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

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Abstract

Cysteinyl leukotrienes (cys-LTs) can mediate Th2 immunity to the house dust mite, *Dermatophagoides farinae* (*Df*), via the type 1 receptor $CysLT_1R$ on dendritic cells (DCs). However, the role of the homologous type 2 receptor $CysLT_2R$ in Th2 immunity is unknown. *Df* sensitization and challenge of $CysLT_2R$-deficient mice showed a marked augmentation of eosinophilic pulmonary inflammation, serum IgE, and Th2 cytokines. Wild-type (WT) mice sensitized by adoptive transfer of *Df*-pulsed $CysLT_2R$-deficient bone marrow-derived DCs (BMDCs) also had a marked increase in *Df*-elicited eosinophilic lung inflammation and Th2 cytokines in restimulated hilar nodes. This response was absent in mice sensitized with *Df*-pulsed BMDCs lacking leukotriene C_4 synthase (LTC_4S), $CysLT_1R$, or both $CysLT_2R/LTC_4S$, suggesting that $CysLT_2R$ negatively regulates LTC_4S- and $CysLT_1R$-dependent DC-mediated sensitization. $CysLT_2R$-deficient BMDCs had increased $CysLT_1R$-dependent LTD_4-induced ERK phosphorylation, whereas N-methyl LTC_4 activation of $CysLT_2R$ on WT BMDCs reduced such signaling. Activation of endogenously expressed $CysLT_1R$ and $CysLT_2R$ occurred over an equimolar range of LTD_4 and N-methyl LTC_4, respectively. Although the baseline expression of cell surface $CysLT_1R$ was not increased on $CysLT_2R$-deficient BMDCs, it was upregulated at 24 h by a pulse of *Df*, as compared to WT or $CysLT_2R/LTC_4S$-deficient BMDCs. Importantly, treatment with N-methyl LTC_4 reduced *Df*-induced $CysLT_1R$ expression on WT BMDCs. Thus, $CysLT_2R$ negatively regulates the development of cys-LT-dependent Th2 pulmonary inflammation by inhibiting both $CysLT_1R$ signaling and *Df*-induced LTC_4S-dependent cell surface expression of $CysLT_1R$ on DCs. Furthermore, these studies highlight how the biologic activity of cys-LTs can be tightly regulated by competition between these endogenously expressed receptors.

Keywords

Knockout mouse; Lipid mediator; G protein-coupled receptor; Asthma; Lung

INTRODUCTION

The cysteinyl leukotrienes (cys-LTs), leukotriene C_4 (LTC_4), LTD_4, and LTE_4, are generated from arachidonic acid through the 5-lipoxygenase and LTC_4 synthase (LTC_4S) pathway (1, 2). They have been implicated in the pathobiology of bronchial asthma on the

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basis of their potent bronchoconstrictive activity at the type 1 cys-LT receptor, $CysLT_1R$ (3). However, $CysLT_1R$, the high-affinity receptor for LTD_4, 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 where $CysLT_1R$ antagonists lower serum total and antigen-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* (*Df*), pharmacologic antagonism of $CysLT_1R$ or genetic deletion of LTC_4S or $CysLT_1R$ attenuates Th2 pulmonary inflammation (9–12). In vitro *Df* stimulation of bone marrow-derived DCs (BMDCs) triggers the robust generation of cys-LTs, and the adoptive transfer of *Df*-pulsed BMDCs from LTC_4S-deficient (*Ltc4s*$^{-/-}$) or $CysLT_1R$-deficient (*Cysltr1*$^{-/-}$) mice to sensitize naïve wild-type (WT) recipients has shown that each protein is needed to prime for Th2 pulmonary inflammation after *Df* challenge (12). These studies highlight the importance of the cys-LT/$CysLT_1R$ axis in DCs at the initiation phase of a Th2 immune response.

$CysLT_2R$ is a G protein-coupled receptor with 37–38% amino acid homology to $CysLT_1R$. $CysLT_2R$ binds both LTC_4 and LTD_4 with ~10-fold less affinity than that of $CysLT_1R$ for LTD_4 in transfected cells (13–15). Evidence of a role for $CysLT_2R$ in the pathobiology of human asthma has been limited by the lack of a specific receptor antagonist. However, a polymorphism in the $CysLT_2R$ gene that produces a mutant $CysLT_2R$ with decreased responsiveness to LTD_4 has been associated with increased atopy, suggesting that $CysLT_2R$ may play a regulatory role in asthma immunobiology (16). $CysLT_2R$ is expressed on human innate immune cells such as mast cells, macrophages, and DCs (5, 17, 18). An in vitro study showed that $CysLT_2R$ could negatively regulate $CysLT_1R$ expression and LTD_4-elicited mitogenic response in human mast cells (17). Whether $CysLT_2R$ can negatively regulate Th2 pulmonary inflammation in vivo has not been addressed.

We previously generated $CysLT_2R$-deficient (*Cysltr2*$^{-/-}$) mice (19), and now provide $CysLT_2R$/LTC_4S-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 *Df* sensitization and challenge. Analysis of the sensitization function by adoptive transfer of *Df*-pulsed BMDCs showed that *Cysltr2*$^{-/-}$ BMDCs generated markedly enhanced responses to *Df* challenge, while the lack of $CysLT_1R$ or LTC_4S or the double deficiency of $CysLT_2R$ and LTC_4S 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 $CysLT_1R$ function, respectively, and assessed $CysLT_1R$-dependent ERK phosphorylation and $CysLT_1R$ expression in BMDCs from relevant deficient strains. We found that pharmacologic inhibition or genetic deficiency of $CysLT_2R$ on BMDCs increased LTD_4-initiated $CysLT_1R$-dependent ERK phosphorylation, whereas N-methyl LTC_4 activation of $CysLT_2R$ on WT BMDCs was inhibitory. Although there was no increase in baseline $CysLT_1R$ cell surface expression on *Cysltr2*$^{-/-}$ BMDCs, they had upregulated *Df*-induced $CysLT_1R$ expression as compared to WT BMDCs. N-methyl LTC_4 activation of $CysLT_2R$ on WT BMDCs reduced $CysLT_1R$ induction. Thus, $CysLT_2R$ negatively regulates both $CysLT_1R$ activation and cell surface expression. The opposing functions of $CysLT_2R$ and $CysLT_1R$ 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.

MATERALS 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), 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 *Df*

Mice received either 1 μg of *Df* (Greer Laboratories, Lenoir, NC) or saline intranasally twice per week for 3 wks as described (11) (Figs. 1 & 2) or 0.5 μg of *Df* on day 0 and 4 and 0.1 μg on day 15 and day 18 (Fig. S1). 2 d after the last injection, mice were killed by 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 *Df*. The LPS levels in batches of *Df* varied by 3.5 fold (from 6.1 EU/μg protein - 21.3 EU/μg protein).

BAL fluid cell analysis

2 d after the last intranasal injection, the trachea was cannulated and BAL fluid was obtained by three repeated lavages with 0.75 ml of Ca^{2+}- and Mg^{2+}-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, and 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 hematoxylin and eosin (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 by a Nikon digital camera DXM 1200 with Nikon ACT-1 (version 2.70) image acquisition software.

Measurement of total IgE and *Df*-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). *Df*-specific IgG1 was measured as described (22). Briefly, 96-well plates were coated with a 5 μg/ml solution of *Df* and incubated with diluted serum followed by alkaline phosphatase-conjugated anti-mouse IgG1 (SouthernBiotech, 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 (LN) cells after ex vivo restimulation with *Df*

2 d after the last intranasal injection, three peribronchial 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 RPMI1640 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 *Df* 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 *Df*-pulsed BMDCs into mice and *Df* challenge

Adoptive transfer of *Df*-pulsed BMDCs into naïve mice was carried out as described (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 *Df* at a concentration of 1×10^6 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 LTC_4 (Cayman Chemical, Ann Arbor, MI) for 10 min before *Df* 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 *Df* 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 in the lungs were performed as described above.

Flow cytometry

Day 7 BMDCs were pulsed with either PBS or 50 μg/ml of *Df* 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 $CysLT_1R$ 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/ml RB34, a custom generated polyclonal rabbit anti-$CysLT_1R$ IgG against a peptide in the 3rd extracellular loop of $CysLT_1R$ (Orbigen) (17) and allophycocyanin-conjugated donkey anti-rabbit IgG. Nonspecific rabbit IgG (Jackson Immunoresearch) was used as a control. For total cell $CysLT_1R$ 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 $CysLT_1R$ 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 $CysLT_1R$ 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 LTD_4

Day 8–9 BMDCs from WT, *Cysltr1*$^{-/-}$, and *Cysltr2*$^{-/-}$ mice were harvested, washed in PBS and resuspended and plated in Hanks' balanced salt solution at 2.5×10^4 cells in a 96-well plate provided with InstantOneELISA™ (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 LTD_4 in ethanol or for 0, 2, 5, 10 min with 300 nM of LTD_4. 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 LTC_4 or HAMI3379 (Cayman Chemical) for 10 min before LTD_4 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 LTD_4 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 Na_3VO_4 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. 4 × SDS/PAGE sample buffer with 10 mM DTT was added to the supernatants and the samples were boiled for 5 min. Twenty microliters (about 8×10^4 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 1:1,000 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's 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, and 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

$CysLT_2R$ negatively regulates *Df*-induced $CysLT_1R$-dependent pulmonary inflammation, serum levels of total IgE and *Df*-specific IgG1, and Th2 cytokine expression

To examine the role of the $CysLT_2R$ in in vivo Th2 immune function, we assessed *Df*-elicited pulmonary inflammation in C57BL/6 WT, *Cysltr1*$^{-/-}$, *Cysltr2*$^{-/-}$, and *Ltc4s*$^{-/-}$ mice. Mice received intranasal injections of 1 μg of *Df* or PBS twice per week for 3 wks and were killed for assays 48 h after the last injection. *Df*-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 to PBS-challenged WT mice (Fig. 1A). *Df*-challenged *Cysltr1*$^{-/-}$ and *Ltc4s*$^{-/-}$ mice showed significantly reduced total cellular infiltration in BAL fluid, as compared to *Df*-challenged WT mice. In contrast, *Df*-challenged *Cysltr2*$^{-/-}$ mice had significant increases in BAL fluid cell numbers of neutrophils, eosinophils, and lymphocytes as compared to *Df*-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 *Df*-challenged *Cysltr2*$^{-/-}$ mice, compared to similarly challenged WT mice, as assessed by periodic acid-Schiff staining, and was absent in *Df*-challenged *Cysltr1*$^{-/-}$ and *Ltc4s*$^{-/-}$ mice (Fig. 1B, PAS). Thus, *Df*-induced pulmonary inflammation is dependent on the integrity of LTC_4S and $CysLT_1R$ and negatively regulated by $CysLT_2R$.

After sensitization and challenge with *Df*, WT mice had significant increases in serum total IgE and *Df*-specific IgG1 as compared to PBS-challenged WT mice ($p < 0.01$) (Fig. 2A). *Cysltr1*$^{-/-}$ and *Ltc4s*$^{-/-}$ mice had significantly less total serum IgE and no increase in *Df*-specific IgG1 as compared to *Df*-challenged WT mice. In contrast, *Df*-challenged *Cysltr2*$^{-/-}$ mice had significantly increased levels of total IgE (~2-fold) and of *Df*-specific IgG1 (~2.5-fold) as compared to *Df*-challenged WT mice. PBS-challenged *Cysltr2*$^{-/-}$ mice also had a significantly increased total IgE level at baseline as compared to PBS-challenged WT mice ($p < 0.05$). Thus $CysLT_2R$ 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 *Df*, 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 *Df* 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 to PBS-challenged WT mice ($p < 0.01$) (Fig. 2B and data not shown). *Df*-challenged *Cysltr1*$^{-/-}$ and *Ltc4s*$^{-/-}$ mice had significantly reduced mRNAs for IL-4, IL-5, and IL-13 as compared to *Df*-challenged WT mice. In contrast, *Df*-challenged *Cysltr2*$^{-/-}$ mice had significant further 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 to *Df*-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 *Df* for 72 h, and cytokine concentrations in the supernatants were measured with ELISAs. The total number of LN cells was similar among *Df*-challenged WT, *Cysltr1*$^{-/-}$, and *Ltc4s*$^{-/-}$ mice (Fig. 2C). In contrast, the total number of LN cells from *Df*-challenged *Cysltr2*$^{-/-}$ mice was significantly increased by 3-fold as compared to *Df*-challenged WT mice. LN cells from *Df* 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 *Df*-challenged *Cysltr1*$^{-/-}$ and *Ltc4s*$^{-/-}$ mice, while the amounts of IL-17A were not changed, as compared to *Df*-challenged WT controls. In contrast, the amounts of IL-5, IL-17A, and IFN-γ were significantly increased in *Df*-challenged *Cysltr2*$^{-/-}$ mice, as compared to *Df*-challenged WT controls. These results suggest that $CysLT_2R$ negatively regulates both *Df*-elicited LN hypertrophy and nodal immune responses.

CysLT$_2$R on BMDCs negatively regulates *Df*-induced LTC$_4$S-dependent sensitization of WT recipients

We have previously demonstrated that the presence of LTC$_4$S and of CysLT$_1$R on *Df*-pulsed BMDCs was critical for their ability to sensitize WT recipients for subsequent *Df* 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 LTC$_4$S-dependent DC sensitization, we generated a *Cysltr2/Ltc4s*$^{-/-}$ mouse strain. We then adoptively transferred 1×10^4 *Df*-pulsed BMDCs from this strain and four other genotypes (WT, *Cysltr1*$^{-/-}$, *Cysltr2*$^{-/-}$, *Ltc4s*$^{-/-}$) to WT recipients, challenged them with 3 μg of *Df* at days 10 and 14, and killed them for assessment at day 16. WT mice sensitized with *Df*-pulsed WT BMDCs generated a significant ($p < 0.05$) increase in total BAL fluid cells, composed of neutrophils, lymphocytes, and eosinophils, as compared to WT mice sensitized with saline-pulsed WT BMDCs (Fig. 3A). WT mice sensitized with *Df*-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 to mice sensitized with *Df*-pulsed WT BMDCs. In contrast, mice sensitized with *Df*-pulsed *Cysltr2*$^{-/-}$ BMDCs responded to challenge with a further significant increase in monocytes/macrophages and eosinophils but not other cell types, as compared to mice sensitized with *Df*-pulsed WT BMDCs. This augmented response after adoptive transfer and challenge was abolished in WT mice sensitized with *Df*-pulsed *Cysltr2/Ltc4s*$^{-/-}$ BMDCs, suggesting that the CysLT$_2$R effect was dependent on LTC$_4$S.

To determine whether the increase in BAL fluid inflammatory cells in mice sensitized with *Df*-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 *Df*. As compared to mice sensitized with saline-pulsed WT BMDCs, there was no significant increase in the total number of LN cells from mice sensitized with *Df*-pulsed WT, *Ltc4s*$^{-/-}$, or *Cysltr1*$^{-/-}$ BMDCs (Fig. 3B). However, mice sensitized with *Df*-pulsed *Cysltr2*$^{-/-}$ BMDCs had significantly increased total LN cell numbers relative to mice receiving *Df*-pulsed WT BMDCs and these numbers were significantly reduced in mice that received *Df*-pulsed *Cysltr2/Ltc4s*$^{-/-}$ BMDCs. These findings suggest the involvement of DC LTC$_4$S in driving the LN hypertrophy in mice receiving *Df*-pulsed *Cysltr2*$^{-/-}$ DCs. The restimulated LN cells from mice receiving *Df*-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 *Df*-pulsed *Cysltr2*$^{-/-}$ BMDCs, as compared to WT mice sensitized with *Df*-pulsed WT BMDCs, and these increased responses were abolished in WT mice sensitized with *Df*-pulsed *Cysltr2/Ltc4s*$^{-/-}$ BMDCs. These results suggest that BMDC CysLT$_2$R negatively regulates both *Df*-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 μg *Df* on days 0 and 4, challenged with 0.1 μg *Df* on days 15 and 18, and sacrificed on day 20. The increase in BAL fluid inflammatory cells (Fig. S1A) and the increase in draining LN cell counts (Fig. S1B) seen in *Cysltr2*$^{-/-}$ mice were absent in *Cysltr2/Ltc4s*$^{-/-}$ mice. LN cells from *Df*-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 CysLT$_2$R negatively regulates *Df*-induced, LTC$_4$S-dependent pulmonary inflammation in mice with either active or adoptive sensitization.

Genetic and pharmacologic approaches demonstrate that $CysLT_2R$ regulates LTD_4-initiated $CysLT_1R$-dependent ERK phosphorylation in BMDCs

To seek a basis for the augmented in vivo function of *Df*-pulsed *Cysltr2*$^{-/-}$ BMDCs, we assessed for upregulation of co-stimulatory molecules, *Df*-induced cys-LT and cytokine generation, and LTD_4/$CysLT_1R$-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 *Df* 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 *Df* pulse (Fig. S2A). *Df*-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 (Fig. S2B). There was no difference in *Df*-elicited TNF-α, IL-6, or IL-10 production among BMDCs of any genotype (Fig. S2B). 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 $CysLT_1R$ activation by LTD_4 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 LTD_4, WT BMDCs showed ERK phosphorylation that was detectable at 2 min, peaked at 5 min, and returned to baseline by 10 min, while *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 to WT BMDCs. Assessment of ERK phosphorylation by Western blot at each time point (Fig. S3) confirmed an increased LTD_4-elicited ERK phosphorylation in *Cysltr2*$^{-/-}$ BMDCs, as compared to WT BMDCs, that was absent in *Cysltr1*$^{-/-}$ BMDCs. In a dose-response comparison from 10–300 nM LTD_4 at 5 min, the *Cysltr2*$^{-/-}$ BMDCs had significantly enhanced ERK phosphorylation at 100 and 300 nM, as compared to WT BMDCs (Fig 4A, right).

The increased $CysLT_1R$ signaling in *Cysltr2*$^{-/-}$ BMDCs suggested to us that $CysLT_2R$ may actively regulate $CysLT_1R$ function in WT cells. To more directly examine this, we stimulated WT BMDCs with a newly described selective $CysLT_2R$ agonist, N-methyl LTC_4 (24), at 300 nM for 10 min and assessed the dose-response to LTD_4-induced ERK phosphorylation at 5 min. WT BMDCs treated with N-methyl LTC_4 had significantly reduced ERK phosphorylation to 100 and 300 nM LTD_4, as compared to cells treated with vehicle alone (Fig. 4B, left). This suppression was specifically mediated through $CysLT_2R$, as there was no reduction in ERK phosphorylation in *Cysltr2*$^{-/-}$ BMDCs treated with N-methyl LTC_4 (Fig. 4B, right). Conversely, pretreatment of WT BMDCs for 10 min with 300 nM HAMI3379, a $CysLT_2R$ antagonist (25), significantly increased LTD_4-induced ERK phosphorylation at 100 and 300 nM LTD_4. This increase was specifically mediated through $CysLT_2R$, as there was no increase in *Cysltr2*$^{-/-}$ BMDCs. Taken together these studies suggest that $CysLT_2R$ activation can negatively regulate $CysLT_1R$ signaling in BMDCs.

Finally, to determine whether $CysLT_2R$ activation in WT BMDCs could negatively regulate *Df*-induced sensitization of WT recipients, WT BMDCs were pulsed with *Df* in the presence or absence of 300 nM N-methyl LTC_4 and transferred 24 h later into WT recipients. After *Df* challenge, mice sensitized with *Df*-pulsed BMDCs in the presence of N-methyl LTC_4 had a significant reduction in BAL fluid eosinophil counts and a significant reduction in LN cellularity, as compared to mice sensitized with *Df*-pulsed BMDCs treated with vehicle alone (Fig. S4). These results indicate that $CysLT_2R$ activation in WT BMDCs can suppress $CysLT_1R$-dependent sensitization of WT recipients to *Df*.

Genetic and pharmacologic approaches demonstrate that $CysLT_2R$ regulates *Df*-induced $CysLT_1R$ expression

To determine whether the enhanced in vivo DC sensitization and enhanced in vitro $CysLT_1R$-dependent ERK phosphorylation observed in *Cysltr2*$^{-/-}$ BMDCs reflected increased cell surface expression of $CysLT_1R$, we assessed expression by flow cytometry on WT, *Cysltr1*$^{-/-}$, and *Cysltr2*$^{-/-}$ BMDCs. At baseline (not shown) or after saline pulse (Fig. 5A, left column) $CysLT_1R$ expression was detectable but not different on WT and *Cysltr2*$^{-/-}$ BMDCs and absent on *Cysltr1*$^{-/-}$ BMDCs. After a 24-h *Df* pulse (Fig. 5A, right column), there was a marginal increase in $CysLT_1R$ expression on WT BMDCs, as compared to saline control, whereas *Cysltr2*$^{-/-}$ BMDCs responded with a significant increase in $CysLT_1R$ that was almost six times that of WT BMDCs (Fig. 5B). WT BMDCs treated for 30 min with N-methyl LTC_4 and then pulsed with *Df* had no upregulation of $CysLT_1R$ at 24 h (Fig. 5C), while *Cysltr2*$^{-/-}$ BMDCs treated with this agonist still showed substantial upregulation of $CysLT_1R$, demonstrating that $CysLT_2R$ activation in WT BMDCs can regulate $CysLT_1R$ cell surface expression in addition to $CysLT_1R$ signaling.

To determine whether the enhanced expression of $CysLT_1R$ on *Cysltr2*$^{-/-}$ BMDCs was ligand-dependent, we assessed $CysLT_1R$ expression on *Df*-pulsed WT, *Cysltr2*$^{-/-}$, and *Cysltr2/Ltc4s*$^{-/-}$ BMDCs. Whereas *Df*-pulsed *Cysltr2*$^{-/-}$ BMDCs responded with a significant increase in $CysLT_1R$ expression, *Cysltr2/Ltc4s*$^{-/-}$ BMDCs showed no increase in $CysLT_1R$, with the level being similar to that observed with saline treatment (Fig. 6A). Thus, LTC_4S was required for the *Df*-induced and $CysLT_2R$-regulated expression of $CysLT_1R$.

To determine if $CysLT_2R$ regulated *Df*-induced expression of $CysLT_1R$ at the level of transcription, we performed quantitative RT-PCR for $CysLT_1R$ in WT and *Cysltr2*$^{-/-}$ BMDCs at 0, 3, 6, 18, and 24 h after the addition of *Df*. There was no induction of $CysLT_1R$ 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 $CysLT_1R$ protein in *Df*-pulsed BMDCs after fixation and permeabilization. $CysLT_1R$ expression with permeabilization was not greater in *Df*-pulsed *Cysltr2*$^{-/-}$ BMDCs as compared to saline-pulsed *Cysltr2*$^{-/-}$ BMDCs (Fig. 6C). $CysLT_1R$ expression was clearly detectable after permeabilization, with staining in *Df*-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 $CysLT_2R$ regulates *Df*-induced trafficking of $CysLT_1R$ 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 $CysLT_1R$ activation on DCs (12). In the present study, we initially found that $CysLT_2R$ profoundly negatively regulates LTC_4S- and $CysLT_1R$-dependent Th2 pulmonary inflammation to dust mite in mice actively sensitized and challenged with *Df*. We then established negative regulation at the level of DC-mediated sensitization by demonstrating the enhanced capacity of *Df*-pulsed *Cysltr2*$^{-/-}$ BMDCs and the reduced capacity of *Cysltr2/Ltc4s*$^{-/-}$ BMDCs or WT BMDCs activated with a $CysLT_2R$ agonist to sensitize WT mice for *Df*-elicited pulmonary inflammation. Although many inflammatory cells are capable of producing and responding to cys-LTs, IgE/FcεRI-independent *Df*-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, while 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 LTC_4S present outside the DC compartment also contributes to pathologic inflammation in response to challenge with *Df*.

$CysLT_2R$ recognizes both LTC_4, the product of LTC_4S, and LTD_4, the metabolite of LTC_4 generated by the action of γ-glutamyl transpeptidase and/or γ-glutamyl leukotrienase to remove glutamic acid from the glutathione moiety (27, 28). Thus, the generation of sequential ligands for the classical $CysLT_2R$ and $CysLT_1R$ may contribute to regulation of the biologic consequences of pathway activation. As studies of human monocyte-derived DCs suggest comparable cell surface staining for $CysLT_1R$ and $CysLT_2R$ (29), it seems possible that LTD_4/$CysLT_1R$ conditioning of DCs for their sensitizing function may be critically regulated by the ratio of LTC_4 to LTD_4 present in tissue and the relative affinities of endogenously expressed $CysLT_2R$ and $CysLT_1R$ for LTC_4 and LTD_4 in DCs.

To understand whether cys-LTs could influence the balance of endogenous $CysLT_1R$ and $CysLT_2R$ 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 LTD_4-elicited, $CysLT_1R$-mediated ERK phosphorylation, as compared to WT controls, that was significant in both dose- and time-dependent analyses. To our surprise, we found that pretreatment of WT BMDCs, but not *Cysltr2*$^{-/-}$ BMDCs, with a selective $CysLT_2R$ ligand, N-methyl LTC_4, significantly reduced ERK phosphorylation to an equimolar dose of LTD_4. The potency of N-methyl LTC_4 (EC_{50} 46.1 ± 8.7 nM) is similar to that of LTC_4 (EC_{50} 38.6 ± 6.1 nM) at heterologously expressed murine $CysLT_2R$ in an assay for calcium flux. N-methyl LTC_4 also has comparable potency to LTC_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 LTC_4 suppression of LTD_4-induced ERK phosphorylation at equimolar dosing suggests that LTC_4 activity at $CysLT_2R$ in immune cells may be relevant over a range of concentrations comparable to LTD_4 activity at $CysLT_1R$. The dominant products recovered at 30 min from *Df*-pulsed BMDCs quantified by enzyme immunoassay with fractions separated by HPLC are LTC_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 $CysLT_1R$-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 LTC_4 generation and its conversion to LTD_4 may determine the magnitude of the $CysLT_1R$-mediated proinflammatory signal. We observed no change in the cell surface expression of $CysLT_1R$ on WT BMDCs during the time period of ERK phosphorylation and conclude that $CysLT_2R$ regulation of $CysLT_1R$ is at the signaling level.

GPR17 is a seven-transmembrane receptor which does not bind cys-LTs (25, 31, 32), but can inhibit $CysLT_1R$ function in transfected cells by interfering with $CysLT_1R$-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 Th2 pulmonary inflammation to *Df* (11, 31). In contrast to the ligand-independent regulation of $CysLT_1R$ by GPR17, we find that negative regulation by $CysLT_2R$ in BMDCs is ligand-dependent. Activation of $CysLT_2R$ in WT BMDCs by the selective agonist N-methyl LTC_4 inhibits $CysLT_1R$-dependent ERK phosphorylation to LTD_4, whereas inhibition of $CysLT_2R$ by the HAMI3379 antagonist

augments LTD_4-induced ERK phosphorylation. Heterologous desensitization of $CysLT_1R$ 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 LTD_4/$CysLT_1R$ activation by its parent compound, LTC_4, acting at $CysLT_2R$.

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). While we could see augmented cell surface $CysLT_1R$ expression on *Df*-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 LTC_4 reduced the *Df*-initiated increase in cell surface $CysLT_1R$ expression on WT BMDC at 24 h, suggesting that a biochemical event suppresses both the LTD_4 and *Df* 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 *Df*-loading, in the absence of augmented transcript or protein in permeabilized *Cysltr2*$^{-/-}$ BMDCs. The findings for $CysLT_2R$ suppression of *Df*-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). Additionally, assessment of *Cysltr2/Ltc4s*$^{-/-}$ BMDCs with *Df*-loading showed no upregulation of cell surface $CysLT_1R$, implying that the ligand for $CysLT_1R$ or the integrity of LTC_4S 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 (from 1.6 nM to 0.5 nM) and for LTD_4-elicited inositol phosphate generation (from 46 nM to 5.6 nM) in COS7 transfectants, supporting the notion that enhanced $CysLT_1R$ signaling promotes human atopy. Furthermore, a polymorphism in the $CysLT_2R$ 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}s for calcium flux (from 8.4 to 16 nM for LTC_4 and from 17 to 66 nM for LTD_4), and reduced inositol phosphate generation in response to LTC_4 or LTD_4 as compared to the WT transfectant. This suggests that a reduction is $CysLT_2R$ 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 Toll-like receptor 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 Shistosoma egg antigen (41) or murine DCs stimulated with lacto-N-

fucopentaose, a Th2-inducing glycan derived from Shistosoma egg antigen (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). While the ERK-dependent function(s) that promotes Th2 immunity is 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used in this paper

BAL	bronchoalveolar lavage
BMDC	bone marrow-derived dendritic cell
cys-LT	cysteinyl leukotriene
$CysLT_1R$ and $CysLT_2R$	type 1 and type 2 cys-LT receptors
DC	dendritic cell
Df	extracts from *Dermatophagoides farinae*
LT	leukotriene
LTC_4S	LTC_4 synthase
LN	lymph node
WT	wild-type

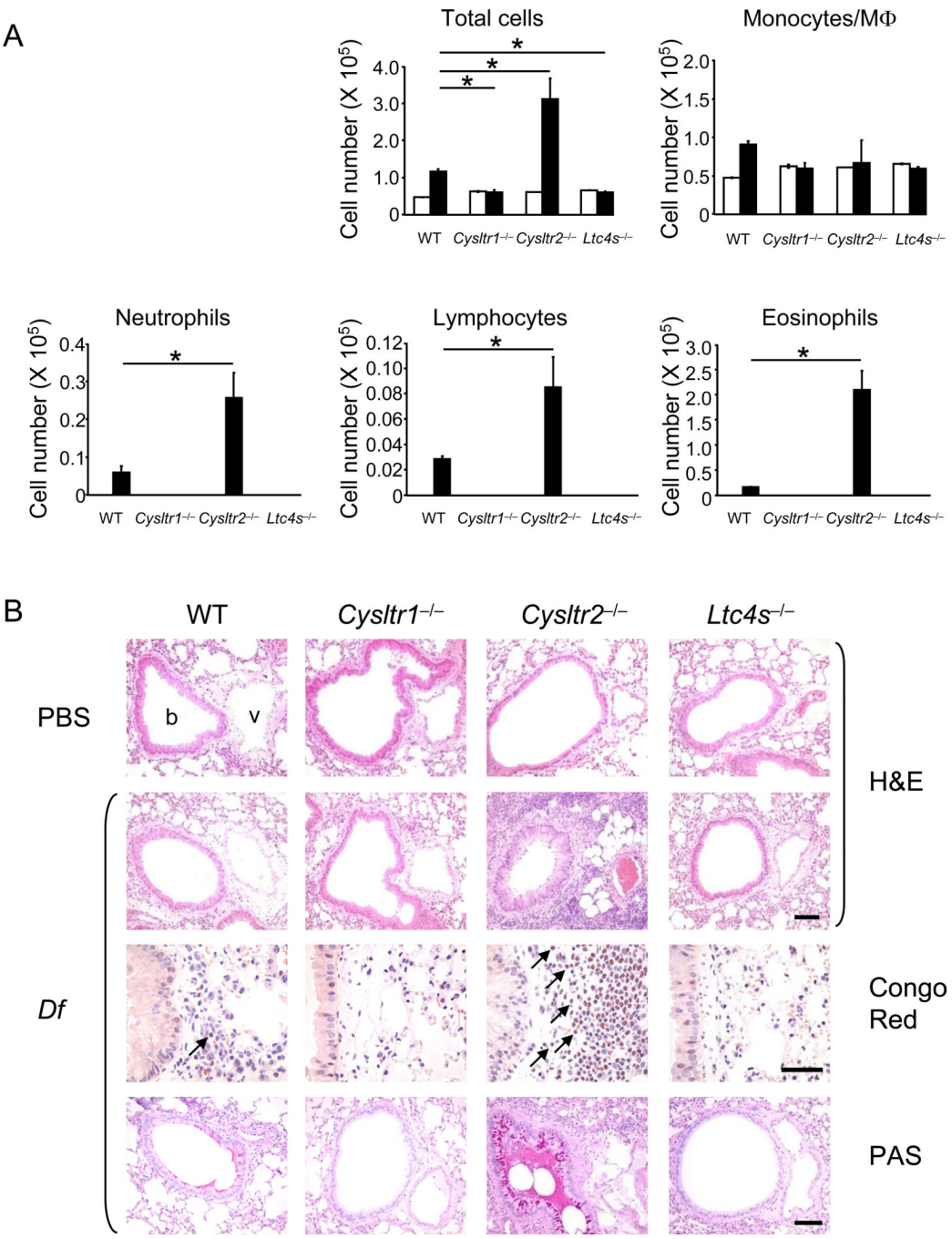

FIGURE 1.

CysLT$_2$R deficiency increases *Df*-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 *Df* (filled columns) or PBS (open columns) by intranasal injection twice per wk for 3 weeks, and BAL was performed 2 days 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 3 independent experiments. *P < 0.05 vs. *Df*-challenged WT. 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). b, bronchi, v, vessels. Eosinophils are indicated

by arrows in Congo red staining, and mucus is stained in purple in PAS. Scale bars = 100 μm for HE and PAS, 50 μm for Congo red.

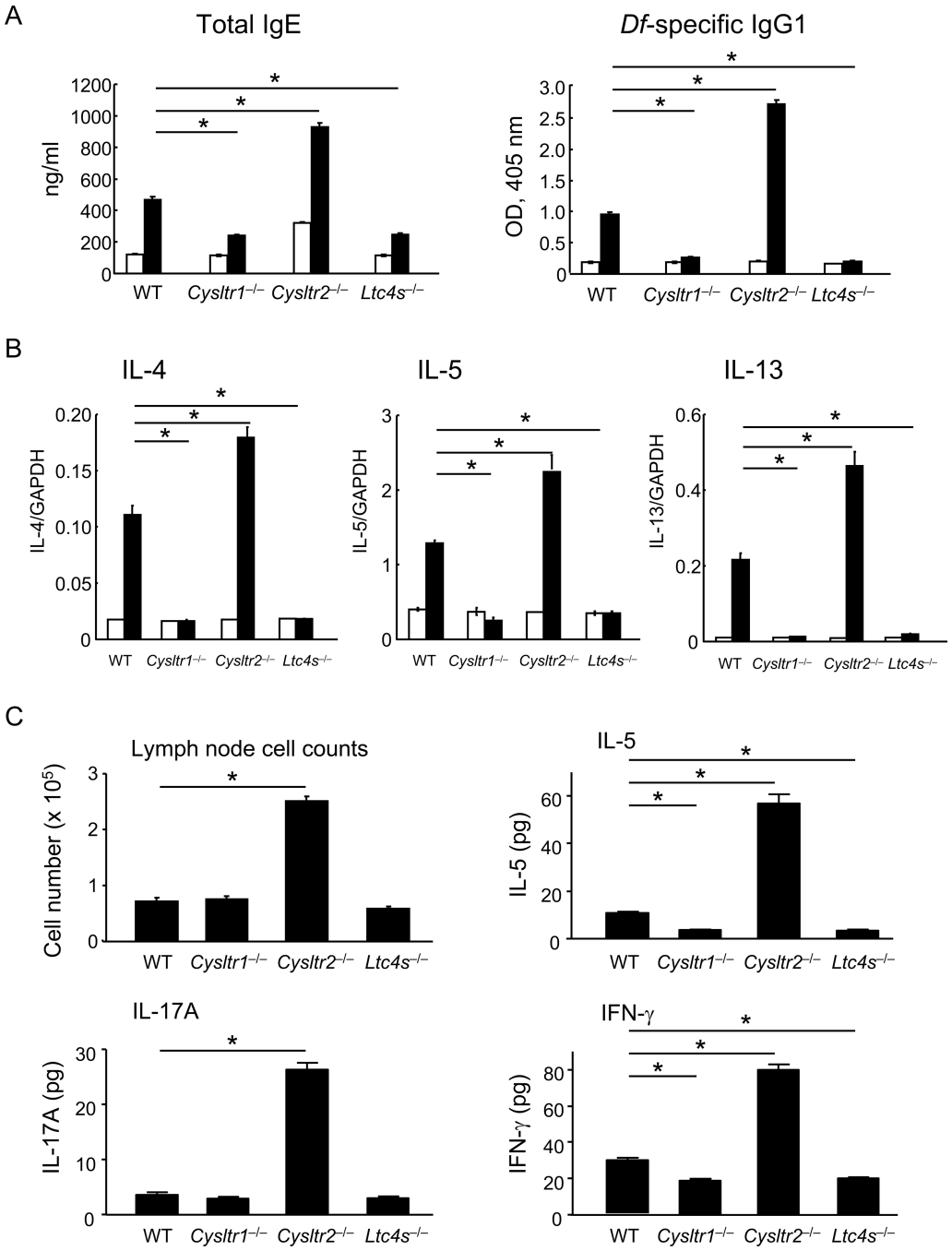

FIGURE 2.

CysLT$_2$R deficiency increases *Df*-induced Th2 immunity. A. Total IgE and *Df*-specific IgG1 in serum of PBS-injected (open columns) or *Df*-challenged (filled columns) are shown. Values are the means ± SEM (n = 8–10) combined from 3 independent experiments. *P < 0.05 vs. *Df*-challenged WT. B. Relative expression of mRNA for IL-4, IL-5, IL-13 to GAPDH in lungs of PBS-injected (open columns) or *Df*-challenged (filled columns) mice from 2 experiments was assessed by quantitative RT-PCR. Values are the means ± SEM (n = 5–7). *P < 0.05 vs. *Df*-challenged WT. C. Peribronchial LN cells were harvested, counted, and restimulated with 20 μg/ml *Df* for 72 h. The concentrations of IL-5, IL-17A, and IFN-γ

in the culture supernatants are shown. Values are the means ± SEM (n = 8–10) combined from the 3 independent experiments depicted in Fig. 1. *P < 0.05 vs. *Df*-challenged WT.

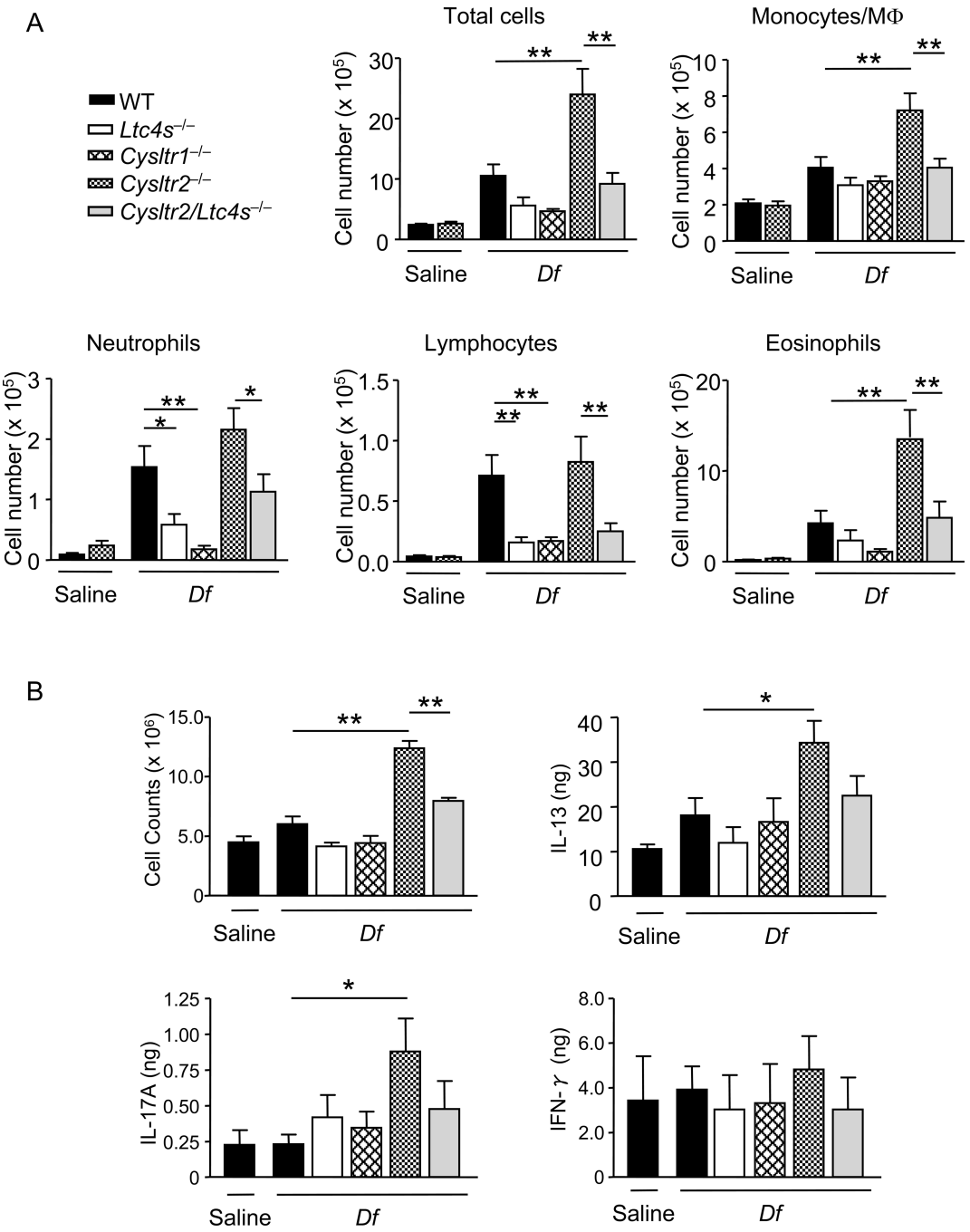

FIGURE 3.

CysLT$_2$R-deficient BMDCs augment *Df*-induced LTC$_4$S-dependent sensitization of WT recipients. BMDCs from WT, *Ltc4s*$^{-/-}$, *Cysltr1*$^{-/-}$, *Cysltr2*$^{-/-}$, and *Cysltr2/Ltc4s* $^{-/-}$ mice were pulsed with saline or *Df* at 50 μg/ml for 24 h, and 10^4 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. Inflammatory cell counts in BAL fluid. Total and differential cell counts for BAL fluid monocytes/macrophages (MΦs), neutrophils, eosinophils, and lymphocytes are shown. Values are the means ± SEM (n = 7–15 per group) combined from 3 independent experiments. *P < 0.05, **P < 0.01. B. Peribronchial LN cells were harvested, counted, and stimulated with 20 μg/

ml *Df* for 72 h. Cytokines in the supernatant were measured by ELISA. Values are the means ± SEM ($n = 5$ for saline and $n = 10–15$ per group for *Df*) combined from 3 independent experiments. $*P < 0.05$, $**P < 0.01$.

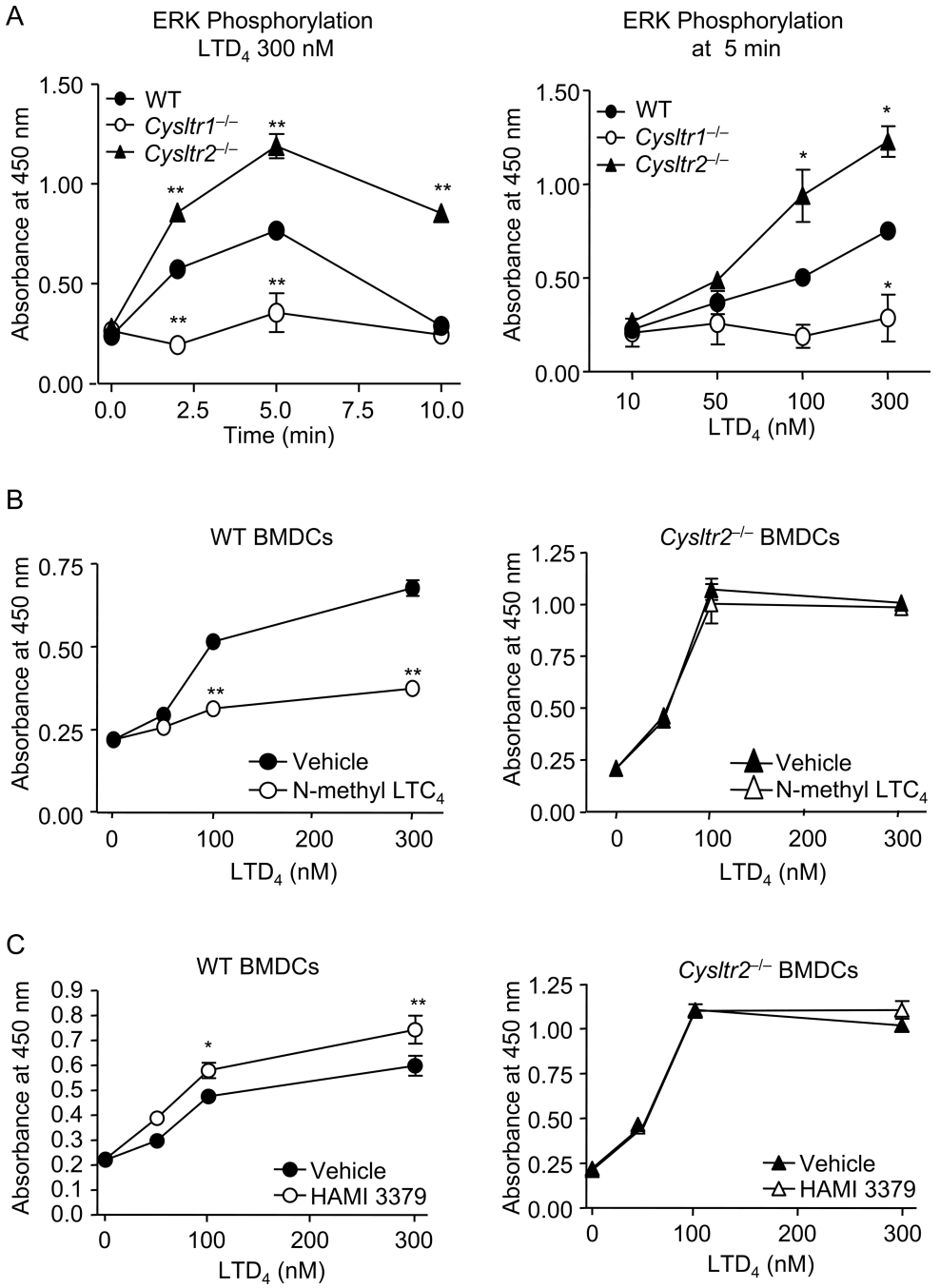

FIGURE 4.

CysLT$_2$R regulates CysLT$_1$R-dependent ERK phosphorylation in BMDCs. A. WT, *Cysltr1*$^{-/-}$, and *Cysltr2*$^{-/-}$ BMDCs (2.5 × 10^4 cells) were stimulated with 300 nM of LTD$_4$ for 0, 2, 5, and 10 min (left) or with 10–300 nM of LTD$_4$ for 5 minutes (right). Cells were then lysed, and phospho-ERK1/2 was quantified by ELISA. Values are the means ± SEM (n = 6 for WT and *Cysltr2*$^{-/-}$ and n = 4 for *Cysltr1*$^{-/-}$) combined from 4 independent experiments. *P < 0.05 and **P < 0.01 vs. WT BMDCs. B. 2.5 × 10^4 WT BMDCs (left) or *Cysltr2*$^{-/-}$ (right) were preincubated with 300 nM N-methyl LTC$_4$ or vehicle control for 10 min and then stimulated with increasing doses of LTD$_4$ for 5 min. Cells were lysed, and phospho-ERK1/2 was quantified by ELISA. Values are the means ± SEM (n = 6 for WT, n

= 4 for *Cysltr2*$^{-/-}$) combined from 3 independent experiments. $^{**}P < 0.01$ vs. 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 LTD_4 for 5 min. Cells were lysed, and phospho-ERK1/2 was quantified by ELISA. Values are the means ± SEM ($n = 6$ for WT, $n = 4$ for *Cysltr2*$^{-/-}$) combined from 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle-treated BMDCs.

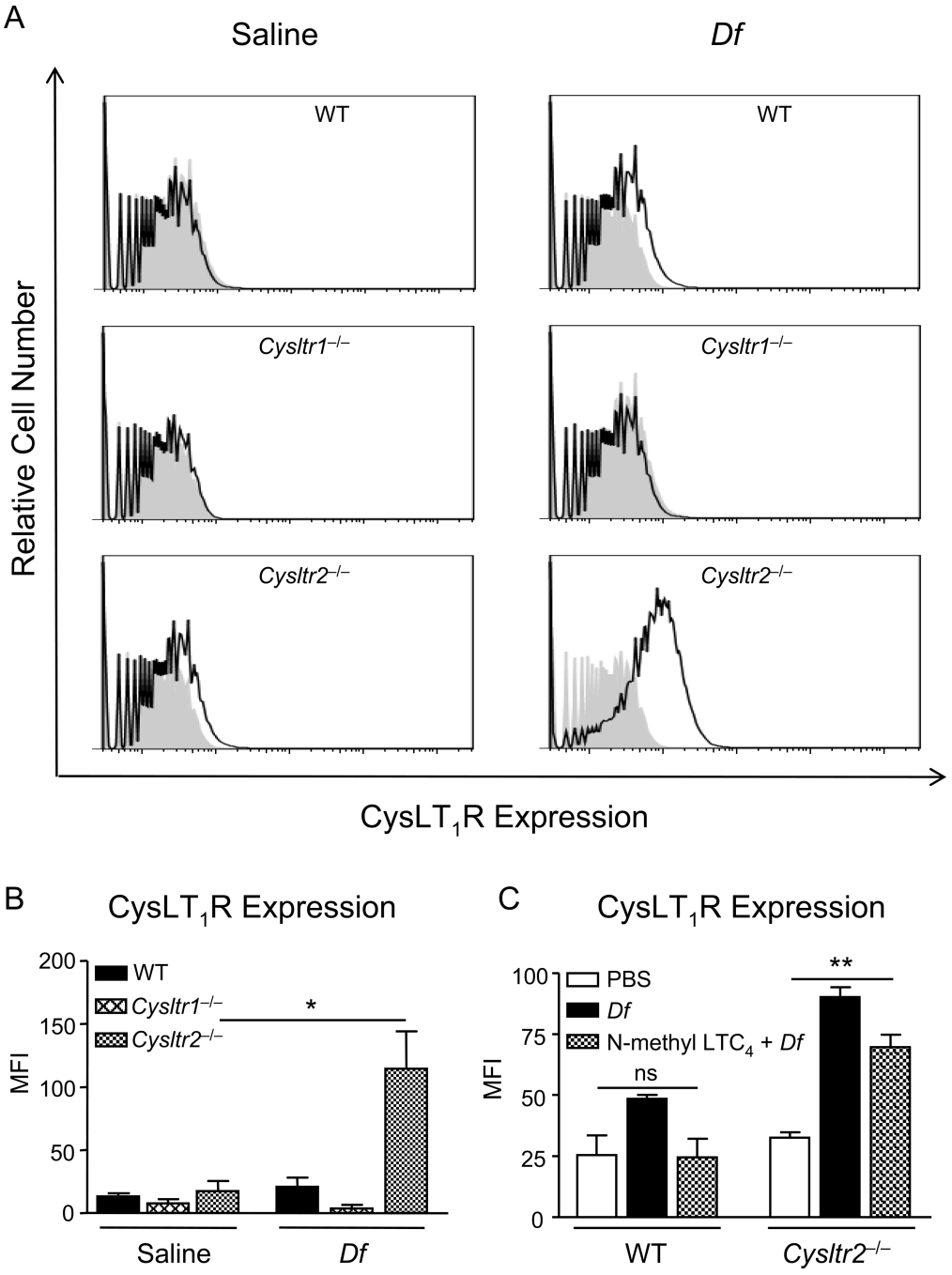

FIGURE 5.

Increased cell surface $CysLT_1R$ expression on *Cysltr2*$^{-/-}$ BMDCs after *Df*-pulsation. WT, *Cysltr1*$^{-/-}$, and *Cysltr2*$^{-/-}$ BMDCs were incubated with PBS or 50 μg/ml *Df* for 24 h, stained for the cell surface expression of $CysLT_1R$, and analyzed by flow cytometry. A. Representative histograms of $CysLT_1R$ expression after PBS (left) or *Df* (right) pulsation. Isotype control staining (shaded histograms) and $CysLT_1R$ (open histograms). B. Mean fluorescence intensities of $CysLT_1R$ staining combined from 3 independent experiments. Values are the means ± SEM. **P* < 0.04 vs. PBS-pulsed *Cysltr2*$^{-/-}$ BMDCs. C. WT and *Cysltr2*$^{-/-}$ BMDCs were preincubated with 300 nM N-methyl LTC_4 or saline for 10 min and then stimulated with PBS or 50 μg/ml *Df* for 24 h, stained for the cell surface expression of

$CysLT_1R$, and analyzed by flow cytometry. Mean fluorescence intensities of $CysLT_1R$ staining combined from 3 independent experiments. Values are the means ± SEM. $^{**}P = 0.01$ vs. PBS-pulsed *Cysltr2*$^{-/-}$ BMDCs.

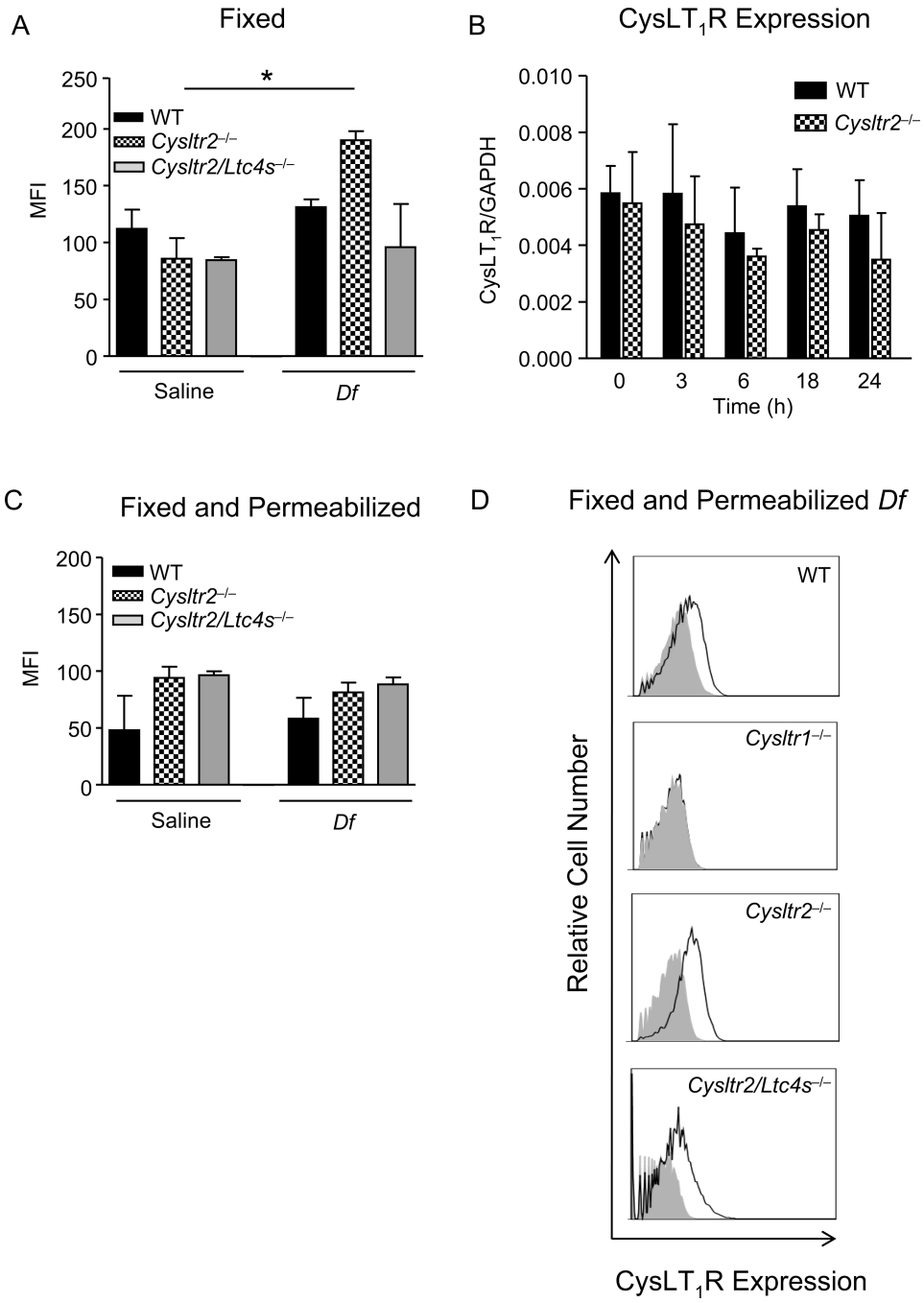

FIGURE 6.

Increased cell surface $CysLT_1R$ expression on *Cysltr2*$^{-/-}$ BMDCs is LTC_4S-dependent. A. WT, *Cysltr2*$^{-/-}$, and *Cysltr2/Ltc4s*$^{-/-}$ BMDCs were incubated with PBS or 50 μg/ml *Df* for 24 h, stained for the cell surface expression of $CysLT_1R$, and analyzed by flow cytometry. Mean fluorescence intensities of $CysLT_1R$ staining combined from 3 independent experiments. Values are the means ± SEM. **P* < 0.05 vs. PBS-pulsed *Cysltr2*$^{-/-}$ BMDCs. B. Relative expression of mRNA for $CysLT_1R$ to GAPDH in *Df*-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 *Df* for 24 h, fixed and permeabilized, stained for total $CysLT_1R$, and analyzed by flow cytometry. Values are the

means ± SEM combined from 3 experiments. D. Representative histograms of $CysLT_1R$ expression after *Df* pulsation in WT, *Cysltr1*$^{-/-}$, *Cysltr2*$^{-/-}$, and *Cysltr2/Ltc4s*$^{-/-}$ BMDCs. Isotype control staining (shaded histograms) and $CysLT_1R$ (open histograms).