T cells ($2 \times$

10^5) were cultured with OVA-pulsed BLT1^{+/+} or BLT1^{-/-} BMDCs (2×10^4) for 72 h in 96-well round-bottom plates.

Measurement of cytokines

Cytokine levels in the BAL fluid and cell culture supernatants were measured by ELISA as previously described (28). IFN-γ, IL-4, IL-5, IL-10, IL-12 (BD Pharmingen), and IL-13 (R&D Systems) ELISAs were performed according to the manufacturer's directions. The limits of detection were 4 pg/ml for IL-4, IL-5, IL-10, IL-12, and IL-13 and 10 pg/ml for IFN-γ.

Establishment of mouse anti-mouse BLT1 mAb

Mouse anti-mouse BLT1 mAbs were generated by immunizing the BLT1^{-/-} mice (BALB/c background) with 300.19 cells expressing murine BLT1-red fluorescence protein as described elsewhere (S. Mathis et al., manuscript in preparation). Hybridoma culture supernatants of clone 3D7 were purified and fractionated over a Mono Q HR column (GE Healthcare). The peak fractions were biotinylated using the EZ-link sulfo-NHS-LC-biotin kit (Pierce) and used in flow cytometry.

Monitoring migration of lung DCs to PBLN

To assess the migration of lung resident DCs from lung to PBLN, mice were anesthetized, and 100 μg of FITC-OVA in 50 μl of PBS were

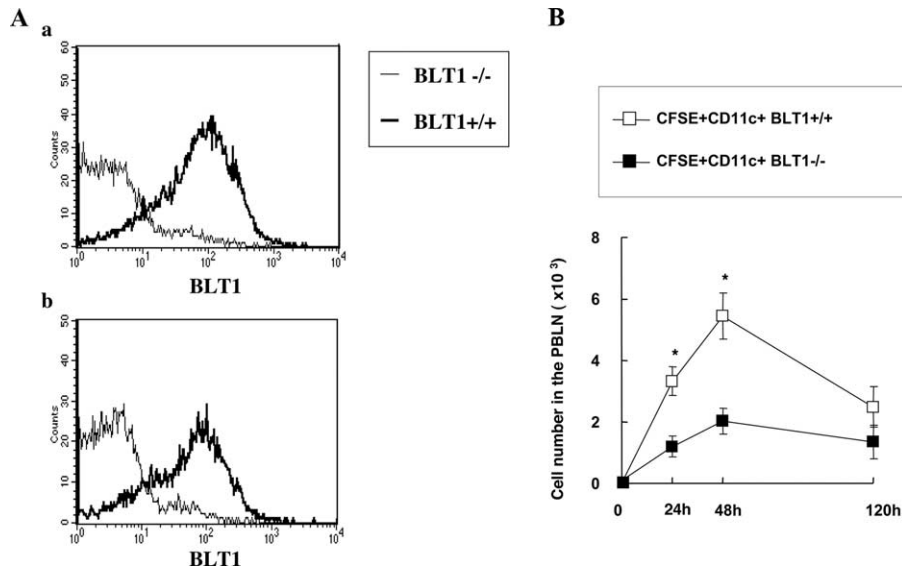


FIGURE 3. BLT1 expression on BMDCs and isolated DCs and accumulation of BLT1^{+/+} or BLT1^{-/-} BMDCs in the PBLN after intratracheal instillation. *A*, BLT1 expression on BMDCs generated in vitro (*a*) and on mononuclear cells isolated from PBLN (*b*) of BLT1^{+/+} mice cells were stained with anti-mouse BLT1 Ab as described in *Materials and Methods*. CD11c⁺ cells were gated on and analyzed for BLT1 expression. Data are representative of three independent experiments. *B*, Kinetics of appearance of transferred BMDCs in PBLN. OVA-pulsed BMDCs were labeled with CFSE and instilled intratracheally as described in *Materials and Methods*. MNCs in PBLNs were isolated and transferred BMDCs were identified as CFSE⁺CD11c⁺ cells. Numbers of transferred CFSE⁺BLT1^{+/+} or CFSE⁺BLT1^{-/-} BMDCs were determined at 24, 48, and 120 h after transfer. *, Significant differences ($p < 0.05$) between BLT1^{-/-} DCs and BLT1^{+/+} DCs.

instilled intratracheally into naive BLT1^{+/+} or BLT1^{-/-} mice. FITC⁺ DCs were traced 24 h later in the PBLN as previously described (33). Samples were analyzed by flow cytometry (FACSCalibur).

Flow cytometry

To evaluate BLT1 expression on BMDCs and lung DCs, mice were sensitized on days 1 and 14 by i.p. injection of 20 μ g of OVA premixed with 2 mg of Al(OH)₃ (Pierce) in 100 μ l of PBS. After sensitization, animals were exposed to aerosolized OVA (1% in saline) for 20 min/day on days 28, 29, and 30. Forty-eight hours after the last OVA challenge (day 32), MNCs from PBLNs and lung tissues were obtained. As a control, animals were injected with 100 μ l of PBS followed by exposure on days 28–30 to aerosolized PBS. Biotinylated mouse anti-mouse BLT1 mAb and allophycocyanin-streptavidin (BD Pharmingen) were used to detect surface expression of murine BLT1. PE-conjugated anti-mouse CCR7 (eBioscience) was used to detect surface expression of murine CCR7. PE-conjugated or allophycocyanin-conjugated anti-mouse CD11c (BD Pharmingen) was used to identify DCs. PE-conjugated anti-mouse Gr-1 and allophycocyanin-conjugated CD11c were used to identify plasmacytoid DCs. The stained cells were washed, resuspended in PBS, and then analyzed by flow cytometry (FACSCalibur) as previously described (32). The numbers of CD11c⁺ and CFSE⁺ cells in BAL, PBLN, and lungs were derived by multiplying the percentage of stained cells by the total number of cells isolated.

Statistical analysis

All results were expressed as the means $\pm$ SEM. ANOVA was used to determine the levels of difference between all groups. Pairs of groups of samples distributed parametrically were compared by an unpaired two-tailed Student *t* test, and those samples distributed nonparametrically were compared by the Mann-Whitney *U* test. The data were pooled from three independent experiments with four mice per group in each experiment ($n = 12$). Significance was assumed at p values of <0.05 .

Results

Mice receiving BLT1^{-/-} BMDCs develop significantly lower AHR compared with the mice receiving BLT1^{+/+} DCs

We first assessed airway responsiveness to inhaled methacholine in mice that received BMDCs intratracheally. Intratracheal injection of OVA-pulsed BLT1^{+/+} BMDCs followed by OVA challenge led to the development of increased AHR in recipient mice,

illustrated by significant increases in RL and decreases in Cdyn, as compared with mice that received nonpulsed BMDCs (Fig. 1). In contrast, mice that received OVA-pulsed BLT1^{-/-} BMDCs developed lower increases in RL and decreases in Cdyn compared with the mice that received OVA-pulsed BLT1^{+/+} BMDCs.

Mice receiving BLT1^{-/-} BMDCs develop significantly lower airway inflammation compared with mice receiving BLT1^{+/+} BMDCs

The numbers and types of inflammatory cells in the airways of the mice that received BLT1^{-/-} and BLT1^{+/+} BMDCs were examined in BAL fluid (Fig. 2A). After intratracheal transfer of OVA-pulsed BLT1^{+/+} BMDCs and OVA challenge, the numbers of eosinophils in BAL fluid increased compared with those of the mice that received nonpulsed BMDCs. Eosinophil numbers in the BAL of mice that received OVA-pulsed BLT1^{-/-} BMDCs were significantly lower than in the mice that received OVA-pulsed BLT1^{+/+} BMDCs.

Lung histology showed an intense infiltration of inflammatory cells in the perivascular tissue area and, to a slightly lesser extent, in the peribronchial spaces in the lungs of mice that received OVA-pulsed BLT1^{+/+} BMDCs. In the mice that received OVA-pulsed BLT1^{-/-} BMDCs, fewer eosinophils were detected in these sites than in wild-type (WT) mice after challenge with OVA. Immunostaining with anti-major basic protein revealed significantly higher numbers of eosinophils in the peribronchial tissue of mice that received OVA-pulsed BLT1^{+/+} BMDCs (mean $\pm$ SEM 112.2 $\pm$ 21.1 cells/mm²) when compared with mice that received OVA-pulsed BLT1^{-/-} BMDCs (65.9 $\pm$ 10.7 cells/mm²; $p < 0.05$) after challenge.

The degree of goblet cell metaplasia and mucus hyperproduction were assessed by PAS staining and quantification of PAS-stained cells. Transfer of OVA-pulsed BLT1^{+/+} BMDCs into naive mice significantly increased the numbers of PAS⁺ cells when challenged with allergen (Fig. 2, *B* and *C*). However, mice that

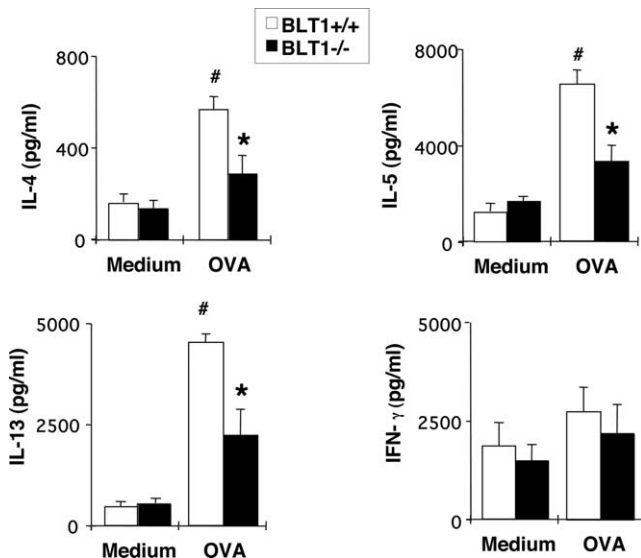


FIGURE 4. Cytokine production is impaired in recipients of BLT1^{-/-} DCs. Cytokine levels were determined by ELISA in cultures of T cells isolated from PBLN, in the presence or absence of OVA (100 μg/ml) as described in *Materials and Methods*. Data represent means ± SEM from three independent experiments conducted in triplicate. *, Significant differences ($p < 0.01$) between mice that received OVA-pulsed BLT1^{+/+} BMDCs (BLT1^{+/+} DCs) vs mice that received nonpulsed BLT1^{+/+} BMDCs (nonpulsed BLT1^{+/+}) or nonpulsed BLT1^{-/-} BMDCs (nonpulsed BLT1^{-/-}). #, Significant differences ($p < 0.05$) between mice that received OVA-pulsed BLT1^{-/-} BMDCs (BLT1^{-/-} DCs) or BLT1^{+/+} DCs.

received OVA-pulsed BLT1^{-/-} BMDCs showed significantly fewer PAS⁺ goblet cells in the airways.

Cytokine levels in BAL fluid

Concentrations of IL-4, IL-5, IL-13, and IFN-γ in the BAL fluid were measured by ELISA. Mice that received OVA-pulsed BLT1^{+/+} BMDCs showed significantly increased levels of IL-4, IL-5, and IL-13 compared with the mice which received nonpulsed BMDCs (Fig. 2D). The levels of IL-5 and IL-13 in the BAL fluid of the mice that received OVA-pulsed BLT1^{-/-} BMDCs were significantly lower than in recipients of OVA-pulsed BLT1^{+/+} BMDCs.

Cytokine production from BMDCs

We evaluated cytokine production in culture supernatants of BLT1^{+/+} or BLT1^{-/-} BMDCs. The levels of IL-12 from OVA-pulsed BLT1^{-/-} BMDCs were similar to the levels from OVA-pulsed BLT1^{+/+} BMDCs following restimulation with OVA (Fig. 2E). The levels of IL-10 in the cultures of OVA-pulsed BLT1^{-/-} BMDCs were also similar to the levels in OVA-pulsed BLT1^{+/+} BMDCs after restimulation with OVA.

Recruitment of DCs into the PBLN

We next determined if BLT1 participates in DC trafficking to PBLN. We first assessed BLT1 expression on established BMDCs and DCs from PBLN. As shown in Fig. 3A, BMDCs expressed BLT1. To determine whether DCs in the PBLN expressed BLT1, MNC were isolated from the PBLN of OVA-sensitized and -challenged mice and then incubated with both anti-BLT1 and anti-CD11c. Gating on the CD11c⁺ population revealed that >80% of the cells stained positively for BLT1. At 24, 48, and 120 h after intratracheal instillation of OVA-pulsed CFSE-labeled BMDCs,

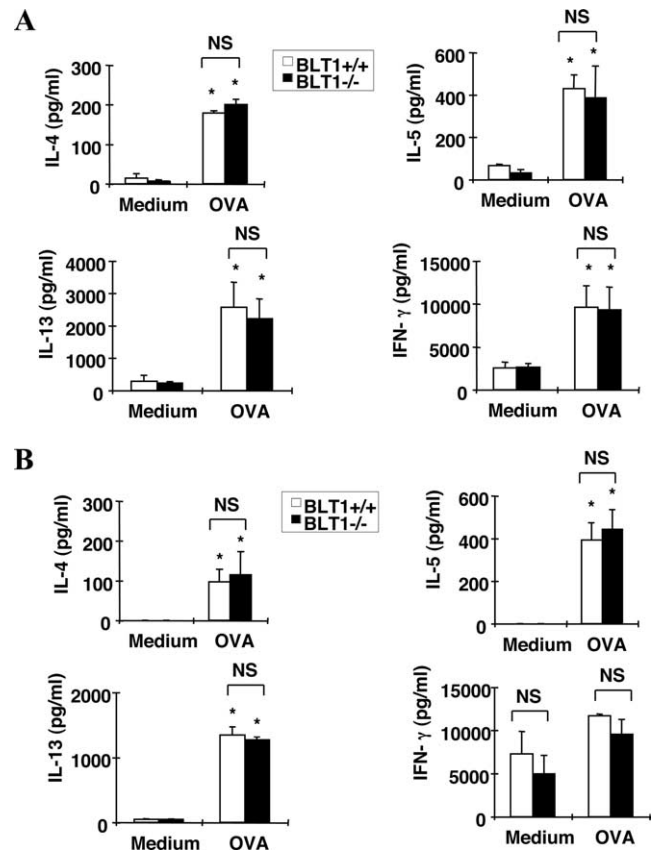


FIGURE 5. Ag-presenting function of BLT1^{-/-} DCs in vitro. *A*, Cytokine levels in culture supernatants from spleen T cells cocultured with OVA-pulsed BLT1^{+/+} or BLT1^{-/-} BMDCs. Spleen T cells from naive mice were isolated, cultured with OVA-pulsed BLT1^{+/+} BMDCs or BLT1^{-/-} BMDCs for 96 h. T cells were then isolated using the T cell isolation kit and cocultured with irradiated naive spleen cells (3000 rads) in the presence or absence of OVA (100 μg/ml) for 24 h as described in *Materials and Methods*. Data represent means ± SEM from three independent experiments conducted in triplicate. *, Significant differences ($p < 0.05$) between mice that received nonpulsed BLT1^{+/+} BMDCs (nonpulsed BLT1^{+/+}) and nonpulsed BLT1^{-/-} BMDCs (nonpulsed BLT1^{-/-}) vs OVA-pulsed BLT1^{+/+} BMDCs (BLT1^{+/+} DCs) or OVA-pulsed BLT1^{-/-} BMDCs (BLT1^{-/-} DCs). *B*, Cytokine levels in culture supernatants from OVA-primed spleen T cells cocultured with BLT1^{+/+} or BLT1^{-/-} DCs. Spleen T cells from sensitized mice were isolated and cocultured with OVA-pulsed BLT1^{+/+} or BLT1^{-/-} BMDCs for 72 h in the presence or absence of OVA as described in *Materials and Methods*. Data represent means ± SEM from three independent experiments conducted in triplicate. *, Significant differences ($p < 0.05$) between nonpulsed BLT1^{+/+} and nonpulsed BLT1^{-/-} vs BLT1^{+/+} DCs or BLT1^{-/-} DCs.

MNC from the PBLN (BAL, and lung) were isolated, and transferred BMDCs were detected as CFSE⁺CD11c⁺ cells. Fig. 3B illustrates the kinetics of appearance of BLT1^{+/+} and BLT1^{-/-} CFSE⁺CD11c⁺ cells in the PBLN. The numbers of OVA-pulsed CFSE⁺BLT1^{-/-} BMDCs recovered from PBLN were significantly lower than from OVA-pulsed CFSE⁺BLT1^{+/+} BMDCs at 24 and 48 h after instillation.

In vitro Th2 cytokine production from PBLN cells is lower in mice that received BLT1^{-/-} BMDCs

To determine whether the differences observed between the two strains of mice in their levels of Th2 cytokines were due to differences in Ag-specific T cell responsiveness, T cells obtained

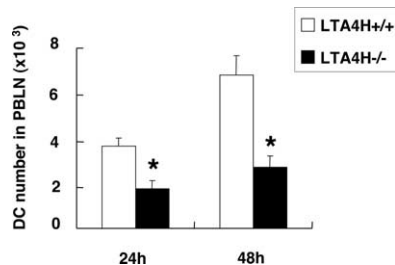


FIGURE 6. Accumulation of DCs in the PBLN of LTA₄H-sufficient or -deficient mice after intratracheal instillation and challenge. OVA-pulsed BMDCs were labeled with CFSE and instilled intratracheally as described in *Materials and Methods*. Transferred BMDCs were identified as CFSE⁺CD11c⁺ cells, and the numbers of cells in the PBLN were determined at 24 and 48 h after transfer as described in *Materials and Methods*. *, Significant differences ($p < 0.05$) between LTA₄H^{+/+} mice that received OVA-pulsed BMDCs and LTA₄H^{-/-} mice that received OVA-pulsed BMDCs from WT (LTA₄H^{+/+}) mice.

from PBLN were isolated and then restimulated in culture with OVA. The proportion of the cells that expressed CD3 exceeded 95%. IL-4, IL-5, and IL-13 production after culture with OVA from mice that received OVA-pulsed BLT1^{-/-} BMDCs was significantly lower than in the mice that received BLT1^{+/+} BMDCs (Fig. 4).

In vitro sensitization of naive T cells with OVA-pulsed BLT1^{+/+} or BLT1^{-/-} BMDCs

Recipients of OVA-pulsed BLT1^{-/-} BMDCs developed lower AHR, airway inflammation, sensitization of T cells as well as the

accumulation of transferred BMDCs in PBLN in vivo than did recipients of OVA-pulsed BLT1^{+/+} BMDCs. To further investigate the mechanism underlying the impaired ability of BLT1^{-/-} DCs to prime T cells, we evaluated the functional activity of BLT1^{+/+} and BLT1^{-/-} BMDCs in vitro. BLT1^{-/-} BMDCs showed normal maturation as judged by surface expression of CD80, CD86, MHC class II, CCR7, and CD40L (data not shown), consistent with a previous study (33). As shown in Fig. 5A, when cultured with OVA-pulsed BLT1^{-/-} BMDCs, naive spleen T cells were able to produce comparable amounts of Th2 cytokines as cultures with OVA-pulsed BLT1^{+/+} BMDCs. Naive T cells cultured with nonpulsed BLT1^{+/+} or BLT1^{-/-} BMDCs did not increase the levels of Th2 cytokines (data not shown). These data suggested that BLT1^{-/-} DCs were not impaired in their ability to act as APCs in vitro.

In vitro stimulation of OVA-primed T cells with OVA-pulsed BLT1^{+/+} or BLT1^{-/-} BMDCs

DCs have been shown to be crucial not only in the sensitization phase but also during the effector phase (9). To determine whether BLT1 expression on DCs is essential for Ag presentation to activated T cells (as may occur in the allergen challenge phase), T cells from OVA-sensitized BLT1^{+/+} mice were isolated and then cultured with OVA-pulsed BLT1^{+/+} or BLT1^{-/-} BMDCs. As shown in Fig. 5B, there were no differences in Th2 cytokine production from T cells obtained from OVA-sensitized mice when cultured with BLT1^{+/+} BMDCs or BLT1^{-/-} BMDCs. Therefore, by all measures, BLT1^{-/-} DCs appeared to have normal Ag-presenting function, at least in vitro.

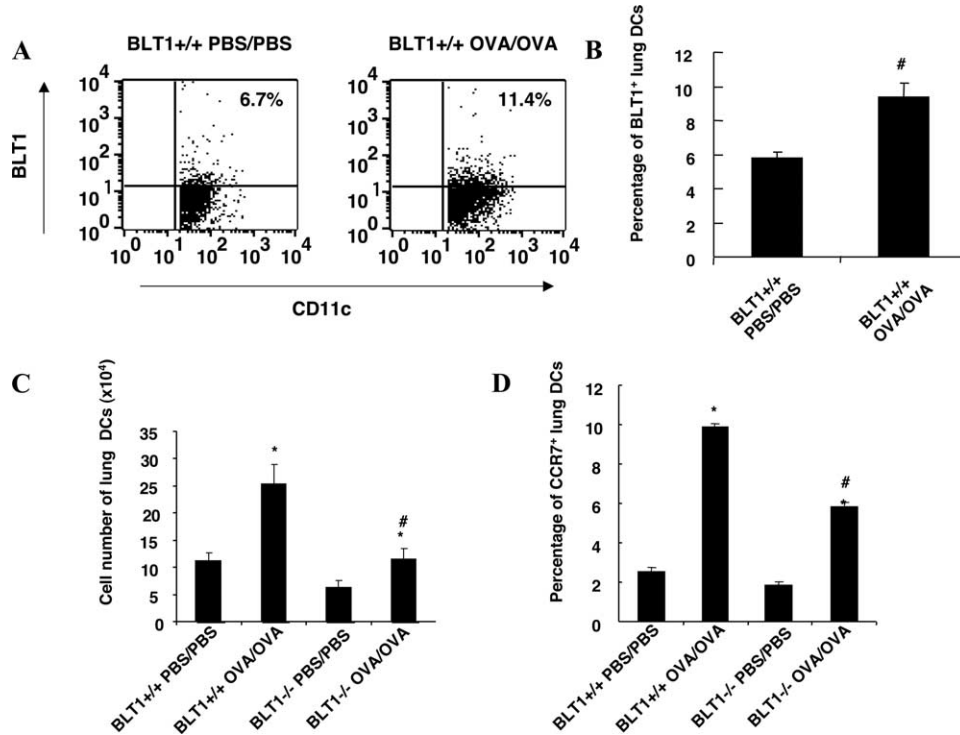


FIGURE 7. BLT1 and CCR7 expression on lung DCs. MNCs isolated from lungs of naive or OVA-sensitized and challenged BLT1^{+/+} mice were stained with anti-mouse BLT1 Ab or CCR7 Ab as described in *Materials and Methods*. CD11c⁺ cells were gated on and analyzed for BLT1 or CCR7 expression. BLT1^{+/+} PBS/PBS, BLT1^{+/+} mice, no sensitization and challenge to OVA; BLT1^{+/+} OVA/OVA, BLT1^{+/+} mice after sensitization and challenge to OVA; BLT1^{-/-} PBS/PBS, BLT1^{-/-} mice, no sensitization and challenge to OVA; BLT1^{-/-} OVA/OVA, BLT1^{-/-} mice after sensitization and challenge to OVA. A, BLT1 expression on lung DCs. Data are representative of three independent experiments. B, Percentages of BLT1⁺ lung DCs. #, $p < 0.05$ compared with BLT1^{+/+} PBS/PBS. C, Numbers of DCs in the lungs. #, $p < 0.05$ compared with BLT1^{+/+} OVA/OVA. *, $p < 0.05$ compared with BLT1^{+/+} PBS/PBS and BLT1^{-/-} PBS/PBS. D, CCR7 expression on lung DCs. #, $p < 0.05$ compared with BLT1^{+/+} OVA/OVA. *, $p < 0.05$ compared with BLT1^{+/+} PBS/PBS and BLT1^{-/-} PBS/PBS.

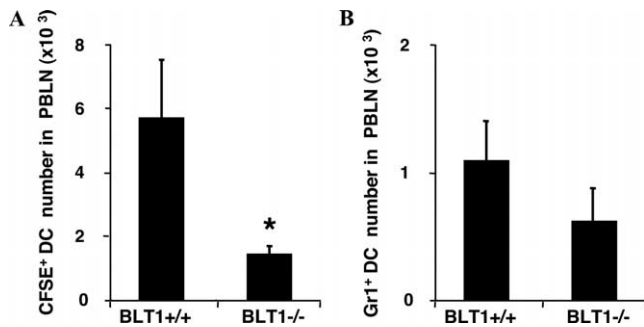


FIGURE 8. Accumulation of lung DCs in the PBLN after intratracheal instillation of FITC-OVA. BLT1^{+/+} or BLT1^{-/-} mice received FITC-OVA by intratracheal instillation and the number of FITC⁺CD11c⁺ cells (A), and the numbers of FITC-Gr-1⁺CD11⁺ cells (B) in the PBLN were determined by flow cytometry analysis 24 h after instillation as described in *Materials and Methods*.

Fewer DC migrate to PBLN when transferred into LTA₄H^{-/-} mice compared with transfer into LTA₄H^{+/+} mice

To determine whether the migration of DCs into PBLN was truly dependent on the LTB₄-BLT1 axis, we compared the accumulation of DCs in the PBLN of LTA₄H^{+/+} and in LTA₄H^{-/-} mice, which lack LTB₄ production in vivo. We generated DCs from the bone marrow of LTA₄H^{+/+} mice and then transferred OVA-pulsed CFSE-labeled BMDCs into LTA₄H^{+/+} mice or LTA₄H^{-/-} mice intratracheally. The numbers of OVA-pulsed CFSE⁺ BMDCs recovered 24 and 48 h later from PBLN of LTA₄H^{-/-} mice were significantly lower than in LTA₄H^{+/+} mice (Fig. 6). The numbers of CFSE⁺ BMDCs recovered in the BAL and lungs were not different in the two groups (data not shown).

Expression of BLT1 on lung DCs

The data illustrate that BMDCs express BLT1 and that the LTB₄-BLT1 pathway can regulate the migration of these cells to the PBLN. We next assessed the expression of BLT1 on lung DCs. As shown in Fig. 7, A and B, lung DCs in naive BLT1^{+/+} mice express BLT1, and following systemic sensitization and challenge with OVA, the percentages of BLT1⁺ lung DCs significantly increased. After sensitization and challenge with OVA, the numbers of DCs in the lung of BLT1^{-/-} also increased, but to a significantly lesser extent than in BLT1^{+/+} mice (Fig. 7C).

Expression of CCR7 on lung DCs

CCR7 has been shown to play an important role in migration of DCs into regional lymph nodes. We determined whether BLT1 affected CCR7 expression on lung DCs. As shown in Fig. 7D, percentages of CCR7⁺ DCs in the lung were significantly increased in BLT1^{+/+} mice after sensitization and challenge with OVA, compared with naive BLT1^{+/+} mice. The percentages of CCR7⁺ DCs in the lungs were significantly lower in BLT1^{-/-} mice than in BLT1^{+/+} mice after sensitization and challenge.

Migration of lung DCs to the PBLN

The preceding data showed that BMDCs require the LTB₄-BLT1 pathway for migration to PBLN. We similarly assessed the migration of resident lung DCs to the PBLN to delineate the role of BLT1 in this process. To follow the migration of resident lung DCs from the lung to the PBLN, we gave mice 100 μg of FITC-OVA by intratracheal instillation. The phenotype and number of FITC-OVA⁺ cells in the PBLN were determined by flow cytometry 24 h after instillation. The numbers of FITC⁺ DCs in the PBLN of BLT1^{-/-} mice were significantly lower than the PBLN of

BLT1^{+/+} mice (Fig. 8A). These data suggest that not only BMDCs but also lung DCs require BLT1 for migration to the PBLN. We also assessed the numbers of FITC⁺Gr-1⁺CD11c⁺ cells in the PBLN 24 h after instillation to determine whether migration of plasmacytoid DCs into PBLN was affected by BLT1 expression. Although lower in the BLT1^{-/-} mice, the numbers of FITC⁺Gr-1⁺CD11c⁺ cells in the PBLN were not significantly different in the two groups (Fig. 8B).

Discussion

Previous studies have shown that LTB₄ plays a central role in the early recruitment of leukocytes and Ag-specific effector memory CD8⁺ T cells and Th2 cytokine production in allergen-induced airway responses (19–24). However, the role for this receptor on DCs in the development of AHR and inflammation has not been defined. Evidence for expression of a functional BLT1 receptor on DCs was provided by Del Prete et al. (34), who showed that murine DCs mobilize calcium and chemotaxis in response to LTB₄ and that BLT1 expression on DC regulates delayed-type hypersensitivity in the skin. Here, we confirm that DCs in the lungs, regional lymph nodes, and airways express BLT1 and that expression of BLT1 on DCs can play an important role in the development of allergen-induced AHR and inflammation. Moreover, after sensitization and challenge, the numbers of lung DCs expressing BLT1 increased significantly over the numbers seen in naive BLT1^{+/+} mice or the numbers from sensitized and challenged BLT1^{-/-} mice. Using a BMDC-dependent model of allergic airway disease, AHR and airway inflammation in mice that received BLT1^{-/-} DCs were significantly reduced compared with the mice that received BLT1^{+/+} DCs. After transfer, migration of BLT1^{-/-} DCs to the regional lymph nodes was significantly impaired compared with transfer of BLT1^{+/+} DCs. In vivo as well as in vitro Th2 cytokine production from PBLN T cells was significantly reduced in the recipients of BLT1^{-/-} DCs compared with mice that received BLT1^{+/+} DCs. These data suggest that BLT1 expression on DCs plays an important role in the development of AHR and inflammation, at least in part facilitated by their ability to migrate to the regional lymph nodes.

The importance of the migration of lung DCs into regional lymph nodes for the development of allergen-induced airway responses has been suggested (35, 36). They found that during the sensitization phase, DCs in the lungs migrate into the regional lymph nodes, where initial priming of T cells occurs. After airway challenge, sensitized T cells produce Th2 cytokines and induce AHR and eosinophilic inflammation in the airways. Therefore, any interference with the migratory capacity of DCs to the regional lymph nodes could result in impairment of the initial sensitization of T cells, and consequently lower eosinophilic inflammation and AHR. In the present study, the absence of BLT1 on DCs resulted in impaired migration to the regional lymph nodes after intratracheal instillation. To confirm that the migration of DCs to the lymph nodes was dependent on the LTB₄-BLT1 pathway, we evaluated the ability of DCs from WT mice to accumulate in the regional lymph nodes of LTA₄H^{-/-} mice, i.e., in an environment where LTB₄ production was completely lacking. DC accumulation in the regional lymph nodes was markedly decreased in the deficient mice, further supporting the notion that recruitment of DCs to the regional lymph nodes in the airways is dependent on the LTB₄-BLT1 pathway. To complement and extend these findings, we tracked lung DC migration after intratracheal instillation of FITC-OVA. In these studies, we confirmed that BLT1 expression on lung DCs plays an important role in their migration to the regional lymph nodes. The impairment associated with the absence of BLT1 expression appeared restricted to DC migratory capacity but

not on other aspects that may have been associated with impaired DC function. BLT1^{-/-} BMDCs showed normal maturation as judged from the expression of surface markers. Further, in vitro, BLT1^{-/-} BMDCs were fully capable of activating naive spleen T cells to produce Th2-type cytokines, similar to BLT1^{+/+} BMDCs.

In the process of DC migration to lymph nodes, chemokine signals acting on the CCR7 are important. In mice homozygous for the paucity of lymph node T cell (*plt*) mutation which lack the CCR7 agonists CCL19 and CCL21, migration was similarly impaired (37). The migration of Ag-pulsed DCs into peripheral tissues has been shown to be dependent on the expression of CCR7 by DCs and the production of CCR7 ligands, CCL19, by stromal cells and mature DCs in the lymph node, and CCL21 by afferent lymphatic cells. Del Prete et al. reported that LTB₄ up-regulates CCR7 expression and function and that this effect is primarily through the enhancement of chemotaxis with only a minor effect on chemokinesis (34). They showed that chemotaxis of BLT1^{-/-} BMDCs in response to CCL19 and CCL21 was significantly impaired compared with BLT1^{+/+} BMDCs, that LTB₄-induced migration of immature DCs to CCL19 and CCL21 was associated with an increase in CCR7 membrane expression, and that LTB₄ up-regulates the migration of mature DCs to CCR7 ligands. In the present study, expression of CCR7 on lung DCs was higher in BLT1^{+/+} mice compared with BLT1^{-/-} mice following sensitization and challenge conditions where LTB₄ levels were also increased (12). Therefore, it appears that LTB₄ can up-regulate CCR7 expression, which in turn, leads to the migration of DCs to regional lymph nodes. Migration of DCs into regional lymph nodes has also been reported to be controlled by other lipid mediators, such as prostaglandins and cysteinyleukotrienes. PGD2 acts on the DP1 receptor expressed by lung DCs and suppresses the migration of lung DCs (38). The leukotriene C₄ transporter, MRP1, regulates DC migration to lymph nodes by transporting leukotriene C₄, which in turn promotes chemotaxis to CCL19 and mobilization of DCs from the epidermis (39).

Von Riet et al. (9) showed that depletion of CD11c⁺ cells during allergen challenge in sensitized mice significantly suppressed features of asthma in mice. These data suggest that during the allergen challenge phase, DCs also migrate from the sites of allergic inflammation to the draining lymph nodes to induce further expansion of recirculating central memory cells or to stimulate naive T cells to become Th2 cells, thus enhancing and sustaining the inflammatory response with new waves of effector T cells (40–42). In the present study, allergen-sensitized T cells restimulated with allergen-pulsed BLT1^{-/-} DCs were able to produce Th2 cytokines similar to those restimulated with BLT1^{+/+} DCs, suggesting that Ag-presenting function during the restimulation phase was not impaired, at least in vitro. Thus, it is more likely that in recipients of BLT1^{-/-} DCs, impaired migration in the allergen challenge phase in vivo might have also contributed to the development of lower levels of AHR, inflammation, and Th2 cytokines compared with these mice which received BLT1^{+/+} DCs.

In this study, we utilized a well-characterized DC-dependent model for development of allergen-induced airway responses and found that in the absence of BLT1 expression on DCs the full spectrum of lung allergic responses was decreased: eosinophilic inflammation; Th2 cytokine production; AHR; and goblet cell metaplasia. These findings are supported by our previous results demonstrating that BLT1^{-/-} mice showed significantly lower AHR and goblet cell metaplasia than did BLT1^{+/+} mice, although they did not show significantly lower eosinophilic inflammation than did BLT1^{-/-} mice when systemically sensitized with OVA together with alum (21). Full reconstitution of all responses in these BLT1^{-/-} recipients was demonstrated

after transfer of Ag-primed BLT1^{+/+} T cells (20). Reconstitution with Ag-primed T cells or T cell subsets likely bypasses some of the requirements for BLT1 expression in the BLT1^{-/-} recipients. Further, in the present study, we did not use any adjuvant for sensitization and in fact bypassed the sensitization phase rendering the system totally dependent on transfer of Ag-pulsed DCs. It has been shown that adjuvant enhances expression of MHC class II and costimulation molecules such as CD80 and CD86 and up-regulates the Ag-presenting function of DCs (43, 44). Therefore, in the earlier study, systemic sensitization to allergen together with alum may have bypassed some aspects of DC function dependent on BLT1 expression, accounting for the differences in eosinophilic inflammation between BLT1^{-/-} and BLT1^{+/+} mice and enabling reconstitution of sensitized BLT1^{-/-} recipients with primed CD8⁺ T cells.

In summary, we have identified the critical role for a functional LTB₄-BLT1 pathway in DCs for the full development of allergen-induced AHR and airway inflammation, and have shown that BLT1 expression on DCs was required for the full development of these responses. Our studies indicate that BLT1 controls migration of DCs into the regional lymph nodes, the initiation of T cell responses leading to Ag-specific Th2 responses, and the development of AHR and airway inflammation. Manipulating DC function through BLT1 may provide a novel approach for controlling allergen-induced AHR and eosinophilic airway disease and has the potential to improve the treatment of asthma.

Acknowledgments

We thank L. N. Cunningham and D. Nabighian (National Jewish Medical and Research Center) for assistance.

Disclosures

The authors have no financial conflict of interest.

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