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Cysteiny1 Leukotriene 2 Receptor on Dendritic Cells Negatively Regulates Ligand-Dependent Allergic Pulmonary Inflammation

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Cysteiny1 leukotrienes (cys-LTs) can mediate Th2 immunity to the house dust mite, Dermatophagoides farinae, via the type 1 receptor CysLT1R on dendritic cells (DCs). However, the role of the homologous type 2 receptor CysLT2R in Th2 immunity is unknown. D. farinae sensitization and challenge of CysLT2R-deficient mice showed a marked augmentation of eosinophilic pulmonary inflammation, serum IgE, and Th2 cytokines. Wild-type (WT) mice sensitized by adoptive transfer of D. farinae-pulsed CysLT2R-deficient bone marrow-derived DCs (BMDCs) also had a marked increase in D. farinae-elicited eosinophilic lung inflammation and Th2 cytokines in restimulated hilar nodes. This response was absent in mice sensitized with D. farinae-pulsed BMDCs lacking leukotriene C4 synthase (LTC4S), CysLT1R, or both CysLT2R/LTC4S, suggesting that CysLT1R negatively regulates LTC4S- and CysLT1R-dependent LTD4-induced ERK phosphorylation, whereas N-methyl LTC4 activation of CysLT1R on WT BMDCs reduced such signaling. Activation of endogenously expressed CysLT1R and CysLT2R occurred over an equimolar range of LTD4 and N-methyl LTC4, respectively. Although the baseline expression of cell surface CysLT1R was not increased on CysLT2R-deficient BMDCs, it had decreased following stimulation of WT BMDCs by D. farinae. Thus, CysLT2R negatively regulates the development of cys-LT-dependent Th2 pulmonary inflammation by inhibiting both CysLT1R signaling and LTC4S, respectively. In mouse models of allergic pulmonary inflammation induced by OVA or by extracts from clinically relevant house dust mite, Dermatophagoides farinae, pharmacologic antagonism of CysLT1R or genetic deletion of LTC4S or CysLT1R attenuates Th2 pulmonary inflammation (9–12). In vitro D. farinae stimulation of bone marrow-derived DCs (BMDCs) triggers the robust generation of cys-LTs, and the adoptive transfer of D. farinae-pulsed BMDCs from LTC4S-deficient (Ltc4s−/−) or CysLT2R-deficient (Cysltr1−/−) mice to sensitize naive wild-type (WT) recipients has shown that each protein is needed to prime for Th2 pulmonary inflammation after D. farinae challenge (12). These studies highlight how the biologic activity of cys-LTs can be tightly regulated by competition between these endogenously expressed receptors. The Journal of Immunology, 2012, 189: 4556–4565.

The online version of this article contains supplemental material.

Abbreviations used in this article: BAL, bronchoalveolar lavage; BMDC, bone marrow-derived dendritic cell; cys-LT, cysteinyl leukotriene; CysLT1R, type 1 cys-LT receptor; CysLT2R, type 2 cys-LT receptor; DC, dendritic cell; LNC, lymph node; LT, leukotriene; LTC4S, LTC4 synthase; WT, wild-type.

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CysLT1R expression and LTD4-elicited mitogenic response in human mast cells (17). Whether CysLT1R can negatively regulate Th2 pulmonary inflammation in vivo has not been addressed.

We previously generated CysLT2R-deficient (Cysltr2−/−) mice (19) and now provide CysLT1R/LTC4S-deficient (Cysltr2/Ltc4s−/−) mice to address whether this receptor can regulate cys-LT-dependent immune responses. We show that Cysltr2−/− mice have markedly increased eosinophilic pulmonary inflammation and Th2 cytokines in response to intranasal D. farinae sensitization and challenge. Analysis of the sensitization function by adoptive transfer of D. farinae-pulsed BMDCs showed that Cysltr2−/− BMDCs generated markedly enhanced responses to D. farinae challenge, whereas the lack of CysLT1R or LTC4S or the double deficiency of CysLT1R and LTC4S significantly suppressed the response well below that of WT BMDCs. We considered that the enhanced responses from Cysltr2−/− BMDCs and reduced responses from Cysltr2/Ltc4s−/− BMDCs may reflect enhanced or reduced CysLT1R function, respectively, and assessed CysLT1R-dependent ERK phosphorylation and CysLT1R expression in BMDCs from relevant deficient strains. We found that pharmacologic inhibition or genetic deficiency of CysLT1R on BMDCs increased LTD4-initiated CysLT1R-dependent ERK phosphorylation, whereas N-methyl LTE4 activation of CysLT1R on WT BMDCs was inhibitory. Although there was no increase in baseline CysLT1R cell surface expression on Cysltr2−/− BMDCs, they had upregulated D. farinae-induced CysLT1R expression as compared with WT BMDCs. N-methyl LTE4 activation of CysLT1R on WT BMDCs reduced CysLT1R induction. Thus, CysLT1R negatively regulates both CysLT1R activation and cell surface expression. The opposing functions of CysLT2R and LTC4S may help to maintain a critical balance of two receptors likely influenced by the local concentrations of mRNA for IL-4, IL-5, IL-13, IL-17A, and IFN-γ were measured relative to GAPDH using the Mx3005P Real-Time PCR System (Agilent Technologies, Santa Clara, CA) with gene-specific primers.

**Materials and Methods**

**Generation of Cysltr2/Ltc4s−/− mice**

Cysltr2−/− and Cysltr2−/− mice generated from C57BL/6 embryonic stem cells (19, 20) were maintained by breeding with C57BL/6 mice (Charles River Laboratories), and N15 and N5 or N6 generations, respectively, were used. Ltc4s−/− mice were obtained from a colony established in our laboratory. The Cysltr2−/− male and Cysltr2−/− females were bred to obtain Cysltr2−/−/Ltc4s−/− males and females. The Cysltr2−/−/Ltc4s−/− mice were further intercrossed to obtain Cysltr2/Ltc4s−/− mice. The Cysltr2/Ltc4s−/− mice were viable and had no apparent abnormalities up to at least 8 mo of age. WT littermates from breeding for Cysltr1−/−, Cysltr2−/−, and Cysltr2/Ltc4s−/− strains were used. Both mutant and WT mice were 8–12 wk old when studied. All animal studies were approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute.

**Active sensitization and challenge with repeated intranasal injections of D. farinae**

Mice received either 1 µg of D. farinae (Greer Laboratories, Lenoir, NC) or saline intranasally twice per week for 3 wk as described (11) (Figs. 1, 2) or 0.5 µg of D. farinae on day 0 and 4 and 0.1 µg on day 15 and day 18 (Supplemental Fig. 1). Two days after the last injection, mice were killed with i.p. injection of pentobarbital. The latter protocol was adjusted for mouse strains used. Both mutant and WT mice were 8–12 wk old when studied. All animal studies were approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute.

**Flow cytometry**

Day 7 BMDCs were pulsed with either PBS or 50 µg/ml of D. farinae (Greer Laboratories, Lenoir, NC). Flow cytometry was performed on day 7 after the last challenge. Cytokine production by peribronchial lymph node cells after ex vivo restimulation with D. farinae

**Measurement of total IgE and D. farinae-specific IgG1**

Sera were collected by cardiac puncture 2 d after the last intranasal injection. Total IgE was determined with an ELISA kit (BD Biosciences, San Jose, CA). D. farinae-specific IgG1 was measured as described (22).

**Cytokine production by peribronchial lymph node cells after ex vivo restimulation with D. farinae**

Two days after the last intranasal injection, three peribronchial lymph nodes (LNs) were excised from each mouse and homogenized. The cell suspensions were filtered through a 70-µm cell strainer, centrifuged at 300 × g for 5 min at room temperature, and resuspended in RPMI 1640 medium containing heat-inactivated 10% FBS. After the total number of cells was counted for each mouse, cells were cultured at 4 × 10^6 cells/ml (100 µl) in the presence of 20 µg/ml D. farinae in a 96-well plate for 72 h. The concentrations of IL-4, IL-5, IL-13, IL-17A, and IFN-γ in the supernatants were measured with ELISA kits (eBiosciences, San Diego, CA).

**Measurement of cytokine mRNA expression in the lung**

Total RNA was isolated from the right lungs with TRIzol reagent (Invi- trogen, Carlsbad, CA) according to the manufacturer’s protocol. Quantities of mRNA for IL-4, IL-5, IL-13, IL-17A, and IFN-γ were measured relative to GAPDH using the Mx3005P Real-Time PCR System (Agilent Technologies, Santa Clara, CA) with gene-specific primers.

**Transfer of D. farinae-pulsed BMDCs into mice and D. farinae challenge**

Adoptive transfer of D. farinae-pulsed BMDCs into naive mice was performed as described previously (11, 12). Bone marrow cells were harvested from femurs and tibiae of each mouse and cultured in RPMI medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, 50 µM 2-ME, and recombinant mouse GM-CSF as described (23). Floating cells were harvested on day 7 and pulsed with either PBS or 50 µg/ml of D. farinae at a concentration of 1 × 10^6 cells/ml in a 35-mm culture dish (Sumitomo Cellight X; Sumitomo Bakelite, Japan) for 24 h. In some experiments, BMDCs were preincubated with N-methyl LTE4 (Cayman Chemical, Ann Arbor, MI) for 10 min before D. farinae stimulation. The next day, the BMDCs were washed twice with PBS and resuspended in PBS; 1 × 10^4 cells in 25 µl were transferred intranasally to recipients. The cells were routinely greater than 85% CD11c+. At days 10 and 14 after DC transfer, recipient mice were challenged with 3 µg D. farinae intranasally; 2 d after the last challenge, mice were killed by i.p. injection of pentobarbital. BAL fluid analysis, lung histology, and assessment of cytokine production were performed as described above.

**Histology**

The lung tissues were excised, and the left lung was fixed and stained as described previously (10). For general morphology, tissue sections were stained with H&E. The extent of cellular infiltration in the bronchovascular bundles was assessed in a blinded manner. Congo red staining was used to identify eosinophils, and periodic acid-Schiff staining was used to assess mucus and goblet cells. The slides were analyzed with a Leica DM LB microscope (Leica Microsystems, Germany). The pictures were taken with a Nikon digital camera DXM 1200 with Nikon ACT-I (version 2.70) image acquisition software.

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CysLT1R deficiency increases *D. farinae*-induced pulmonary inflammation. (A) Inflammatory cell counts in BAL fluid. For active sensitization and challenge, WT, *Cysltr1*−/−, *Cysltr2*−/−, and *Ltc4s*−/− mice received 1 μg of *D. farinae* (filled columns) or PBS (open columns) by intranasal injection twice per week for 3 wk, and BAL was performed 2 d after the last injection. Total and differential cell counts for monocytes/macrophages (MΦs), neutrophils, eosinophils, and lymphocytes are shown. Values are the means ± SEM (n = 8–10) combined from three independent experiments. (B) Histologic analyses of the lung. After BAL, lung tissues were fixed with paraformaldehyde and stained with H&E, Congo red, or periodic acid-Schiff (PAS). Eosinophils are indicated by arrows in Congo red staining, and mucus is stained in purple in PAS. Scale bars, 100 μm for H&E and PAS, 50 μm for Congo red. *p < 0.05 versus *D. farinae*-challenged WT; b, bronchi; v, vessels.

ml RB34, a custom generated polyclonal rabbit anti-CysLT1R IgG against a peptide in the third extracellular loop of CysLT1R (Orbigen) (17) and allophycocyanin-conjugated donkey anti-rabbit IgG. Nonspecific rabbit IgG (Jackson ImmunoResearch) was used as a control. For total cell CysLT1R expression, BMDCs were fixed with 100% ice cold methanol at −20 °C for 5 min, permeabilized with saponin (eBiosciences), and stained as above. Analyses were performed on a FACSCanto II flow cytometer (BD Biosciences), and data were analyzed with the FlowJo 7.5.

Cys-LT and cytokine measurement

Cys-LTs in the supernatants of stimulated cells were measured by enzyme immunoassay according to the manufacturer’s protocol (Cayman Chemical). TNF-α, IL-6, and IL-10 were measured by ELISA (eBiosciences). Measurement of CysLT1R transcript in BMDCs

Total RNA was isolated from day 7 BMDCs with TRIzol reagent (Invitrogen), according to the manufacturer’s protocol. Quantities of mRNA for CysLT1R transcript were measured relative to GAPDH using the Mx3005P Real-Time PCR System (Agilent Technologies) with gene-specific primers.

Quantification of phospho ERK in response to LTD4

Day 8–9 BMDCs from WT, *Cysltr1*−/−, and *Cysltr2*−/− mice were harvested, washed in PBS, and resuspended and plated in HBSS at 2.5 × 10^4 cells in a 96-well plate provided with InstantOne ELISA (eBiosciences). Cells were incubated at 37 °C for 1–2 h prior to the addition of ligand to allow for equilibration. Cells were then incubated for 5 min with various concentrations of LTD4 in ethanol or for 0, 2, 5, and 10 min with 300 nM of LTD4. Cells were then lysed in the 96-well plate by adding lysis buffer provided in the InstantOne ELISA kit and shaking at ~300 rpm for 10 min at room temperature. Phospho ERK1/2 was quantified with InstantOne ELISA according to the manufacturer’s protocol. In some experiments, cells were preincubated with N-methyl LTC4 or HAMI3379 (Cayman Chemical) for 10 min before LTD4 stimulation.

For ERK analysis by Western blot, day 7–8 BMDCs were harvested, washed in PBS, and seeded at 5 × 10^5 cells per 100 μl of serum-free media in 1.5-ml Eppendorf tubes. The cells were stimulated with vehicle (ethanol) or LTD4 at 300 nM in 100 μl of serum-free medium for 0, 2, 5, and 10 min at 37 °C. The cells were then placed on ice, centrifuged, and lysed in 0.1 ml of 50 mM Tris-HCl (pH 8.0) buffer containing 0.15 M NaCl, 0.5% Triton X-100, 0.05% Tween 20, a protease inhibitor mixture (Roche), 10 mM NaN3, and 1 mM Na2VO4 on ice for 20 min. The samples were centrifuged for 5 min at 14,000 × g at 4 °C and the supernatants were transferred to new Eppendorf tubes; 4x SDS/PAGE sample buffer with 10 mM DTT was added to the supernatants, and the samples were boiled for 5 min. Twenty microliters (~8 × 10^5 cells) per sample were resolved by SDS-PAGE on a NuPAGE Novex 10% Bis-Tris gel (Invitrogen) with MOPS running buffer under reducing conditions and transferred to a polyvinylidene fluoride membrane (Bio-Rad). The membranes were incubated with a 1:1000 dilution of rabbit anti-phospho p44/p42 (Cell Signaling) and HRP-conjugated donkey anti-rabbit IgG (Pierce) and developed by ECL (SuperSignal Pico; Pierce) according to the manufacturer’s instructions.

Statistical analysis

Results were expressed as means ± SEM. Student unpaired, two-tailed t test was used for the statistical analysis in cases in which the variance was homogeneous, and Welch’s test was used when the variance was heterogeneous. To compare between multiple genotypes, one-way ANOVAs were used. To compare multiple genotypes over doses or time, two-way ANOVAs were used with Bonferroni posttests. A value of p < 0.05 was considered significant.
Results

CysLT R negatively regulates D. farinae-induced Th2 immunity. CysLT R-dependent pulmonary inflammation, serum levels of total IgE, and D. farinae-specific IgG1, and Th2 cytokine expression

To examine the role of the CysLT R in Th2 immune function in vivo, we assessed D. farinae-elicted pulmonary inflammation in C57BL/6 WT, Cysltr1−/−, Cysltr2−/−, and Ltc4s−/− mice. Mice received intranasal injections of 1 µg of D. farinae or PBS twice per week for 3 wk and were killed for assays 48 h after the last injection. D. farinae-challenged WT mice had a significantly increased total cellular infiltration in BAL fluid (p < 0.01) compared with monocytes/macrophages, neutrophils, eosinophils, and lymphocytes as compared with PBS-challenged WT mice (Fig. 1A). D. farinae-challenged Cysltr1−/− and Ltc4s−/− mice showed significantly reduced total cellular infiltration in BAL fluid, as compared with D. farinae-challenged WT mice. In contrast, D. farinae-challenged Cysltr2−/− mice had significant increases in BAL fluid cell numbers of neutrophils, eosinophils, and lymphocytes as compared with D. farinae-challenged WT mice.

Histologic analysis of the bronchovascular bundles in the lung showed a modest cellular infiltration in WT mice that was largely absent in Cysltr1−/− and Ltc4s−/− mice, but was marked in Cysltr2−/− mice (Fig. 1B; H&E). Congo red staining revealed that the eosinophilic infiltration present in WT mice was more prominent in Cysltr2−/− mice and virtually absent in Cysltr1−/− or Ltc4s−/− mice (Fig. 1B; Congo red). Goblet cell metaplasia with mucus production was particularly prominent in D. farinae-challenged Cysltr2−/− mice, compared with similarly challenged WT mice, as assessed by periodic acid-Schiff staining, and was absent in D. farinae-challenged Cysltr1−/− and Ltc4s−/− mice (Fig. 1B; PAS). Thus, D. farinae-induced pulmonary inflammation is dependent on the integrity of LTC4S and CysLT1R and negatively regulated by CysLT2R.

After sensitization and challenge with D. farinae, WT mice had significant increases in serum total IgE and D. farinae-specific IgG1 as compared with PBS-challenged WT mice (p < 0.01; Fig. 2A). Cysltr1−/− and Ltc4s−/− mice had significantly less total serum IgE and no increase in D. farinae-specific IgG1 as compared with D. farinae-challenged WT mice. In contrast, D. farinae-challenged Cysltr2−/− mice had significantly increased levels of total IgE (∼2-fold) as compared with similarly challenged WT mice. D. farinae-challenged Cysltr2−/− mice also had a significantly increased total IgE level at baseline as compared with PBS-challenged WT mice (p < 0.05). Thus, CysLT R negatively regulates Th2-mediated Ig responses at baseline as well as with sensitization and challenge.

To assess the T cell cytokine expression profile in the lungs after sensitization and challenge with D. farinae, total RNA was isolated from the right lungs of WT, Cysltr1−/−, Cysltr2−/−, and Ltc4s−/− mice, and quantitative RT-PCR for IL-4, IL-5, IL-13, IL-17A, and IFN-γ was performed. In WT mice, sensitization and challenge
with *D. farinae* significantly increased the expression of mRNAs for IL-4, IL-5, and IL-13, but not of mRNAs for IL-17A or IFN-\(\gamma\), as compared with PBS-challenged WT mice (\(p<0.01\); Fig. 2B and data not shown). *D. farinae*-challenged Cysltr1\(^{-/-}\) and Ltc4s\(^{-/-}\) mice had significantly reduced mRNAs for IL-4, IL-5, and IL-13 as compared with *D. farinae*-challenged WT mice. In contrast, *D. farinae*-challenged Cysltr2\(^{-/-}\) mice had significant additional increases in expression of IL-4, IL-5, and IL-13 (Fig. 2B), but not IL-17A and IFN-\(\gamma\) (data not shown), as compared with *D. farinae*-challenged WT mice.

To determine whether the profile of T cell cytokine production was similar in the thoracic draining LNs, the LN cells were dissociated, counted, and restimulated with *D. farinae* for 72 h, and cytokine concentrations in the supernatants were measured with ELISAs. The total number of LN cells was similar among *D. farinae*-challenged WT, Cysltr1\(^{-/-}\), and Ltc4s\(^{-/-}\) mice (Fig. 2C). In contrast, the total number of LN cells from *D. farinae*-challenged Cysltr2\(^{-/-}\) mice was significantly increased by 3-fold as compared with *D. farinae*-challenged WT mice. LN cells from *D. farinae*-sensitized and challenged WT mice generated IL-5, IL-17A, and IFN-\(\gamma\) (Fig. 2C), which were undetectable in mice treated with PBS (data not shown). The amounts of IL-5 and IFN-\(\gamma\) were significantly reduced in *D. farinae*-challenged Cysltr1\(^{-/-}\) and Ltc4s\(^{-/-}\) mice, whereas the amounts of IL-17A were not changed, as compared with *D. farinae*-challenged WT controls. In contrast, the amounts of IL-5, IL-17A, and IFN-\(\gamma\) were significantly increased in *D. farinae*-challenged Cysltr2\(^{-/-}\) mice, as compared with *D. farinae*-challenged WT controls. These results suggest that CysLT\(_2\)R negatively regulates both *D. farinae*-elicited LN hypertrophy and nodal immune responses.

**CysLT\(_2\)R on BMDCs negatively regulates *D. farinae*-induced Ltc4s\(^{-/-}\)-dependent sensitization of WT recipients**

We have previously demonstrated that the presence of LTC4S and of CysLT\(_2\)R on *D. farinae*-pulsed BMDCs was critical for their ability to sensitize WT recipients for subsequent *D. farinae*-elicited pulmonary inflammation (12). To determine whether the exaggerated Th2 responses observed in actively sensitized and challenged Cysltr2\(^{-/-}\) mice (Figs. 1, 2) involved CysLT\(_2\)R regulation of LTC4S-dependent DC sensitization, we generated a Cysltr2\(/Ltc4s^{−/−}\) mouse strain. We then adoptively transferred 1 \(\times\) 10\(^6\) *D. farinae*-pulsed BMDCs from this strain and four other genotypes (WT, Cysltr1\(^{-/-}\), Cysltr2\(^{-/-}\), Ltc4s\(^{-/-}\)) to WT recipients, challenged them with 3 \(\mu\)g of *D. farinae* at days 10 and 14, and killed them for assessment at day 16. WT mice sensitized with *D. farinae*-pulsed WT BMDCs generated a significant (\(p<0.05\)) increase in total BAL fluid cells composed of neutrophils, lymphocytes, and eosinophils, as compared with WT mice sensitized with saline-pulsed WT BMDCs (Fig. 3A). WT mice sensitized with *D. farinae*-pulsed Ltc4s\(^{-/-}\) or Cysltr1\(^{-/-}\) BMDCs had significantly decreased BAL fluid neutrophils and lymphocytes and a trend to reduced eosinophils that was not significant, as compared with mice sensitized with *D. farinae*-pulsed WT BMDCs. In contrast, mice sensitized with *D. farinae*-pulsed Cysltr2\(^{-/-}\) BMDCs responded to challenge with a further significant increase in monocytes/macrophages and eosinophils, but not other cell types.

**FIGURE 3.** CysLT\(_2\)R-deficient BMDCs augment *D. farinae*-induced Ltc4s\(^{-/-}\)-dependent sensitization of WT recipients. BMDCs from WT, Ltc4s\(^{-/-}\), Cysltr1\(^{-/-}\), Cysltr2\(^{-/-}\), and Cysltr2\(/Ltc4s^{−/−}\) mice were pulsed with saline or *D. farinae* at 50 \(\mu\)g/ml for 24 h, and 10\(^6\) cells were administered intranasally to sensitize WT recipients. Recipients were challenged with 3 \(\mu\)g of *D. farinae* intranasally at days 10 and 14 and were killed at day 16 for analyses. (A) Inflammatory cell counts in BAL fluid. Total and differential cell counts for BAL fluid monocytes/macrophages (MPs), neutrophils, eosinophils, and lymphocytes are shown. Values are the means \(\pm\) SEM (n = 7–15 per group) combined from three independent experiments. \(*p<0.05\), \(**p<0.01\). (B) Peribronchial LN cells were harvested, counted, and stimulated with 20 \(\mu\)g/ml *D. farinae* for 72 h. Cytokines in the supernatant were measured by ELISA. Values are the means \(\pm\) SEM (n = 5 for saline and n = 10–15 per group for *D. farinae*) combined from three independent experiments. \(*p<0.05\), \(**p<0.01\).
types, as compared with mice sensitized with D. farinae-pulsed WT BMDCs. This augmented response after adoptive transfer and challenge was abolished in WT mice sensitized with D. farinae-pulsed Cysltr2/Ltc4s+/- BMDCs, suggesting that the CysLT1R effect was dependent on Ltc4S.

To determine whether the increase in BAL fluid inflammatory cells in mice sensitized with D. farinae-pulsed Cysltr2+/- BMDCs included augmented immune responses in the LNs, we assessed total cell numbers of the draining LN and their potential cytokine production by restimulation with D. farinae. As compared with mice sensitized with saline-pulsed WT BMDCs, there was no significant increase in the total number of LN cells from mice sensitized with D. farinae-pulsed WT, Ltc4s+/-, or Cysltr1+/- BMDCs (Fig. 3B). However, mice sensitized with D. farinae-pulsed Cysltr2+/- BMDCs had significantly increased total LN cell numbers relative to mice receiving D. farinae-pulsed WT BMDCs, and these numbers were significantly reduced in mice that received D. farinae-pulsed Cysltr2/Ltc4s+/- BMDCs. These findings suggest the involvement of DC Ltc4S in driving the LN hypertrophy in mice receiving D. farinae-pulsed Cysltr2+/- DCs.

The restimulated LN cells from mice receiving D. farinae-pulsed WT BMDCs generated IL-13, IL-17A, and IFN-γ (Fig. 3B). The amounts of IL-13 and IL-17A, but not IFN-γ, were significantly increased in WT mice sensitized with D. farinae-pulsed Cysltr2+/- BMDCs, as compared with WT mice sensitized with D. farinae-pulsed WT BMDCs, and these increased responses were abolished in WT mice sensitized with D. farinae-pulsed Cysltr2/Ltc4s+/- BMDCs. These results suggest that BMDC CysLT2R negatively regulates both D. farinae-elicted LN hypertrophy and associated Th2 and Th17 immune responses.

To determine whether the protection seen in WT mice sensitized with Cysltr2/Ltc4s+/- BMDCs could be extended to a direct sensitization model, WT, Ltc4s+/-, and Cysltr2+/- mice were injected intranasally with 0.5 μg D. farinae on days 0 and 4, challenged with 0.1 μg D. farinae on days 15 and 18, and sacrificed on day 20. The increase in BAL fluid inflammatory cells (Supplemental Fig. 1A) and the increase in draining LN cell counts (Supplemental Fig. 1B) seen in Cysltr2+/- mice were absent in Cysltr2/Ltc4s+/- mice. LN cells from D. farinae-sensitized and challenged Cysltr2+/- mice had robust generation of IL-13 and IL-17A, which was significantly reduced in Cysltr2/Ltc4s+/- mice. The impaired response in Cysltr2/Ltc4s+/- mice was similar to that in Ltc4s+/- mice. The findings suggest that CysLT1R negatively regulates D. farinae-induced, Ltc4S-dependent pulmonary inflammation in mice with either active or adoptive sensitization.

Genetic and pharmacologic approaches demonstrate that CysLT1R regulates LTD4-initiated CysLT1R-dependent ERK phosphorylation in BMDCs

To seek a basis for the augmented in vivo function of D. farinae-pulsed Cysltr2+/- BMDCs, we assessed for upregulation of co-stimulatory molecules, D. farinae-induced cys-LT and cytokine generation, and LTD4/CysLT1R-dependent ERK phosphorylation. There was no difference in the numbers of bone marrow-derived CD11c+ cells generated from the different genotypes and the numbers used for sensitization after a D. farinae pulse were the same. There was also no difference in expression levels of CD80, CD86, CD40, OX40L, and MHC class II by flow cytometric analysis of CD11c+ BMDCs from WT and Cysltr2+/- mice with either PBS or D. farinae pulse (Supplemental Fig. 2A). D. farinae-elicted cys-LT production by Cysltr2+/- BMDCs was comparable to that of WT and Cysltr1+/- BMDCs. There was no cys-LT production by BMDCs from Ltc4s+/- and Cysltr2/Ltc4s+/- mice, as expected (Supplemental Fig. 2B). There was no difference in D. farinae-elicted TNF-α, IL-6, or IL-10 production among BMDCs of any genotype (Supplemental Fig. 2B). There was no IL-4, TGF-β, IL-23p19, or IL-12p70 detected in this response (data not shown).

We next assessed ERK phosphorylation in response to CysLT1R activation by LTD4 as described by Jiang et al. (17) by comparing WT and Cysltr2+/- BMDCs and using Cysltr1+/- BMDCs as a control for specificity. After stimulation with 300 nM of LTD4, WT BMDCs showed ERK phosphorylation that was detectable at 2 min, peaked at 5 min, and returned to baseline by 10 min, whereas Cysltr1+/- BMDCs did not show a response (Fig. 4A, left). ERK phosphorylation in Cysltr2+/- BMDCs was significantly increased at each of these time points, as compared with WT BMDCs. Assessment of ERK phosphorylation by Western blot at each time point (Supplemental Fig. 3) confirmed an in-

![Figure 4](http://www.jimmunol.org/DownloadedFrom)
increased LTD₄-elicited ERK phosphorylation in Cysltr2⁻/⁻ BMDCs, as compared with WT BMDCs, that was absent in Cysltr1⁻/⁻ BMDCs. In a dose-response comparison from 10–300 nM LTD₄ at 5 min, the Cysltr2⁻/⁻ BMDCs had significantly enhanced ERK phosphorylation at 100 and 300 nM, as compared with WT BMDCs (Fig 4A, right).

The increased CysLT₁R signaling in Cysltr2⁻/⁻ BMDCs suggested to us that CysLT₁R might actively regulate CysLT₁R function in WT cells. To more directly examine this possibility, we stimulated WT BMDCs with a newly described selective CysLT₁R agonist, N-methyl LTC₄ (24), at 300 nM for 10 min and assessed the dose response to LTD₄-induced ERK phosphorylation at 5 min. WT BMDCs treated with N-methyl LTC₄ had significantly reduced ERK phosphorylation to 100 and 300 nM LTD₄, as compared with cells treated with vehicle alone (Fig. 4B, left). This suppression was specifically mediated through CysLT₁R, as there was no reduction in ERK phosphorylation in Cysltr2⁻/⁻ BMDCs treated with N-methyl LTC₄ (Fig. 4B, right). Conversely, pretreatment of WT BMDCs for 10 min with 300 nM HAMI3379, a CysLT₂Ra antagonist (25), significantly increased LTD₄-induced ERK phosphorylation at 5 min. This increase was specifically mediated through CysLT₂R, as there was no increase in Cysltr2⁻/⁻ BMDCs. Taken together these studies suggest that CysLT₂R activation in WT BMDCs can suppress CysLT₁R signaling in BMDCs.

Finally, to determine whether CysLT₂R activation in WT BMDCs could negatively regulate D. farinae-induced sensitization of WT recipients, WT BMDCs were pulsed with D. farinae in the presence or absence of 300 nM N-methyl LTC₄ and transferred 24 h later into WT recipients. After D. farinae challenge, mice sensitized with D. farinae-pulsed BMDCs in the presence of N-methyl LTC₄ had a significant reduction in BAL fluid eosinophil counts and a significant reduction in LN cellularity, as compared with mice sensitized with D. farinae-pulsed BMDCs treated with vehicle alone (Supplemental Fig. 4). These results indicate that CysLT₂R activation in WT BMDCs can suppress CysLT₁R-dependent sensitization of WT recipients to D. farinae.

**FIGURE 5.** Increased cell surface CysLT₁R expression on Cysltr2⁻/⁻ BMDCs after D. farinae pulsation. WT, Cysltr1⁻/⁻, and Cysltr2⁻/⁻ BMDCs were incubated with PBS or 50 μg/ml D. farinae for 24 h, stained for the cell surface expression of CysLT₁R, and analyzed by flow cytometry. (A) Representative histograms of CysLT₁R expression after PBS (left) or D. farinae (right) pulsation. Isotype control staining (shaded histograms) and CysLT₁R (open histograms). (B) Mean fluorescence intensities of CysLT₁R staining combined from three independent experiments. Values are the means ± SEM. *p < 0.04 versus PBS-pulsed Cysltr2⁻/⁻ BMDCs. (C) WT and Cysltr2⁻/⁻ BMDCs were preincubated with 300 nM N-methyl LTC₄ or saline for 10 min and then stimulated with PBS or 50 μg/ml D. farinae for 24 h, stained for the cell surface expression of CysLT₁R, and analyzed by flow cytometry. Mean fluorescence intensities of CysLT₁R staining combined from three independent experiments. Values are the means ± SEM. **p = 0.01 versus PBS-pulsed Cysltr2⁻/⁻ BMDCs.
and then pulsed with *D. farinae* had no upregulation of CysLT1R at 24 h (Fig. 5C), whereas Cysltr2−/− BMDCs treated with this agonist still showed substantial upregulation of CysLT1R, demonstrating that CysLT-R activation in WT BMDCs can regulate CysLT1R cell surface expression in addition to CysLT1R signaling.

To determine whether the enhanced expression of CysLT1R on Cysltr2−/− BMDCs was ligand-dependent, we assessed CysLT1R expression on *D. farinae*-pulsed WT, Cysltr2−/−, and Cysltr2/Ltc4s−/− BMDCs. Whereas *D. farinae*-pulsed Cysltr2−/− BMDCs responded with a significant increase in CysLT1R expression, Cysltr2/Ltc4s−/− BMDCs showed no increase in CysLT1R, with the level being similar to that observed with saline treatment (Fig. 6A). Thus, LTC4S was required for the *D. farinae*-induced and CysLT1R-regulated expression of CysLT1R.

To determine whether CysLT2R regulated *D. farinae*-induced expression of CysLT1R at the level of transcription, we performed quantitative RT-PCR for CysLT1R in WT and Cysltr2−/− BMDCs at 0, 3, 6, 18, and 24 h after the addition of *D. farinae*. There was no induction of CysLT1R transcript in either WT or Cysltr2−/− BMDCs at any time point (Fig. 6B). Because redistribution to the plasma membrane was another possibility, we assessed expression of CysLT1R protein in *D. farinae*-pulsed BMDCs after fixation and permeabilization. CysLT1R expression with permeabilization was not greater in *D. farinae*-pulsed Cysltr2−/− BMDCs as compared with saline-pulsed Cysltr2−/− BMDCs (Fig. 6C). CysLT1R expression was clearly detectable after permeabilization, with staining in *D. farinae*-pulsed WT, Cysltr2−/−, and Cysltr2/Ltc4s−/− BMDCs that was absent in Cysltr1−/− BMDCs (Fig. 6D). The absence of induced transcript, and of an increase in mean fluorescence intensities in permeabilized Cysltr2−/− BMDCs, suggests that CysLT1R regulates *D. farinae*-induced trafficking of CysLT1R to the cell surface.

**Discussion**

Despite recent attention, the pathways activated in DCs that program Th2 responses to allergens are poorly understood. We have previously established that the potent sensitizing capacity of house dust mite for mice is mediated, in part, by its ability to trigger Dectin-2-dependent cys-LT generation and CysLT1R activation on DCs (12). In the current study, we initially found that CysLT-R profoundly and negatively regulates LTC4S- and CysLT1R-dependent Th2 pulmonary inflammation to dust mite in mice actively sensitized and challenged with *D. farinae*. We then established negative regulation at the level of DC-mediated sensitization by demonstrating the enhanced capacity of *D. farinae*-pulsed Cysltr2−/− BMDCs and the reduced capacity of Cysltr2/Ltc4s−/− BMDCs or WT BMDCs activated with a CysLT-R agonist to sensitize WT mice for *D. farinae*-elicited pulmonary inflammation. Although many inflammatory cells are capable of producing and responding to cys-LTs, IgE/FcεRI-independent *D. farinae*-induced cys-LT generation during sensitization/priming is restricted to Dectin-2–expressing DCs and macrophages (26) (our own observations), highlighting the importance of inflammatory mediator generation from these cells. Notably, although WT mice sensitized with Cysltr2/Ltc4s−/− BMDCs were protected from pulmonary inflammation, the degree of protection in directly sensitized and challenged Cysltr2/Ltc4s−/− mice was greater, suggesting that LTC4S present outside the DC compartment also contributes to pathologic inflammation in response to challenge with *D. farinae*.

CysLT1R recognizes both LTC4, the product of LTC4S, and LTD4, the metabolite of LTC4 generated by the action of γ-glutamyl transpeptidase or γ-glutamyl leukotrienease to remove glutamic acid from the glutathione moiety (27, 28). Thus, the generation of sequential ligands for the classical CysLT1R and CysLT-R may contribute to regulation of the biologic consequences of pathway activation. Because studies of human monocyte-derived DCs suggest comparable cell surface staining for CysLT1R and CysLT-R (29), it seems possible that LTD4/CysLT-R conditioning of DCs for their sensitizing function may be critically regulated by the ratio of LTC4 to LTD4 present in tissue and the relative affinities of endogenously expressed CysLT1R and CysLT-R for LTC4 and LTD4 in DCs.

To understand whether cys-LTs could influence the balance of endogenous CysLT1R and CysLT1R function, we turned to in vitro studies of receptor function on BMDCs, supplementing genetic approaches with pharmacologic tools. We found that Cysltr2−/− BMDCs had enhanced LTD4-elicited CysLT1R-mediated ERK phosphorylation, as compared with WT controls, which was significant in both dose- and time-dependent analyses. To our sur-

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**FIGURE 6.** Increased cell surface CysLT1R expression on Cysltr2−/− BMDCs is LTC4S-dependent. (A) WT, Cysltr2−/−, and Cysltr2/Ltc4s−/− BMDCs were incubated with PBS or 50 μg/ml *D. farinae* for 24 h, stained for the cell surface expression of CysLT1R, and analyzed by flow cytometry. Mean fluorescence intensities of CysLT1R staining combined from three independent experiments. Values are the means ± SEM. *p < 0.05 versus PBS-pulsed Cysltr2−/− BMDCs. (B) Relative expression of mRNA for CysLT1R to GAPDH in *D. farinae*-pulsed WT or Cysltr2−/− BMDCs. Values are the means ± SEM combined from two experiments. (C) WT, Cysltr2−/−, and Cysltr2/Ltc4s−/− BMDCs were incubated with PBS or 50 μg/ml *D. farinae* for 24 h, fixed and permeabilized, stained for total CysLT1R, and analyzed by flow cytometry. Values are the means ± SEM combined from three experiments. (D) Representative histograms of CysLT1R expression after *D. farinae* pulsation in WT, Cysltr2−/−, Cysltr2−/−, and Cysltr2/Ltc4s−/− BMDCs. Isotype control staining (shaded histograms) and CysLT1R (open histograms).
prise, we found that pretreatment of WT BMDCs, but not Cysltr2^{−/−} BMDCs, with a selective CysLT_{2R} ligand (N-methyl LTE_{4}) significantly reduced ERK phosphorylation to an equimolar dose of LTD_{4}. The potency of N-methyl LTE_{4} (EC_{50} 46.1 ± 8.7 nM) is similar to that of LTE_{4} (EC_{50} 38.6 ± 6.1 nM) at heterologous expressed murine CysLT_{2R} in an assay for calcium flux. N-methyl LTE_{4} also has comparable potency to LTE_{4} at human CysLT_{2R} for eliciting calcium flux or β-arrestin-2 binding in transfected cells, and for eliciting a vascular leak in transgenic mice overexpressing human CysLT_{2R} (24). Our finding for N-methyl LTE_{4} suppression of LTD_{4}-induced ERK phosphorylation at equimolar dosing suggests that LTD_{4} activity at CysLT_{2R} and in immune cells may be relevant over a range of concentrations comparable to LTD_{4} activity at CysLT_{2R}. The dominant products recovered at 30 min from D. farinae-pulsed BMDCs quantified by enzyme immunoassay with fractions separated by HPLC are LTE_{4} and LTE_{4} in a 1:3 ratio (data not shown). There was little detectable LTD_{4} as expected from its rapid conversion to LTE_{4}, as seen during smooth muscle contraction in vitro or during induced vascular leak in vivo (21, 30). Nonetheless, when we treated WT BMDCs with the CysLT_{2R} antagonist HAMI3379, we observed a small but significant increase in LTD_{4}-initiated CysLT_{2R}-dependent ERK phosphorylation, suggesting that LTD_{4} activity at CysLT_{2R} may also be germane. If each ligand can act at CysLT_{2R}, the timing of LTE_{4} generation and its conversion to LTD_{4} may determine the magnitude of the CysLT_{2R}-mediated proinflammatory signal. We observed no change in the cell surface expression of CysLT_{2R} on WT BMDCs during the time period of ERK phosphorylation and conclude that CysLT_{2R} regulation of CysLT_{2R} is at the signaling level.

GPR17 is a seven-transmembrane receptor that does not bind cys-LTs (25, 31, 32), but can inhibit CysLT_{2R} function in transfected cells by interfering with CysLT_{2R}-LTD_{4} binding without requiring its own ligand activation (31). Knockdown of GPR17 in BM-derived macrophages resulted in enhanced LTD_{4}-initiated calcium flux and GPR17-deficient mice had augmented CysLT_{1R}-dependent ERK phosphorylation to LTD_{4}, whereas inhibition of CysLT_{2R} by the selective antagonist N-methyl LTE_{4} inhibits CysLT_{2R}-dependent ERK phosphorylation to LTD_{4}, whereas inhibition of CysLT_{2R} by the HAMI3379 antagonist augments LTD_{4}-induced ERK phosphorylation. Heterologous desensitization of CysLT_{2R} has been reported for other G protein-coupled receptors, including P2Y_{6}, BLT_{1}, EP_{2}, and EP_{4} (33, 34). Our study extends this concept to the regulation of LTD_{4}/CysLT_{2R} activation by its parent compound, LTE_{4}, acting at CysLT_{R}.

Heterodimerization of CysLT_{2R} with CysLT_{1R} has been demonstrated with human mast cells by fluorescence lifetime imaging microscopy, and knock down of CysLT_{2R} in those cells enhanced their expression of cell surface CysLT_{1R} (17). Although we could see augmented cell surface CysLT_{1R} expression on D. farinae-pulsed Cysltr2^{−/−} BMDCs by 24 h, we did not detect basal differences in CysLT_{1R} expression between WT and Cysltr2^{−/−} BMDCs that would account for their differences in LTD_{4}-elicited, CysLT_{1R}-dependent ERK phosphorylation. Furthermore, we found that treatment with the selective CysLT_{2R} agonist N-methyl LTE_{4} reduced the D. farinae-initiated increase in cell surface CysLT_{2R} expression on WT BMDC at 24 h, suggesting that a biochemical event suppresses both the LTD_{4} and D. farinae responses.

Our studies suggest that CysLT_{2R} regulates CysLT_{1R} expression at the level of receptor trafficking, as we found upregulation of cell surface CysLT_{1R} with D. farinae loading, in the absence of augmented transcript or protein in permeabilized Cysltr2^{−/−} BMDCs. The findings for CysLT_{2R} suppression of D. farinae-induced CysLT_{1R} may reflect reduced anterograde trafficking or recycling to the cell surface or enhanced internalization, each of which have been reported to control G protein coupled receptor activation (35–37). In addition, assessment of Cysltr2/Ltc4s^{−/−} BMDCs with D. farinae loading showed no upregulation of cell surface CysLT_{2R}, implying that the ligand for CysLT_{2R} or the integrity of LTC_{4}S is required for trafficking of this receptor on the cell membrane.

Opposing roles in the generation of Th2 immunity are found in studies of the human CysLT_{1R} and CysLT_{2R} genes in relation to bronchial asthma. A study of 112 subjects from Tristan da Cunha revealed a CysLT_{1R} mutation at G300S to be associated with atopy and asthma in females (38). This mutation decreases the EC_{50} for LTD_{4}-elicited calcium flux (1.6-0.5 nM) and for LTD_{4}-elicited inositol phosphate generation (46.5-6.8 nM) in COS7 transfectants, supporting the notion that enhanced CysLT_{1R} signaling promotes human atopy. Furthermore, a polymorphism in the CysLT_{1R} gene, resulting in a single amino acid substitution M201V, was found in this same population to be associated with atopy (16). In vitro experiments with transient (16) and stable (39) transfectants revealed that the M201V mutant had reduced affinities for LTC_{4} and LTD_{4} increased EC_{50} for calcium flux (8.4-16 nM for LTC_{4} and 17-66 nM for LTD_{4}), and reduced inositol phosphate generation in response to LTC_{4} or LTD_{4} compared with the WT transfectant. This finding suggests that a reduction is CysLT_{1R} function may also promote Th2 immunity in humans, and that the opposing actions of CysLT_{1R} and CysLT_{2R} observed here in the mouse are germane to human disease.

Our studies of the balance between cys-LT receptors highlight their role in regulating both developing Th2 responses and ERK phosphorylation, which has been associated with a Th2-inducing function in DCs. We did not find cys-LT regulation of canonical cytokines associated with Th1, Th2, or Th17 immunity such as TNF-α, IL-12p70, IL-4, IL-10, IL-6, or IL-23. The TLR-2 agonist Pam-3-cys induces DCs to instruct the generation of IL-5 and IL-13 producing CD4^{+} T cells in human in vitro or murine in vivo experiments (40, 41). Activation of murine splenic CD11c^{+} cells with Pam-3-cys triggers ERK phosphorylation, IL-10 production, and suppression of IL-12p70, which is dramatically reduced in ERK1-deficient DCs or in DCs treated with the MEK1/2 inhibitor U0126 (40). This same pattern of ERK phosphorylation and cytokine production is seen in human DCs stimulated with Schistosoma egg Ag (41) or murine DCs stimulated with lacto-N-fucopentaose, a Th2-inducing glycan derived from Schistosoma egg Ag (42). Selective ERK phosphorylation (in the absence of p38 MAPK or JNK phosphorylation) is also triggered in human monocye-derived DCs by the peanut allergen Ara h 1 (43). Although the ERK-dependent functions that promote Th2 immunity are yet to be identified, our data suggest that this signaling can be enhanced or inhibited by CysLT_{1R} or CysLT_{2R} activation, respectively, and that DC-dependent Th2 immune responses to aeroallergens are not merely a default pathway (44), but can be finely regulated by the cys-LT pathway.

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Disclosures
The authors have no financial conflicts of interest.
References


Fig. S1. CysLT₂R deficiency negatively regulates Df-induced LTC₄S-dependent pulmonary inflammation. A. Inflammatory cell counts in BAL fluid. For active sensitization and challenge, WT, Ltc4s⁻/⁻, Cysltr2⁻/⁻, and Cysltr2/ Ltc4s⁻/⁻ mice were intranasally injected with 0.5 μg of Df on days 0 and 4, and 0.1 μg of Df on days 15, and 18. BAL was performed 2 days after the last injection. Total and differential cell counts for monocytes/macrophages (MΦs), neutrophils, eosinophils, and lymphocytes are shown. B. Peribronchial LN cells from the same experiment were harvested, counted, and restimulated with 20 μg/ml Df for 72 h. The concentrations of IL-13 and IL-17A in the culture supernatants are shown. Values are the means ± SEM (n = 4-5) from a representative experiment that was repeated 1 time. *P < 0.05, **P < 0.01.
Fig. S2

A

PBS  \( \text{Df} \)

Rat IgG1

CD86

CD40

OX40L

MHCI\( \text{I} \)

Hamster IgG

CD80

B

\begin{align*}
\text{Cys-LTs (pg/ml)} & \quad \text{WT} \quad \text{Ltc4s}^{-/-} \quad \text{Cysltr1}^{-/-} \quad \text{Cysltr2}^{-/-} \quad \text{Cysltr2/Ltc4s}^{-/-} \\
0 & \quad 10 \quad 50 \quad \text{Df (\mu g/ml)}
\end{align*}

\begin{align*}
\text{TNF-\( \alpha \) (pg/ml)} & \\
0 & \quad 50 \quad \text{Df (\mu g/ml)}
\end{align*}

\begin{align*}
\text{IL-6 (pg/ml)} & \\
0 & \quad 50 \quad \text{Df (\mu g/ml)}
\end{align*}

\begin{align*}
\text{IL-10 (pg/ml)} & \\
0 & \quad 50 \quad \text{Df (\mu g/ml)}
\end{align*}

Fig. S2. (A) WT and Cysltr2\( ^{-/-} \) BMDCs have comparable cell surface expression of costimulatory markers. WT and Cysltr2\( ^{-/-} \) BMDCs were grown to day 7, pulsed with PBS or 50 \( \mu \text{g/ml} \) Df for 24 h, harvested, stained for the cell surface expression of CD11c, MHC class II, CD80, CD86, CD40, and OX40L, and analyzed by flow cytometry. Representative histograms are shown of costimulatory marker expression on CD11c\( ^{+} \) WT (thin black lines) and Cysltr2\( ^{-/-} \) (thick black lines) BMDCs. (B) WT, Cysltr1\( ^{-/-} \), and Cysltr2\( ^{-/-} \) BMDCs have comparable production of cys-LTs, TNF-\( \alpha \), IL-6, and IL-10 in response to Df. WT, Ltc4s\( ^{-/-} \), Cysltr1\( ^{-/-} \), Cysltr2\( ^{-/-} \), and Cysltr2/Ltc4s\( ^{-/-} \) BMDCs were stimulated with the indicated concentrations of Df for 30 min for cys-LTs and with 0 and 50 \( \mu \text{g/ml} \) Df for 4 h for cytokine productions. The supernatants were analyzed by ELISA. Values are the means \( \pm \) SEM combined from two experiments.
Fig. S3. CysLT2R regulates CysLT1R-dependent ERK phosphorylation in BMDCs. WT, Cysltr2−/−, and Cysltr1−/− BMDCs (5.0 x 10^5 cells) were stimulated with 300 nM of LTD4 for 0, 2, 5, and 10 min. Cells were then lysed, and phospho-ERK1/2 (P-ERK) and total ERK were quantified by Western blot analysis using rabbit polyclonal anti-phospho p44/p42 ERK1/2 antibody or polyclonal anti-total p44/p42 ERK1/2 antibody and horseradish peroxidase-conjugated donkey anti-rabbit IgG. Results are from a single experiment repeated 3 times with BMDCs from different animals.
Fig. S4. N-methyl LTC₄ treatment of Df-pulsed BMDCs suppresses eosinophilic pulmonary inflammation and LN hypertrophy. WT BMDCs were pulsed with saline or Df at 50 μg/ml for 24 h in the presence of 300 nM N-methyl LTC₄ or vehicle control. 10⁴ cells were administered intranasally to sensitize WT recipients. Recipients were challenged with 3 μg of Df intranasally at days 10 and 14 and were killed at day 16 for analyses. A. BAL fluid eosinophils. B. Peribronchial LN cell counts. Values are the mean ± SEM (n = 5) from a representative experiment, repeated 1 time. **P < 0.01.