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

Nora A. Barrett,¹ James M. Fernandez,¹ Akiko Maekawa, Wei Xing, Li Li, Matthew W. Parsons, K. Frank Austen, and Yoshihide Kanaoka

Cysteinyl 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 CysLT₂R negatively regulates LTC₄S- and CysLT₁R-dependent DC-mediated sensitization. CysLT₂R-deficient BMDCs had increased CysLT₁R-dependent LTD₄-induced ERK phosphorylation, whereas N-methyl LTC₄ activation of CysLT₂R 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 was upregulated at 24 h by a pulse of *D. farinae*, compared with WT or CysLT₂R/LTC₄S-deficient BMDCs. Importantly, treatment with N-methyl LTC₄ reduced *D. farinae*-induced CysLT₁R expression on WT BMDCs. Thus, CysLT₂R negatively regulates the development of cys-LT-dependent Th2 pulmonary inflammation by inhibiting both CysLT₁R signaling and *D. farinae*-induced LTC₄S-dependent cell surface expression of CysLT₁R on DCs. Furthermore, 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: 000–000.

The cysteinyl leukotrienes (cys-LTs), leukotriene (LT) C₄, LTD₄, and LTE₄, are generated from arachidonic acid through the 5-lipoxygenase and LTC₄ synthase (LTC₄S) pathway (1, 2). They have been implicated in the pathobiology of bronchial asthma on the basis of their potent bronchoconstrictive activity at the type 1 cys-LT receptor, CysLT₁R (3). However, CysLT₁R, the high-affinity receptor for LTD₄, is expressed not only on human smooth muscle cells of the airway and microvasculature but also on hematopoietic cells, such as mast cells, macrophages, eosinophils, basophils, and dendritic cells (DCs), that participate in

innate and adaptive immune responses (4–6). Indeed, an influence of cys-LTs in Th2 immunity has been observed in clinical trials in which CysLT₁R antagonists lower serum total and Ag-specific IgE in children with asthma or food allergy (7, 8).

In mouse models of allergic pulmonary inflammation induced by OVA or by extracts from the 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 CysLT₁R-deficient (*Cyslt1*^{−/−}) 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 the importance of the cys-LT/CysLT₁R axis in DCs at the initiation phase of a Th2 immune response.

CysLT₂R is a G protein-coupled receptor with 37–38% amino acid homology to CysLT₁R. CysLT₂R binds both LTC₄ and LTD₄ with ~10-fold less affinity than that of CysLT₁R for LTD₄ in transfected cells (13–15). Evidence of a role for CysLT₂R in the pathobiology of human asthma has been limited by the lack of a specific receptor antagonist. However, a polymorphism in the CysLT₂R gene that produces a mutant CysLT₂R with decreased responsiveness to LTD₄ has been associated with increased atopy, suggesting that CysLT₂R might have a regulatory role in asthma immunobiology (16). CysLT₂R is expressed on human innate immune cells such as mast cells, macrophages, and DCs (5, 17, 18). An in vitro study showed that CysLT₂R could negatively regulate

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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; LN, 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 CysLT₂R can negatively regulate Th2 pulmonary inflammation *in vivo* has not been addressed.

We previously generated CysLT₂R-deficient (*Cysltr2*^{-/-}) mice (19) and now provide CysLT₂R/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 CysLT₂R 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 CysLT₂R on BMDCs increased LTD₄-initiated CysLT₁R-dependent ERK phosphorylation, whereas N-methyl LTC₄ activation of CysLT₂R 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 LTC₄ activation of CysLT₂R on WT BMDCs reduced CysLT₁R induction. Thus, CysLT₂R negatively regulates both CysLT₁R activation and cell surface expression. The opposing functions of CysLT₂R and CysLT₁R on DCs at the level of the allergen sensitization reveal a critical balance of two receptors likely influenced by the local generation of their ligands.

Materials and Methods

Generation of *Cysltr2/Ltc4s*^{-/-} mice

Cysltr1^{-/-} 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 established from 129 derived embryonic stem cells (21) and were backcrossed onto the C57BL/6 background, and N12 generations were used. To generate *Cysltr2/Ltc4s*^{-/-} mice, *Cysltr2*^{-/-} males and *Ltc4s*^{-/-} 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*^{-/-}, *Ltc4s*^{-/-}, 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 a comparable eosinophil response in the bronchoalveolar lavage (BAL) fluid of WT mice with a different batch of *D. farinae*. The LPS levels in batches of *D. farinae* varied by 3.5-fold (from 6.1–21.3 EU/µg protein).

BAL fluid cell analysis

Two days after the last intranasal injection, the trachea was cannulated and BAL fluid was obtained by three repeated lavages with 0.75 ml of Ca²⁺-free and Mg²⁺-free PBS with 1 mM EDTA. The BAL fluid was centrifuged at 500 × g for 5 min. Cells were resuspended in 0.2 ml of PBS with 1% BSA, and the total cells were counted manually with a hemocytometer. For the

differential cell counts of macrophages, neutrophils, eosinophils, and lymphocytes, the cells were cytospun onto a glass slide and stained with Diff-Quik. Cell types in a total of 200 cells were identified by morphologic criteria.

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 LB2 microscope (Leica Microsystems, Germany). The pictures were taken with a Nikon digital camera DXM 1200 with Nikon ACT-1 (version 2.70) image acquisition software.

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). Briefly, 96-well plates were coated with a 5-µg/ml solution of *D. farinae* and incubated with diluted serum followed by alkaline phosphatase-conjugated anti-mouse IgG1 (Southern Biotech, Birmingham, AL) and p-nitrophenyl phosphate substrate (Sigma-Aldrich, St. Louis, MO).

Measurement of cytokine mRNA expression in the lung

Total RNA was isolated from the right lungs with TRIzol reagent (Invitrogen, 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.

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 × 10⁶ 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).

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 × 10⁶ cells/ml in a 35-mm culture dish (Sumilon Celltight X; Sumitomo Bakelite, Japan) for 24 h. In some experiments, BMDCs were preincubated with N-methyl LTC₄ (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 × 10⁴ 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.

Flow cytometry

Day 7 BMDCs were pulsed with either PBS or 50 µg/ml of *D. farinae* for 24 h. Cells were harvested, washed, blocked in PBS containing 1 mM EDTA and 1% mouse IgG (Jackson ImmunoResearch, West Grove, PA), and stained with either rat anti-mouse CD11c-PECy7, MHC class II-PE, CD80-PE, CD86-PE, CD40-PE, OX40L-PE, or isotype controls (BD Biosciences). For CysLT₁R cell surface expression, BMDCs were fixed with 100% ice cold methanol at -20°C for 5 min. Cells were washed with PBS, blocked with 10% donkey serum, and incubated serially with 10 µg/

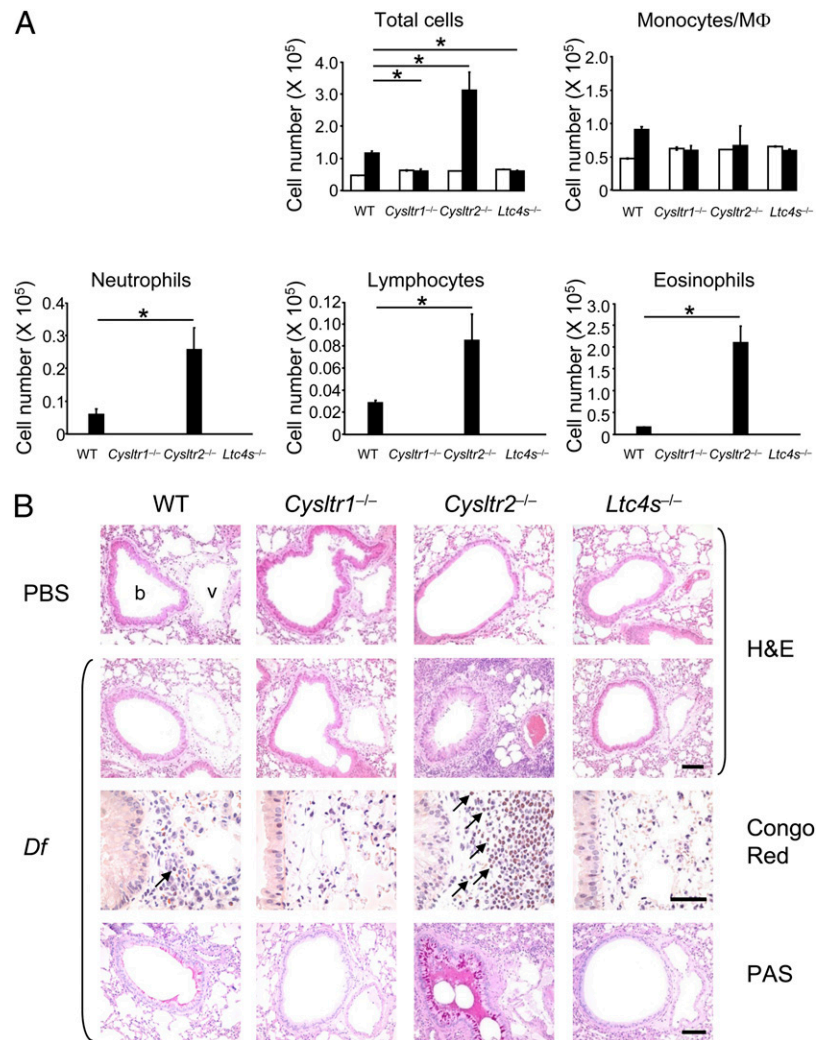


FIGURE 1. CysLT₂R deficiency increases *D. fariniae*-induced pulmonary inflammation. **(A)** Inflammatory cell counts in BAL fluid. For active sensitization and challenge, WT, *Cysltr1*^{-/-}, *Cysltr2*^{-/-}, and *Ltc4s*^{-/-} mice received 1 μg of *D. fariniae* (filled columns) or PBS (open columns) 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. fariniae*-challenged WT; b, bronchi; v, vesicles.

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 × 10⁴ 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 × 10⁵ 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 NaF, and 1 mM Na₃VO₄ 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 × 10⁴ 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 polyclonal anti-phospho p44/p42 ERK1/2 Ab or polyclonal anti-total p44/p42 ERK1/2 Ab (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.

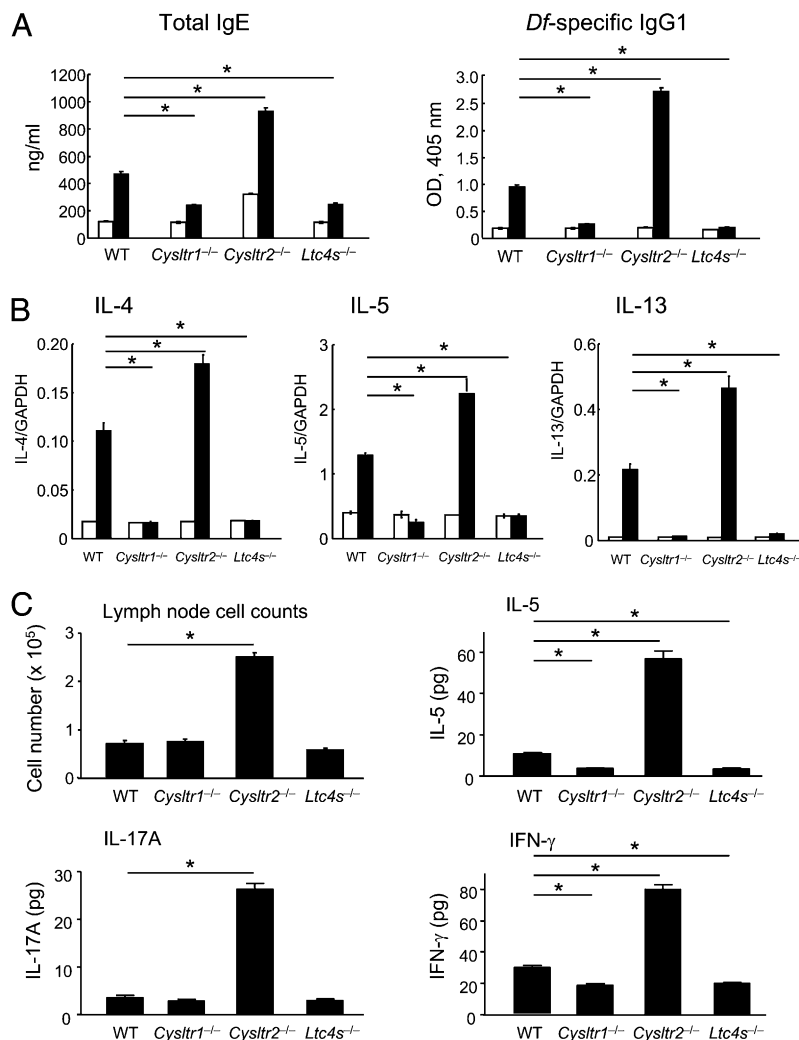


FIGURE 2. CysLT₂R deficiency increases *D. farinae*-induced Th2 immunity. **(A)** Total IgE and *D. farinae*-specific IgG1 in serum of PBS-injected (open columns) or *D. farinae*-challenged (filled columns) are shown. Values are the means $\pm$ SEM ($n = 8-10$) combined from three independent experiments. $*p < 0.05$ versus *D. farinae*-challenged WT. **(B)** Relative expression of mRNA for IL-4, IL-5, IL-13 to GAPDH in lungs of PBS-injected (open columns) or *D. farinae*-challenged (filled columns) mice from two experiments was assessed by quantitative RT-PCR. Values are the means $\pm$ SEM ($n = 5-7$). $*p < 0.05$ versus *D. farinae*-challenged WT. **(C)** Peribronchial LN cells were harvested, counted, and restimulated with 20 μ g/ml *D. farinae* for 72 h. The concentrations of IL-5, IL-17A, and IFN- γ in the culture supernatants are shown. Values are the means $\pm$ SEM ($n = 8-10$) combined from the three independent experiments depicted in Fig. 1. $*p < 0.05$ versus *D. farinae*-challenged WT.

Results

CysLT₂R negatively regulates *D. farinae*-induced *CysLT₁R*-dependent pulmonary inflammation, serum levels of total IgE and *D. farinae*-specific IgG1, and Th2 cytokine expression

To examine the role of the CysLT₂R in Th2 immune function in vivo, we assessed *D. farinae*-elicited pulmonary inflammation in C57BL/6 WT, *Cysltr1*^{-/-}, *Cysltr2*^{-/-}, and *Ltc4s*^{-/-} mice. Mice received intranasal injections of 1 μ g 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 ($p < 0.01$) composed of 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) and of *D. farinae*-specific IgG1 (~ 2.5 -fold) as compared with *D. farinae*-challenged WT mice. PBS-challenged *Cysltr2*^{-/-} mice also had a significantly increased total IgE level at baseline as compared with PBS-challenged WT mice ($p < 0.05$). Thus, CysLT₂R 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- γ , 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- γ (data not shown), as compared with *D. farinae*-challenged WT mice.

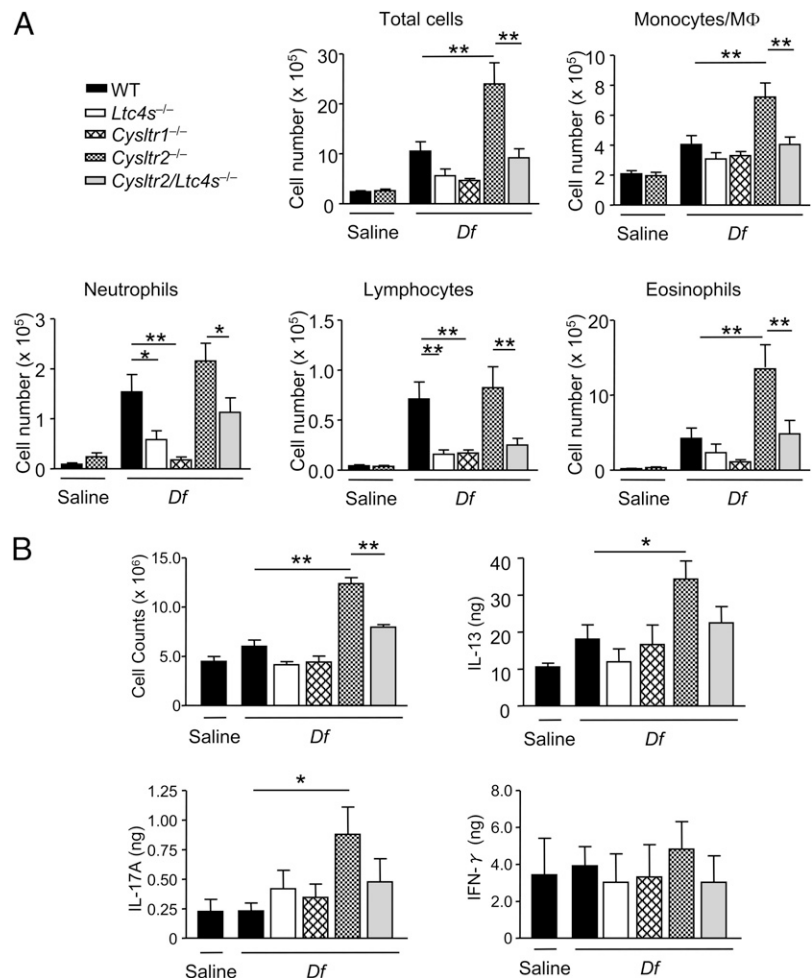
To determine whether the profile of T cell cytokine production was similar in the thoracic draining LNs, the LN cells were dissociated, counted, and restimulated with *D. farinae* for 72 h, and cytokine concentrations in the supernatants were measured with ELISAs. The total number of LN cells was similar 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- γ (Fig. 2C), which were undetectable in mice treated with PBS (data not shown). The amounts of IL-5 and IFN- γ 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- γ were significantly increased in *D. farinae*-challenged *Cysltr2*^{-/-} mice, as compared with *D. farinae*-challenged WT controls. These results

suggest that CysLT₂R negatively regulates both *D. farinae*-elicited LN hypertrophy and nodal immune responses.

CysLT₂R on BMDCs negatively regulates D. farinae-induced LTC₄S-dependent sensitization of WT recipients

We have previously demonstrated that the presence of LTC₄S and of CysLT₁R 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 CysLT₂R regulation of LTC₄S-dependent DC sensitization, we generated a *Cysltr2/Ltc4s*^{-/-} mouse strain. We then adoptively transferred 1×10^4 *D. farinae*-pulsed BMDCs from this strain and four other genotypes (WT, *Cysltr1*^{-/-}, *Cysltr2*^{-/-}, *Ltc4s*^{-/-}) to WT recipients, challenged them with 3 μ 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

FIGURE 3. CysLT₂R-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 μ g/ml for 24 h, and 10^4 cells were administered intranasally to sensitize WT recipients. Recipients were challenged with 3 μ 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 (M Φ s), 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 μ 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 CysLT₂R 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*-elicited 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*^{-/-}, *Cysltr2*^{-/-}, *Cysltr2/Ltc4s*^{-/-} were injected intranasally with 0.5 μ g *D. farinae* on days 0 and 4, challenged with 0.1 μ g *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 CysLT₂R negatively regulates *D. farinae*-induced, LTC₄S-dependent pulmonary inflammation in mice with either active or adoptive sensitization.

Genetic and pharmacologic approaches demonstrate that CysLT₂R 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 costimulatory 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*-elicited 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*-elicited 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-

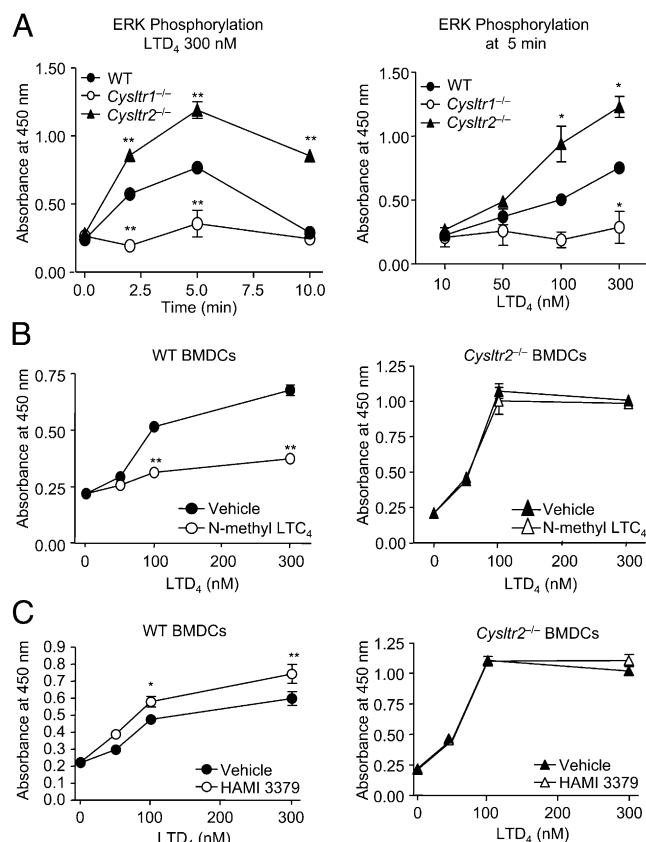


FIGURE 4. CysLT₂R regulates CysLT₁R-dependent ERK phosphorylation in BMDCs. (A) WT, *Cysltr1*^{-/-}, and *Cysltr2*^{-/-} BMDCs (2.5×10^4 cells) were stimulated with 300 nM of LTD₄ for 0, 2, 5, and 10 min (left) or with 10–300 nM of LTD₄ for 5 min (right). Cells were then lysed, and phospho-ERK1/2 was quantified by ELISA. Values are the means $\pm$ SEM ($n = 6$ for WT and *Cysltr2*^{-/-}; $n = 4$ for *Cysltr1*^{-/-}) combined from four independent experiments. * $p < 0.05$; ** $p < 0.01$ versus WT BMDCs. (B) 2.5×10^4 WT BMDCs (left) or *Cysltr2*^{-/-} (right) were preincubated with 300 nM N-methyl LTC₄ or vehicle control for 10 min and then stimulated with increasing doses of LTD₄ for 5 min. Cells were lysed, and phospho-ERK1/2 was quantified by ELISA. Values are the means $\pm$ SEM ($n = 6$ for WT; $n = 4$ for *Cysltr2*^{-/-}) combined from three independent experiments. ** $p < 0.01$ versus vehicle-treated BMDCs. (C) 2.5×10^4 WT BMDCs (left) or *Cysltr2*^{-/-} (right) were preincubated with 300 nM HAMI3379 or vehicle control for 10 min and then stimulated with increasing doses of LTD₄ for 5 min. Cells were lysed, and phospho-ERK1/2 was quantified by ELISA. Values are the means $\pm$ SEM ($n = 6$ for WT; $n = 4$ for *Cysltr2*^{-/-}) combined from three independent experiments. * $p < 0.05$; ** $p < 0.01$ versus vehicle-treated BMDCs.

creased 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 CysLT₂R signaling in *Cysltr2*^{-/-} BMDCs suggested to us that CysLT₂R might actively regulate CysLT₁R function in WT cells. To more directly examine this possibility, we stimulated WT BMDCs with a newly described selective CysLT₂R 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 CysLT₂R, 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 CysLT₂R antagonist (25), significantly increased LTD₄-induced ERK phosphorylation at 100 and 300 nM LTD₄. This increase was specifically mediated through CysLT₂R, as there was no increase in *Cysltr2*^{-/-} BMDCs. Taken together these studies suggest that CysLT₂R activation can negatively regulate CysLT₁R signaling in BMDCs.

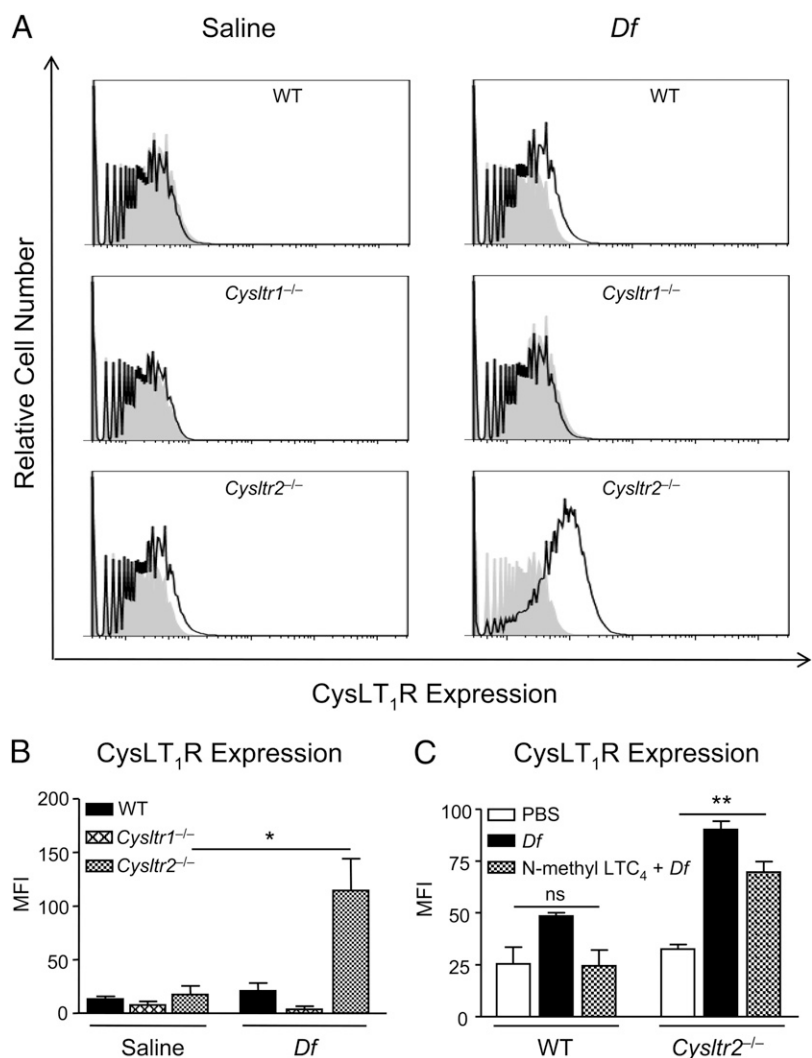
Finally, to determine whether CysLT₂R activation in WT BMDCs could negatively regulate *D. farinae*-induced sensitiza-

tion 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*.

Genetic and pharmacologic approaches demonstrate that CysLT₂R regulates *D. farinae*-induced CysLT₁R expression

To determine whether the enhanced in vivo DC sensitization and enhanced in vitro CysLT₁R-dependent ERK phosphorylation observed in *Cysltr2*^{-/-} BMDCs reflected increased cell surface expression of CysLT₁R, we assessed expression by flow cytometry on WT, *Cysltr1*^{-/-}, and *Cysltr2*^{-/-} BMDCs. At baseline (not shown) or after saline pulse (Fig. 5A, left column) CysLT₁R expression was detectable but not different on WT and *Cysltr2*^{-/-} BMDCs and absent on *Cysltr1*^{-/-} BMDCs. After a 24-h *D. farinae* pulse (Fig. 5A, right column), there was a marginal increase in CysLT₁R expression on WT BMDCs, as compared with saline control, whereas *Cysltr2*^{-/-} BMDCs responded with a significant increase in CysLT₁R that was almost 6-fold that of WT BMDCs (Fig. 5B). WT BMDCs treated for 30 min with N-methyl LTC₄

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 CysLT₂R 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 CysLT₂R-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 CysLT₂R 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 aeroallergens 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 CysLT₂R 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 CysLT₂R 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 production 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*.

CysLT₂R recognizes both LTC₄, the product of LTC₄S, and LTD₄, the metabolite of LTC₄ generated by the action of γ-glutamyl transpeptidase or γ-glutamyl leukotrienase to remove glutamic acid from the glutathione moiety (27, 28). Thus, the generation of sequential ligands for the classical CysLT₂R and CysLT₁R 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 CysLT₂R (29), it seems possible that LTD₄/CysLT₁R 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 CysLT₂R and CysLT₁R for LTC₄ and LTD₄ in DCs.

To understand whether cys-LTs could influence the balance of endogenous CysLT₁R and CysLT₂R 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-

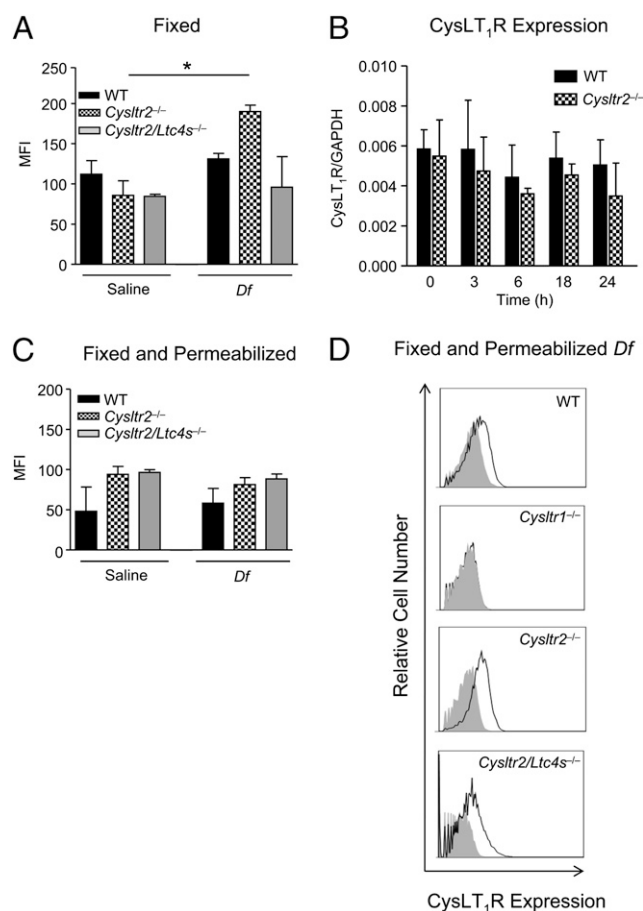


FIGURE 6. Increased cell surface CysLT₁R expression on *Cysltr2*^{-/-} BMDCs is LTC₄S-dependent. **(A)** WT, *Cysltr2*^{-/-}, and *Cysltr2/Ltc4s*^{-/-} BMDCs were incubated with PBS or 50 μg/ml *D. farinae* for 24 h, stained for the cell surface expression of CysLT₁R, and analyzed by flow cytometry. 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, *Cysltr1*^{-/-}, *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 CysLT₂R ligand (N-methyl LTC₄) significantly reduced ERK phosphorylation to an equimolar dose of LTD₄. The potency of N-methyl LTC₄ (EC₅₀ 46.1 ± 8.7 nM) is similar to that of LTC₄ (EC₅₀ 38.6 ± 6.1 nM) at heterologous expressed murine CysLT₂R in an assay for calcium flux. N-methyl LTC₄ also has comparable potency to LTC₄ at human CysLT₂R for eliciting calcium flux or β-arrestin-2 binding in transfected cells, and for eliciting a vascular leak in transgenic mice overexpressing human CysLT₂R (24). Our finding for N-methyl LTC₄ suppression of LTD₄-induced ERK phosphorylation at equimolar dosing suggests that LTC₄ activity at CysLT₂R in immune cells may be relevant over a range of concentrations comparable to LTD₄ activity at CysLT₁R. The dominant products recovered at 30 min from *D. farinae*-pulsed BMDCs quantified by enzyme immunoassay with fractions separated by HPLC are LTC₄ and LTE₄ in a 1:3 ratio (data not shown). There was little detectable LTD₄ as expected from its rapid conversion to LTE₄, 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 CysLT₂R antagonist HAMI3379, we observed a small but significant increase in LTD₄-initiated CysLT₁R-dependent ERK phosphorylation, suggesting that LTD₄ activity at CysLT₂R may also be germane. If each ligand can act at CysLT₂R, the timing of LTC₄ generation and its conversion to LTD₄ may determine the magnitude of the CysLT₁R-mediated proinflammatory signal. We observed no change in the cell surface expression of CysLT₁R on WT BMDCs during the time period of ERK phosphorylation and conclude that CysLT₂R regulation of CysLT₁R is at the signaling level.

GPR17 is a seven-transmembrane receptor that does not bind cys-LTs (25, 31, 32), but can inhibit CysLT₁R function in transfected cells by interfering with CysLT₁R-LTD₄ binding without requiring its own ligand activation (31). Knockdown of GPR17 in BM-derived macrophages resulted in enhanced LTD₄-initiated calcium flux and GPR17-deficient mice had augmented CysLT₁R-dependent Th2 pulmonary inflammation to *D. farinae* (11, 31). In contrast to the ligand-independent regulation of CysLT₁R by GPR17, we find that negative regulation by CysLT₂R in BMDCs is ligand-dependent. Activation of CysLT₂R in WT BMDCs by the selective agonist N-methyl LTC₄ inhibits CysLT₁R-dependent ERK phosphorylation to LTD₄, whereas inhibition of CysLT₂R by the HAMI3379 antagonist augments LTD₄-induced ERK phosphorylation. Heterologous desensitization of CysLT₁R has been reported for other G protein-coupled receptors, including P2Y₆, BLT₁, EP₂, and EP₄ (33, 34). Our study extends this concept to the regulation of LTD₄/CysLT₁R activation by its parent compound, LTC₄, acting at CysLT₂R.

Heterodimerization of CysLT₂R with CysLT₁R has been demonstrated with human mast cells by fluorescence lifetime imaging microscopy, and knock down of CysLT₂R in those cells enhanced their expression of cell surface CysLT₁R (17). Although we could see augmented cell surface CysLT₁R expression on *D. farinae*-pulsed *Cysltr2*^{-/-} BMDCs by 24 h, we did not detect basal differences in CysLT₁R expression between WT and *Cysltr2*^{-/-} BMDCs that would account for their differences in LTD₄-elicited, CysLT₁R-dependent ERK phosphorylation. Furthermore, we found that treatment with the selective CysLT₂R agonist N-methyl LTC₄ reduced the *D. farinae*-initiated increase in cell surface CysLT₁R expression on WT BMDC at 24 h, suggesting that a biochemical event suppresses both the LTD₄ and *D. farinae* responses.

Our studies suggest that CysLT₂R regulates CysLT₁R expression at the level of receptor trafficking, as we found upregulation of

cell surface CysLT₁R with *D. farinae* loading, in the absence of augmented transcript or protein in permeabilized *Cysltr2*^{-/-} BMDCs. The findings for CysLT₂R suppression of *D. farinae*-induced CysLT₁R 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 CysLT₁R, implying that the ligand for CysLT₁R or the integrity of LTC₄S 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 CysLT₁R and CysLT₂R genes in relation to bronchial asthma. A study of 112 subjects from Tristan da Cunha revealed a CysLT₁R mutation at G300S to be associated with atopy and asthma in females (38). This mutation decreases the EC₅₀ for LTD₄-elicited calcium flux (1.6–0.5 nM) and for LTD₄-elicited inositol phosphate generation (46–5.6 nM) in COS7 transfectants, supporting the notion that enhanced CysLT₁R signaling promotes human atopy. Furthermore, a polymorphism in the CysLT₂R 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 LTC₄ and LTD₄, increased EC₅₀s for calcium flux (8.4–16 nM for LTC₄ and 17–66 nM for LTD₄), and reduced inositol phosphate generation in response to LTC₄ or LTD₄ compared with the WT transfectant. This finding suggests that a reduction in CysLT₂R function may also promote Th2 immunity in humans, and that the opposing actions of CysLT₁R and CysLT₂R 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 UO126 (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 monocyte-derived DCs by the peanut allergen Ara h1 (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 CysLT₁R or CysLT₂R 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.

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Fig. S1

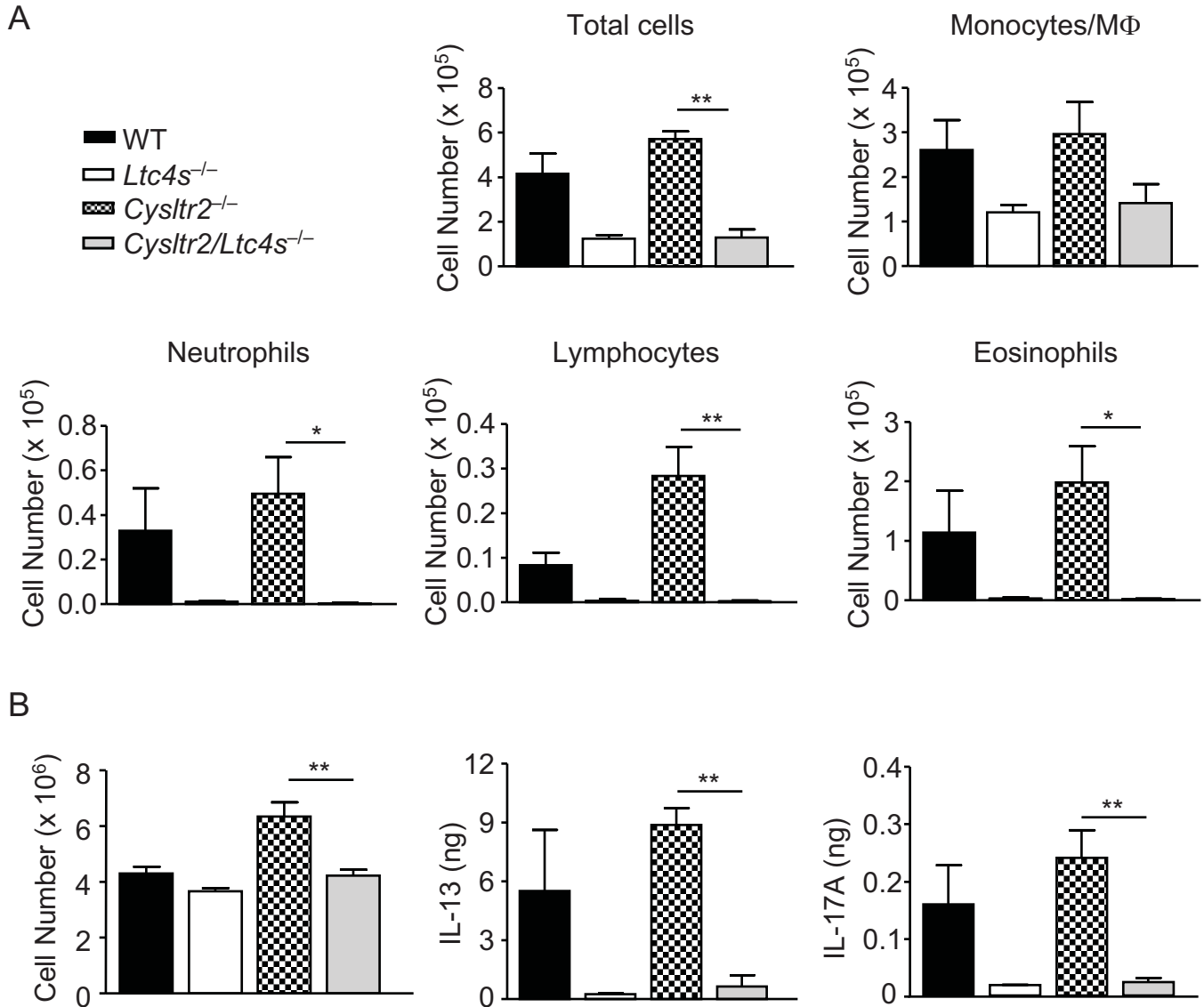
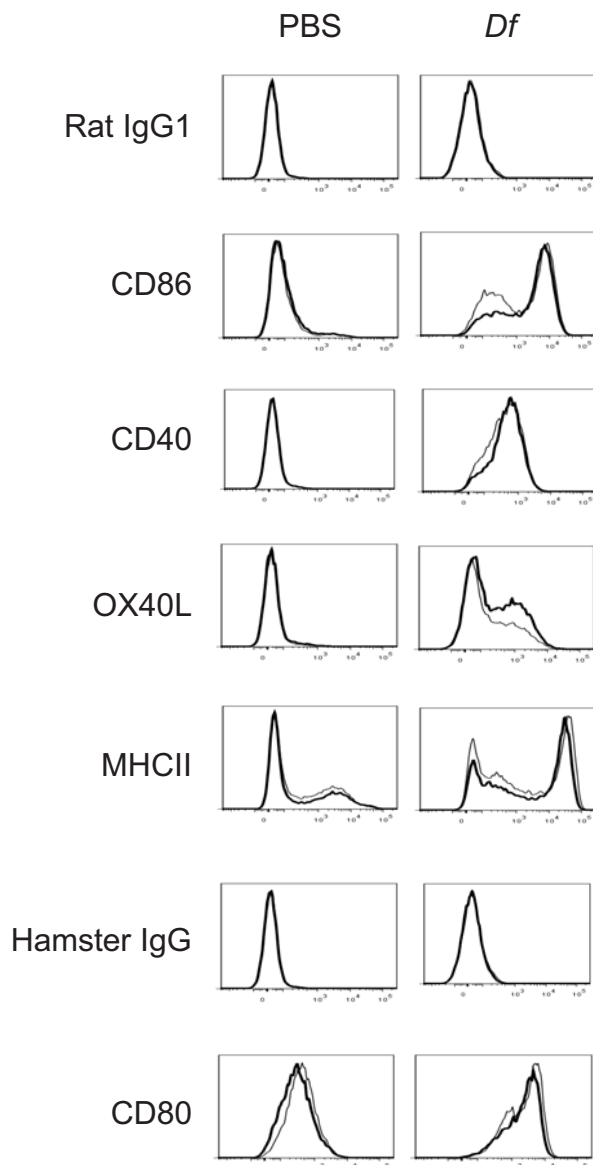


Fig. S1. CysLT₂R deficiency negatively regulates *Df*-induced LTC₄S-dependent pulmonary inflammation. A. Inflammatory cell counts in BAL fluid. For active sensitization and challenge, WT, *Ltc4s*^{-/-}, *Cysltr2*^{-/-}, and *Cysltr2/Ltc4s*^{-/-} mice were intranasally injected with 0.5 µg of *Df* on 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



B

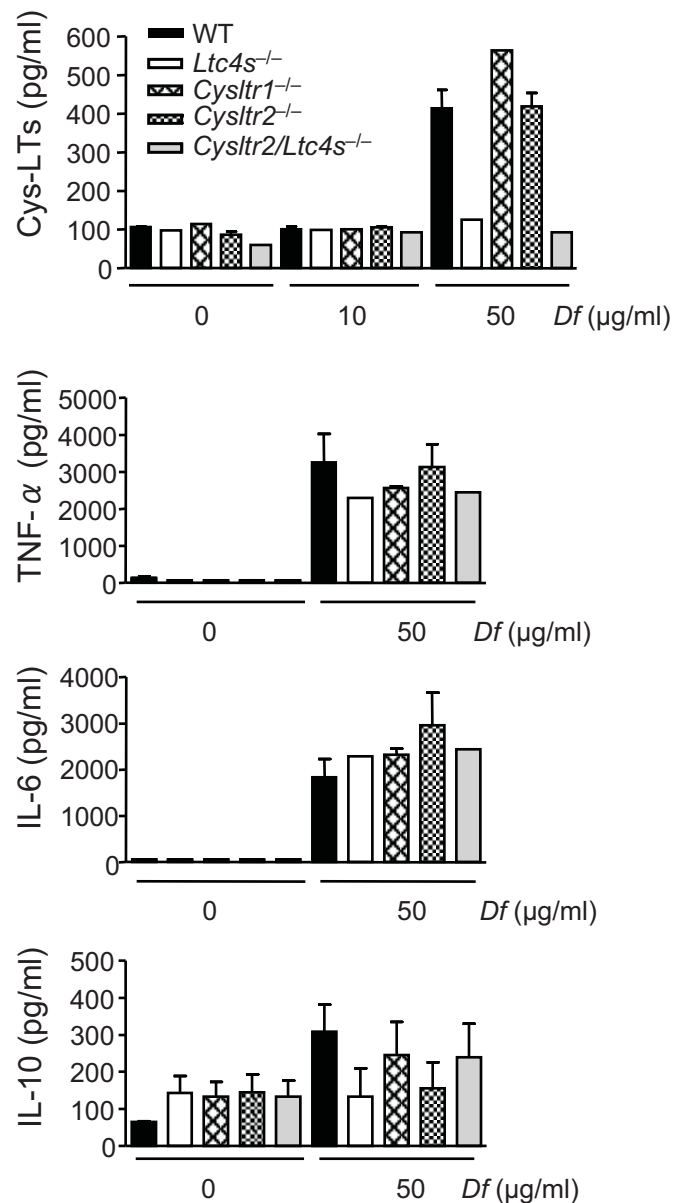


Fig. S2. (A) WT and *Cysltr2*^{-/-} BMDCs have comparable cell surface expression of costimulatory markers. WT and *Cysltr2*^{-/-} BMDCs were grown to day 7, pulsed with PBS or 50 μg/ml *Df* for 24 h, harvested, stained for the cell surface expression of CD11c, MHC class II, CD80, CD86, CD40, and OX40L, 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-α, 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 μg/ml *Df* for 4 h for cytokine productions. The supernatants were analyzed by ELISA. Values are the means ± SEM combined from two experiments.

Fig. S3

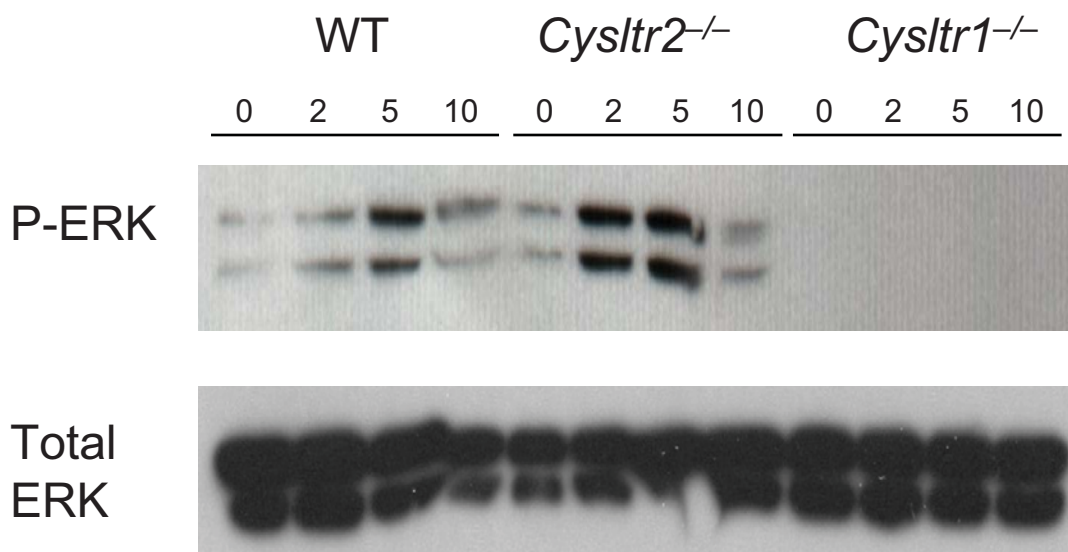


Fig. S3 . CysLT₂R regulates CysLT₁R-dependent ERK phosphorylation in BMDCs. WT, *Cysltr2*^{-/-}, and *Cysltr1*^{-/-} BMDCs (5.0×10^5 cells) were stimulated with 300 nM of LTD₄ for 0, 2, 5, and 10 min. Cells were then lysed, and phospho-ERK1/2 (P-ERK) and total ERK were quantified by Western blot analysis 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

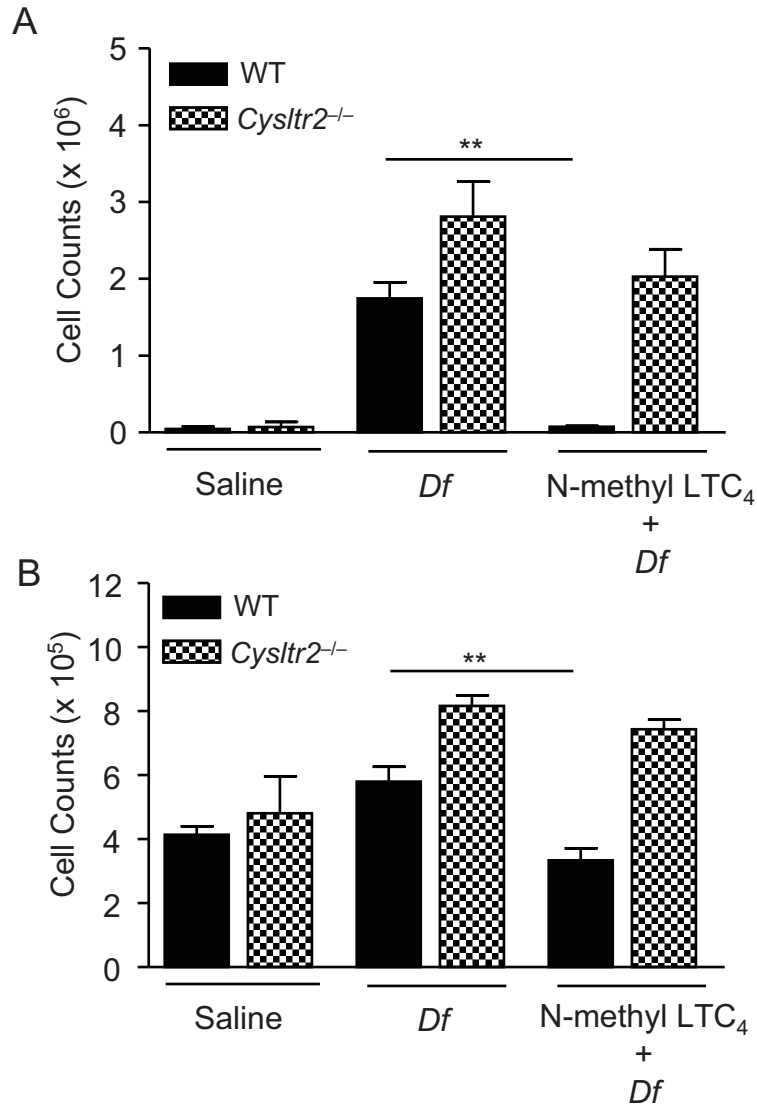


Fig. S4. N-methyl LTC₄ treatment of *Df*-pulsed BMDCs suppresses eosinophilic pulmonary inflammation and LN hypertrophy. WT BMDCs were pulsed with saline or *Df* at 50 µg/ml for 24 h in the presence of 300 nM N-methyl LTC₄ or vehicle control. 10⁴ cells were administered intranasally to sensitize WT recipients. 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.