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Type 2 cysteinyl leukotriene receptors drive IL-33-dependent type 2 immunopathology and aspirin sensitivity*

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Abstract

Cysteinyl leukotrienes (cysLTs) facilitate mucosal type 2 immunopathology by incompletely understood mechanisms. Aspirin-exacerbated respiratory disease (AERD), a severe asthma subtype, is characterized by exaggerated eosinophilic respiratory inflammation and reactions to aspirin, each involving the marked overproduction of cysLTs. Here we demonstrate that the type 2 cysLT receptor ($CysLT_2R$), which is not targeted by available drugs, is required in two different models to amplify eosinophlilic airway inflammation via induced expression of IL-33 by lung epithelial cells. Endogenously generated cysLTs induced eosinophilia and expanded group 2 innate lymphoid cells (ILC2s) in AERD-like *Ptges*$^{-/-}$ mice. These responses were mitigated by deletions of either *Cysltr2* or leukotriene C_4 synthase (*Ltc4s*). Administrations of either LTC_4 (the parent cysLT) or the selective $CysLT_2R$ agonist N-methyl LTC_4 to allergen sensitized WT mice markedly boosted ILC2 expansion and IL-5/IL-13 generation in a $CysLT_2R$-dependent manner. Expansion of ILC2s and IL-5/IL-13 generation reflected $CysLT_2R$-dependent production of IL-33 by alveolar type 2 cells, which engaged in a bilateral feed-forward loop with ILC2s. Deletion of *Cysltr1* blunted LTC_4-induced ILC2 expansion and eosinophilia but did not alter IL-33 induction. Pharmacological blockade of $CysLT_2R$ prior to inhalation challenge of *Ptges*$^{-/-}$ mice with aspirin blocked IL-33-dependent mast cell activation, mediator release, and changes in lung function. Thus, $CysLT_2R$ signaling IL-33-dependent ILC2 expansion and IL-33-driven mast cell activation that are necessary for induction of type 2 immunopathology and aspirin sensitivity. $CysLT_2R$-targeted drugs may interrupt these processes.

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Author contributions

T. Liu designed the experiments and performed them with C. Feng and D. Garofalo, and analyzed the results with J. Boyce. N. Barrett conducted the analyses of IL-33 immunolocalization and identified the effect of $CysLT_2R$ blockade on type 2 immunity with E. Yoshimoto. Y. Kanaoka developed the compound KO mouse strains. J. Boyce conceived of the study and composed the manuscript with N. Barrett and T. Liu.

Keywords

Leukotrienes; type 2 immunity; asthma

Introduction

Mucosal type 2 immune responses elicit tissue eosinophilia, goblet cell metaplasia, and IgE synthesis. Such responses defend against intestinal helminths (1) and facilitate epithelial repair following respiratory viral infections (2, 3). Danger signals from pathogens or allergens (e.g., fungal or helminthic proteases, glycans, endotoxin) initiate type 2 responses and associated immunopathology by activating or damaging barrier cells, which generate and release of innate type 2 cytokines IL-33, IL-25, thymic stromal lymphopoietin (TSLP)) (4). These barrier-derived cytokines facilitate durable type 2 adaptive immune responses through their effects on dendritic cells (DCs) (5), and directly activate innate hematopoietic tissue resident effector cells (e.g., mast cells (MCs), macrophages, group 2 innate lymphoid cells (ILC2s)) and conventional T cells (6) to generate IL-5, IL-13, and other cytokines that induce eosinophilic inflammation (4). Prolonged or exaggerated activation of these pathways elicits organ dysfunction and contributes to disease. Epithelial barrier cell nuclei consitituvely contain IL-33 that is released during necrosis (7), but these same cells can also upregulate IL-33 expression and actively secrete IL-33 during inflammation (8, 9). Increases in steady-state tissue levels of IL-33 and its receptor, suppressor of tumorogenicity 2 (ST2), are associated with chronic, non-resolving eosinophilic inflammation and end-organ dysfunction, as observed in refractory chronic rhinosinusitis and severe asthma (10, 11). Animal models suggest that endogenously generated alarmins (12, 13) and/or autocrine feed-forward amplification systems (14, 15) can prolong/enhance IL-33 production by structural cells, and/or induce IL-33 expression de novo by hematopietic cells such as macrophages (16). Due to the potential of IL-33 to drive immunopathology, the mechanisms and pathways responsible for its upregulation with severe disease are fundamentally important. However, these pathways and mechanisms are incompletely understood.

Cysteinyl leukotrienes (cysLTs) are potent lipid inflammatory mediators that abound in allergic inflammation and play validated roles in asthma (17, 18). They form in myeloid cells by 5-lipoxygenase (5-LO)-mediated oxidation of arachidonic acid to leukotriene A_4 (LTA_4), which is converted to LTC_4 by conjugation to reduced glutathione by leukotriene C_4 synthase (LTC_4S)(19). After its export from the cells of origin (20), extracellular enzymes convert LTC_4 to LTD_4 (21), a powerful but unstable smooth muscle spasmogen, and then LTD_4 to LTE_4, a stable metabolite (22). Three G protein coupled receptors bind the cysLTs. The type 1 cysLT receptor ($CysLT_1R$) binds LTD_4 with high affinity and in preference to LTC_4 when expressed in heterologous cells (23), whereas the type 2 cysLT receptor ($CysLT_2R$) binds LTC_4 and LTD_4 equally and with lower affinity (24). $CysLT_3R$ (also designated GPR99), binds LTE_4 in preference to LTC_4 or LTD_4, although it can mediate responses to all three ligands in vivo (25). Mice lacking LTC_4S (*Ltc4s*$^{-/-}$ mice) show reduced eosinophilia, IgE production, and goblet cell metaplasia compared with WT controls when sensitized and challenged with dust mite allergens or ovalbumin (OVA) (26, 27), indicating a role for cysLTs in type 2 immunity. Intransal administration of LTD_4 to

Alternaria-challenged mice induces ILC2 proliferation in vivo, and induces secretion of IL-4, IL-5 and IL-13 by ILCs sorted from the lungs of *Alternaria* challenged mice (28). Both LTC_4 and LTD_4 activate lung ILC2s by a direct $CysLT_1R$-dependent mechanism in which $CysLT_2R$ is dispensable (29, 30). LTC_4 and LTD_4 also synergize with IL-33 to induce cytokine generation by lung ILC2s, again via $CysLT_1R$ (29, 30). Although respiratory mucosal epithelial cells also express cysLT receptors (particularly $CysLT_2R$ and $CysLT_3R$) (31, 32), it is unknown whether cysLTs can also participate in upstream regulation of IL-33 expression by barrier cells. Hypothetically, such an effect could synergize with direct $CysLT_1R$-driven ILC2 activation to promote type 2 immunopathology in circumstances where cysLTs are abundant.

Aspirin exacerbated respiratory disease (AERD) is the prototypical disorder in which markedly elevated levels of cysLTs accompany robust type 2 respiratory immunopathology. AERD affects ~7% of all asthmatics and a significantly higher proportion (15–30%) of those with severe disease (33, 34). The dysregulated basal production of LTC_4 (35, 36) increases further and abruptly in response to the ingestion of nonselective cyclooxygenase (COX) inhibitors (36, 37). The increase in cysLTs results in an idiosyncratic respiratory reaction associated with cryptic, cysLT-dependent mast cell activation (38). We previously demonstrated that nasal polyps from subjects with AERD, which are especially rich in eosinophils (39), contain markedly more IL-33 protein than tissues from aspirin tolerant controls (40), indicating dysregulated innate type 2 inflammation. Moreover, lung IL-33 levels and eosinophilic inflammation are markedly increased in AERD-like prostaglandin E_2-deficient (*Ptges*$^{-/-}$) mice compared with WT controls when primed by repetitive low-dose inhalation of an extract (*Df*) from the house dust mite *Dermatophagoides farinae*. Deletion of *Ltc4s* prevents the increases in both lung IL-33 expression and eosinophilic inflammation in *Ptges*$^{-/-}$ mice, indicating that these features depend on endogenous cysLTs (40, 41). Moreover, blockade of LTC_4 production, IL-33, or ST2 each prevent increases in airway resistance (R_L) and mast cell activation in response to challenges of *Ptges*$^{-/-}$ mice with nonselective COX inhibitors, suggesting an essential, proximal role for cysLTs in driving IL-33 release (and consequent mast cell activation) during aspirin challenges (40, 41). The cysLT receptor(s) and cell type(s) responsible for the induced expression of IL-33 and enhanced type 2 immunopathology in AERD are not known.

In the current study, we demonstrate in two different in vivo models that cysLTs drive ILC2 expansion and activation not only directly through $CysLT_1R$ signaling, but also indirectly through $CysLT_2R$-mediated IL-33 expression by type 2 alveolar (AT2) cells. These effects are synergistic, and each is essential for cysLT-facilitated type 2 immunopathology. Moreover, “unbraking” of LTC_4 synthesis by lysine-aspirin (Lys-ASA) challenges of *Ptges*$^{-/-}$ mice reveals that $CysLT_2R$ signaling mediates IL-33-dependent mast cell activation and pathognomonic changes in airway resistance that are characteristic of AERD. $CysLT_2R$-mediated feed-forward systems may amplify type 2 immunopathology in general, may explain the severe, non-resolving nature of respiratory inflammation in AERD, and may offer several potential targets for therapy.

Materials and Methods

Reagents

Df was obtained from Greer Laboratories (XPB81D3A25; Lenoir, NC). Ovalbumin and PBS were obtained from Sigma-Aldrich (St. Louis, MO). The mMCP-1 EIA kit was purchased from eBiosciences (San Diego, CA). LTC_4, LTD_4, LTE_4, and N-Me-LTC_4 were from Cayman Chemical (Ann Arbor, MI). Histamine, TXB_2, PGD_2, and cysLT EIA kits were from Cayman. IL-5, IL-13, ICAM-1, and VCAM-1 EIA kits were from R&D systems (Minneapolis, MN). CXCL7 EIA kit was purchased from Abcam (Cambridge, MA). The HMGB1 EIA kit was from LifeSpan (Providence, RI). The following antibody reagents were purchased from the indicated vendors: Polyclonal goat anti-mouse IL-33 (R&D systems), Polyclonal rabbit anti-human proSPC (Millipore, Billerica, MA), Donkey anti-Goat IgG (H+L) Secondary Antibody, Alexa Fluor® 488 (Invitrogen, Carlsbad, CA), Donkey anti-rabbit IgG(H+L) Secondary Antibody, Alexa Fluor®594 (Invitrogen), DAKO Serum-Free Protein Block (Agilent, Santa Clara, CA), DAKO Target Retrieval (Agilent). FITC anti-mouse CD11c, FITC anti-mouse/human CD11b, FITC anti-mouse IgE, FITC anti-mouse CD3ε, FITC anti-mouse CD19, FITC anti-mouse CD8a, FITC anti-mouse NK-1.1, FITC anti-mouse Ly-6G/Ly-6C (Gr-1), APC anti-mouse CD45, APC/Cy7 anti-mouse/human CD44, PerCP/Cy5.5 anti-mouse CD90.2, PerCP/Cy5.5 anti-mouse IL-33Rα (IL1RL1, ST2), PE anti-mouse CD278 (ICOS), APC-anti-mouse CD41, PE/Cy7-anti-mouse CD62P, PE-anti-HMGB1, anti-HMGB1, anti-mouse-CD90.2, anti-mouse-CD4, anti-mouse-NK1.1, anti-mouse CD16/32, and isotype controls were all from BioLegend (San Diego, CA). A549 cells were purchased from the American Type Culture Collection.

Mice

C57BL/6 mice lacking mPGES-1 (*ptges*$^{-/-}$ mice) were from Dr. Shizuo Akira (Osaka University, Japan) (42). The mice were intercrossed with *Ltc4s*$^{-/-}$ mice (43), *Cysltr1*$^{-/-}$ (44), *Cysltr2*$^{-/-}$ (45), and *Cysltr3*$^{-/-}$ (25) to generate respective DKO strains. All of the mice and wild type C57BL/6 controls were housed at Charles River (Wilmington, MA). Six- to 8-wk-old male were used. All animal studies were approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute (Protocol 03-042).

Immunization and challenge

To study potentiation of airway inflammation by exogenous cysLTs, mice were sensitized by intraperitoneal (i.p.) injections on days 0 and 5 with Alum-precipitated chicken egg OVA (10 μg). On days 16-18, the mice received intranasal challenge of 2.2 nmol LTC_4 or vehicle. On days 17-19, mice were challenged by inhalation of 0.1% OVA (46). Twenty-four hours after the final OVA aerosol challenge, the mice were euthanized and exsanguinated. The lungs were lavaged three times with 0.7 ml PBS/5 mM EDTA. Bronchoalveolar lavage (BAL) fluid cells were cytocentrifuged onto slides, stained with Diff-Quick (Fisher Diagnostics, Middletown, VA), and differentially counted.

To study the effect of endogenous cysLTs, airway inflammation was induced by intranasal administration of *Df* (Greer, XPB81D3A25; containing <0.005 EU/mL of endotoxin, 3 μg dissolved in 30 μl PBS) to mice after anesthesia with isoflurane in a bell jar system on days

0, 4, 7, 10, 14, and 17 as described elsewhere (41). Control mice were treated with an equal volume of PBS alone. Mice were euthanized for studies 24 h after the last *Df* treatment. The dose of *Df* was titrated previously to elicit a sizable cysLT-dependent increment in inflammation in *Ptges*$^{-/-}$ mice over the minimal response of WT C57BL/6 controls (47).

Flow cytometry

Mice lungs (right lobes) were transferred into 6 well dish and teased tissue apart with forceps. Then the tissues were digested at RT for 45 min in 2 ml of dispase (2 U/ml), followed by adding 0.5 mg DNAse/mouse to the mixture and incubated for 10 min at RT with gently rocking on a shaker to 200 rpm. Cells were filtered through 70 μm nylon meshes, and pelleted by centrifugation for 10 min at 350 g at 4 ºC. RBC lysis was performed by resuspending the pellet in 2 ml 1x RBC lysis buffer (Biolegend) and incubating on ice for 4 min, terminated by addition of 13 ml DMEM. Cells were centrifuged for 10 min 350 g at 4 ºC, and then washed twice with FACS buffer (0.5% BSA in PBS). 1×10^6 cells were stained with antibodies in 100 μl FACS buffer for 20 min on ice in dark. The cells were washed and resuspended in 300 μl of 1% paraformaldehyde in PBS prior to analysis on a FACSCanto flow cytometer (BD Biosciences). ILC2s were quantitated as Lin-,CD45+,CD44+,CD278+,CD90.2+ cells in the lymphocyte gate . In the depletion experiments involving anti-CD90.2, anti-ST2 was substituted for anti-CD90.2 to enumerate ILC2s within the Lin-,CD45+,CD44+,CD278+ cells. The numbers of ILC2s measured by both sets of criteria were similar (not shown).

IL-33 staining

Lungs were fixed in 4% PFA (wt./vol) for 24 h, and embedded in paraffin. Four μm tissue sections were deparaffinized by heating to 60º C for 30 minutes and washing serially with xylene; ETOH at 100%, 95%, 70%, and 50%; and ddH2O. Slides were quenched with 1 mg/ml sodium borohydride solution on ice for 10 min. For target retrieval, slides were incubated in DAKO target retrieval citrate buffer at 95º C for 30 min, cooled for 20 min at RT, and washed with ddH20 and PBS. Slides were blocked with DAKO Protein Block + 5% donkey serum for 60 minutes, incubated with 100 μL of goat anti-mouse IL-33 at 1 μg/ml and Polyclonal rabbit anti-human proSPC at 1:750 overnight at 4° C, and with donkey secondary Abs at 1:500 with Hoechst nuclear dye at 1:10,000 in PBS-T at RT for 1 h. Images were acquired with a Nikon Eclipse E800 microscope at 20X and analyzed with Image J (NIH). To count IL-33+ cells, the threshold gray value of WT PBS-treated mice was set at 45. The mean number of IL-33+ cells per image and the mean intensity of IL-33+ staining was averaged from three images per mouse. The specificity of staining was validated using WT and *Il33*$^{-/-}$ mice (not shown).

Measurement of airway resistance

Airway resistance (R_L) in response to Lys-ASA was assessed with an Invasive Pulmonary Function Device (Buxco, Sharon, CT) as described elsewhere (41). Briefly, mice were anesthetized 24 h after the last *Df* challenge, and a tracheotomy was performed. After allowing for R_L to reach a stable baseline, Lys-ASA (12 μl of 100 mg/ml) was delivered to the lung *via* nebulizer, and R_L was recorded for 45 min. This dose was based on the peak effect of Lys-ASA on R_L in *Ptges*$^{-/-}$ mice, which generally is reached between 30-45 min

after administration (41). The results were expressed as the peak R_L as a percentage change from baseline.

Cell depletions, antibody blockade and CysLT$_2$R antagonism

Mice were given i.p. injections with polyclonal goat anti-mouse IL-33 (3.6 μg/mouse), anti-CD90.2 (50 μg/mouse), anti-NK1.1 (50 μg/mouse), anti-CD4 (50 μg/mouse), or equivalent doses of isotype controls were administered on days 16 and 18 of the OVA protocol. In the Lys-ASA challenge experiments, mice were given i.p. injections of anti-HMGB1 antibody (25 μg/mouse) 1 day before the challenge. HAMI-3379 (0.2mg/kg) or buffer control (ethanol) were given by i.p. on 1 and 2 days before the challenge. In some experiments, HAMI-3379 was administered daily for 17 days during *Df* induction of lung inflammation.

Statistical analysis

Data are expressed as mean ±SEM from at least 10 mice from at least two experiments, except where otherwise indicated. Analyses were performed with Prism software (Graphpad). Differences between two treatment groups were assessed using Student t test, and differences among multiple groups were assessed using one-way ANOVA and Bonferroni post hoc test. $P<0.05$ was considered statistically significant.

Results

CysLT$_2$R is essential for type 2 immunopathology induced by endogenous cysLTs in PGE$_2$-deficient mice

To identify the receptors through which endogenously generated cysLTs drive the features of type 2 immunopathology in *Ptges*$^{-/-}$ mice, we intercrossed the parent *Ptges*$^{-/-}$ strain (42) with *Cysltr1*$^{-/-}$ (44), *Cysltr2*$^{-/-}$ (45), and *Cysltr3*$^{-/-}$ (25) strains to generate respective strains of receptor double knockout (DKO) mice. All three receptor DKO strains, along with WT and *Ptges/Ltc4s*$^{-/-}$ DKO mice, were treated with six doses of intranasal *Df* to induce type 2 pulmonary inflammation. Twenty-four hours after the final dose of *Df*, BAL fluid was collected and single lung cell suspensions were analyzed by flow cytometry. *Df* weakly induced BAL fluid eosinophilia (Fig. 1A) and lung ILC2 expansion (Fig. 1B) in WT mice in this low-dose protocol, but strongly induced these parameters in *Ptges*$^{-/-}$ mice (Fig. 1A, 1B). *Df*-treated *Ptges*$^{-/-}$ mice displayed significantly stronger expression of IL-13 mRNA in the lung than did WT controls (Supplemental Fig. 1A). Levels of IL-5 and IL-13 proteins were at or below limits of detection by ELISA of whole lung lysates.

When compared with *Df*-treated *Ptges*$^{-/-}$ mice, *Ptges/Ltc4s*$^{-/-}$ DKO mice and each of the receptor DKO strains showed significantly diminished BAL fluid eosinophilia 24 hours after treatment with *Df* (Fig. 1A). The *Ptges/Ltc4s*$^{-/-}$ and *Ptges/Cysltr2*$^{-/-}$ DKO strains showed the greatest decreases, with significant reductions in eosinophil numbers compared to both the parent *Ptges*$^{-/-}$ strain and to the *Ptges/Cysltr1*$^{-/-}$ and *Ptges/Cysltr3*$^{-/-}$ DKO strains. Deletion of *Ltc4s* eliminated the *Df*-induced increase in ILC2s observed in *Ptges*$^{-/-}$ single KO mice, whereas the *Cysltr1*, *Cysltr2*, and *Cyslt3r* in the DKO strains also all showed significantly reduced the numbers of lung ILC2s relative to the *Ptges*$^{-/-}$ strain (Fig. 1B). Deletions of *Ltc4s*, *Cysltr2*, and *Cysltr3* all sharply reduced the *Df*-induced expression of

IL-13 mRNA in the lungs of *Df*-treated *Ptges*$^{-/-}$ mice, whereas deletion of *Cysltr1* did not (Supplemental Fig. 1A). None of the receptor deletions affected expression of the other receptors (Supplemental Fig. 1B), though *Cysltr3* transcripts were below detection limits in all strains. Deletion of *Ltc4s*, but not of any of the receptors, significantly reduced the levels of cysLTs detected in BAL fluid at 24 hours after the last dose of *Df* (Supplemental Fig. 1C). The numbers of BAL fluid eosinophils (Supplemental Fig. 2A) and lung ILC2s (Supplemental Fig. 2B) in PGE_2-sufficient *Cysltr1*$^{-/-}$, *Cyslt2r*$^{-/-}$ and *Cyslt3r*$^{-/-}$ single KO mice were low and not different from the WT controls.

Exogenous LTC_4 induces eosinophilia and expands lung ILC2s in PGE_2-sufficient mice by a mechanism requiring $CysLT_2R$

To verify that $CysLT_2R$ signaling also drives features of type 2 immunopathology in a context where PGE_2 synthesis was unimpaired, we used a reductionist model in which intranasal cysLTs are administered to ovalbumin (OVA)-sensitized WT mice 12 h prior to low dose (0.1%) OVA challenges on three successive days (46). We administered LTC_4, LTD_4, LTE_4, or N-methyl LTC_4, a hydrolysis-resistant synthetic cysLT that stimulates $CysLT_2R$ in preference to $CysLT_1R$ (48) (2.2 nmol each) to respective cohorts of naïve and OVA sensitized mice. LTC_4, N-methyl LTC_4, and (to a lesser extent) LTE_4 each increased OVA-induced BAL fluid total cell counts, eosinophil percentages, and total eosinophils in sensitized mice (Fig. 2A). In contrast, LTD_4 did not induce eosinophilia (Fig. 2A). Despite differences in the capacity to induce eosinophilia, all three cysLTs increased the numbers of lung ILC2s to comparable extents, as did N-methyl LTC_4 (Fig. 2B). LTC_4 and N-methyl LTC_4 significantly increased the quantities of IL-5 and IL-13 proteins in the lysates of lungs from OVA-challenged mice, whereas LTD_4 and LTE_4 were less active (Fig. 2C). LTC_4 and N-methyl LTC_4 -induced increases in total cells and total eosinophils were reduced by >80% in *Cysltr2*$^{-/-}$ mice compared to WT controls (Fig. 3A). ILC2 expansion (Fig 3B) and expressions of IL-5 and IL-13 (Fig. 3C) were reduced in *Cysltr2*$^{-/-}$ mice as well. In naive WT mice, intranasal LTC_4 weakly induced airway inflammation (0 and 4.4 ± 1.2% BAL fluid eosinophils in PBS- and LTC_4-challenged mice, $P = 0.01$) and ILC2 expansion (1.74 ± .09 and 2.28 ±.11 × 10^4 cells/lung in PBS- and LTC_4-challenged mice, $P = 0.006$, $n = 5$, not shown).

$CysLT_2R$ signaling drives IL-33 expression

Compared with WT controls, *Df*-treated *Ptges*$^{-/-}$ mice display sharply increased levels of IL-33 in the lung. These levels are reduced in *Ptges/Ltc4s*$^{-/-}$ mice, implying a dependence on endogenous cysLTs (40). Because IL-33 can expand lung ILC2s and synergize with $CysLT_1R$ signaling to drive IL-5/IL13 production (30), we examined lysates of whole lung for IL-33 protein in each DKO strain to determine which cysLT receptors might account for the induction of IL-33 expression in *Ptges*$^{-/-}$ mice. *Df* treatment induced significantly higher levels of lung IL-33 protein in *Ptges*$^{-/-}$ mice than WT controls, and also strongly induced IL-33 expression in the *Ptges/Cysltr1*$^{-/-}$ and *Ptges/Cysltr3*$^{-/-}$ DKO mice. In sharp contrast, *Df* failed to significantly induce IL-33 expression in either the *Ptges/Cyslt2r*$^{-/-}$ or *Ptges/Ltc4s*$^{-/-}$ DKO strains (Fig. 4A). None of the strains displayed significant induction of TSLP protein, and IL-25 was undetectable (not shown). To exclude potential epistatic influences of *Cysltr2* deletion on IL-33 induction, we treated *Ptges*$^{-/-}$ mice with a $CysLT_2R$ selective

antagonist, HAMI-3379, to pharmacologically block $CysLT_2R$ during *Df* priming. Compared with vehicle treatment, the daily administration of HAMI-3379 during the *Df* exposure period prevented BAL fluid eosinophilia (Fig. 4B) and the upregulation of IL-33 protein (Fig. 4C). Immunofluorescent staining (Fig. 4D) revealed that the nuclei of surfactant protein C (SPC)+ cells were the dominant site of IL-33 immunoreactivity. Compared with saline-treated controls, *Df*-treated *Ptges*$^{-/-}$ mice displayed a 3-fold increases in the numbers of IL-33+ cells. These increases were sharply reduced in the lungs of *Df*-treated *Ptges/Ltc4s*$^{-/-}$ and *Ptges/Cysltr2*$^{-/-}$ DKO mice (Fig. 4D,4E). IL-33 expression on a per cell basis was also increased in *Df*-treated *Ptges*$^{-/-}$ mice, as indicated by the intensity of nuclear staining, and decreased in both *Df*-treated *Ptges/Ltc4s*$^{-/-}$ and *Ptges/Cysltr2*$^{-/-}$ mice (Fig. 4E). Similar results were observed in HAMI-3379-treated *Ptges*$^{-/-}$ mice (not shown). There were rare CD45+IL-33+ cells with cytoplasmic IL-33 expression. These cells were not different in number or intensity across *Df*-treated *Ptges*$^{-/-}$, *Ptges/Ltc4s*$^{-/-}$ and *Ptges/Cysltr2*$^{-/-}$ DKO mouse strains, and were not altered by HAMI-3379. Deletions of *Ltc4s*, *Cysltr1*, *Cysltr2*, or *Cysltr3* failed to alter the weak IL-33 expression, ILC2 expansion, or lung eosinophilia in PGE_2-sufficient mice (Supplemental Fig. 2C).

To verify that LTC_4-induced $CysLT_2R$ signaling could also promote IL-33 expression in PGE_2-sufficient mice, we examined the lungs of WT mice treated with exogenous cysLTs. The administration of LTC_4, N-Methyl LTC_4, and (to a lesser degree) LTE_4 to WT OVA-sensitized mice increased the levels of lung IL-33 protein (Fig. 4F), again localizing to SPC+ cells (not shown). As was the case for eosinophilia, LTD_4 failed to elicit IL-33 expression. Compared to WT mice, *Cysltr2*$^{-/-}$ mice showed reduced induction of IL-33 expression in response to both LTC_4 and N-methyl LTC_4 (Fig. 4G). The administration of LTC_4 to *Df*-primed WT mice also significantly increased both lung eosinophilia and lung IL-33 levels (Supplemental Fig. 3).

To determine whether $CysLT_2R$ signaling could directly induce IL-33 expression in AT2 cells, we stimulated a human AT2 cell line, A549, with LTC_4 or with N-methyl LTC_4. Some samples were treated simultaneously with IL-13 (2 ng/ml), which upregulates the expression of IL-33 mRNA in A549 cells (14). Neither LTC_4 nor N-methyl LTC_4 significantly increased expression levels of IL-33 mRNA when provided alone or in combination with IL-13 (not shown).

$CysLT_1R$ and $CysLT_2R$ signaling synergize to promote LTC_4-induced ILC2 expansion and immunopathology

To distinguish between the contributions of $CysLT_1R$, $CysLT_2R$, and $CysLT_3R$ to LTC_4-induced immunopathology, we compared the responses of OVA-sensitized WT, *Cysltr1*$^{-/-}$, *Cysltr2*$^{-/-}$ and *Cysltr3*$^{-/-}$ single KO mice to exogenous LTC_4. Deletions of either $CysLT_1R$ or $CysLT_2R$ both reduced the numbers of total cells and eosinophils in the BAL fluids of LTC_4-challenged mice (Fig. 5A), and sharply reduced the numbers of ILC2s (Fig. 5B). The deletion of $CysLT_2R$ was significantly more efficacious than the deletion of $CysLT_1R$ for both responses. In contrast to its effects on eosinophilia and ILC2 numbers, deletion of $CysLT_1R$ did not alter the induction of IL-33 expression, whereas deletion of $CysLT_2R$ did (Fig. 5C). $CysLT_1R$ deletion modestly reduced the levels of lung IL-5 protein, but not IL-13

protein, whereas $CysLT_2R$ deletion substantially reduced both (Fig. 5D). Notably, deletion of $CysLT_3R$ had no effect on the induction of eosinophilia, ILC2 expansion, IL-33 induction, or IL-5/IL-13 expression in response to exogenous LTC_4 (Fig. 5A–D). In contrast, deletion of $CysLT_3R$ abrogated the LTE_4-induced increments in inflammation, ILC2 expansion, and IL-5 expression in OVA sensitized mice without completely eliminating the modest IL-33 increase (not shown).

$CysLT_2R$-driven immunopathology requires both ILC2s and CD4+ T cells, but only ILC2s engage in a feed-forward loop with IL-33

We used Ab-based neutralization and cell depletion strategies to determine whether LTC_4-elicited IL-33 was necessary for the accompanying increase in airway eosinophilia, and to identify the lymphocyte subsets most essential to covey the responses. Treatment of the mice with anti-IL-33 eliminated the LTC_4-induced increment in lung IL-33 (Supplemental Fig 4), blocked the LTC_4-induced increase in eosinophilia by >80% (Fig. 6A), and reduced the numbers of ILC2s by ~40% (Fig. 6B). Anti-IL-33 also prevented the LTC_4-induced increases in both IL-5 and IL-13 proteins in the lung (Fig. 6C). The administration of an anti-CD90.2 Ab, which depletes ILC2s (3), NK cells, and T cells (49), completely prevented the LTC_4-induced potentiation of BAL fluid total cells and eosinophils (Fig. 6D) and expansion of ILC2s by >80% (Fig. 6E), while also depleting NK1.1+ NK cells (not shown) compared with an isotype control Ab. In contrast, selective NK cell depletion with anti-NK1.1 (95% depletion, not shown) had no effect on cellularity, eosinophilia (Fig. 6D) or ILC2 expansion (Fig. 6E). The increases in IL-5 and IL-13 elicited by LTC_4 were eliminated by anti-CD90.2, but not by anti-NK1.1 or anti-CD4 (Fig. 6F), indicating ILC2s as the most likely relevant target. Because ILC2s can sustain lung IL-33 expression in a feed-forward loop (14), we examined the lungs of the Ab-treated mice for the levels of IL-33 protein. Notably, anti-CD90.2 prevented the LTC_4-induced induced expression of IL-33 completely, whereas anti-NK.1.1 had no effect (Fig. 6G). Although anti-CD4 also prevented the LTC_4-mediated increases in total BAL fluid cells and eosinophils (Fig. 6D), it did not alter LTC_4-induced expansion of ILC2s (Fig. 6E) or IL-33 expression (Fig. 6G).

$CysLT_2R$-mediated pathways are necessary for aspirin sensitivity

We next sought to determine whether $CysLT_2R$ was necessary for aspirin sensitivity in *Df*-primed $Ptges^{-/-}$ mice. Reactions to Lys-ASA in this model depend on unbraked cysLT production, cysLT-driven IL-33 release, and consequent mast cell activation. *Df*-primed $Ptges^{-/-}$ mice were treated intraperitoneally with HAMI-3379 at 24 h before and again immediately before challenge by inhalation of Lys-ASA. Lys-ASA challenged mice treated with vehicle or isotype control Ab exhibited sharply increased R_L (Fig. 7A), along with increases in cysLTs (Fig. 7B), and MC activation products (mouse mast cell protease -1 (mMCP-1), histamine, and PGD_2) (Fig. 7C–E). Blockade of $CysLT_2R$ attenuated the Lys-ASA-induced increase in R_L (Fig. 7A), along with increases in BAL fluid cysLTs (Fig. 7B) and the MC activation signatures mMCP-1, histamine, and PGD_2 (Fig. 7C–E). Administration of anti-CD90.2 24 h prior to the Lys-ASA challenge attenuated both the physiologic response and the release of mediators (Fig. 7F–J).

Discussion

Long recognized as constrictors of human airway smooth muscle in asthma (50, 51), cysLTs are now known to also act directly on cellular effectors of type 2 immunity (27, 28, 30). *Ltc4s*$^{-/-}$ mice display markedly impaired eosinophilic pulmonary inflammation and type 2 recall responses to OVA (26) and dust mite (27), supporting the biological importance of these immunologic effects. LTC_4 and LTD_4 can both elicit $CysLT_1R$-dependent ILC2 proliferation and nuclear factor of activated T cells (NFAT) activation that strongly synergizes with IL-33 to drive IL-5 and IL-13 expression (30). Although LTE_4 is inactive on ILC2s in vitro (30), it does activate lung ILC2s in vivo by a mechanism that resists $CysLT_1R$ blockade (28). Thus, cysLTs may affect ILC2 homeostasis by eliciting synergy between direct $CysLT_1R$-dependent and heretofore uncharacterized indirect mechanisms. Although ILC2s express both *Cysltr1* and *Cysltr2* transcripts, only *Cysltr1*$^{-/-}$ ILC2s show impaired proliferation or activation with direct stimulation by cysLTs (30). No prior studies had demonstrated a role for $CysLT_2R$ in ILC2 homeostasis. Although $CysLT_2R$ binds LTC_4 and LTD_4 equally avidly when expressed in heterologous cell systems (24), it exhibits a clear preference for activation by LTC_4 over LTD_4 in platelets (46) and dermal fibroblasts (52), suggesting a functional modification of the receptor when expressed in its native context, or in settings where the other receptors are also present. The relatively low affinity of $CysLT_2R$ for LTC_4 (EC50 10-70 nM (24))(53) suggests that it can mediate autocrine signals (54), or can function in paracrine signaling when local concentrations of LTC_4 are elevated due to increased synthesis. Such increased synthesis is a hallmark of AERD, where severe respiratory dysfunction is accompanied by marked tissue eosinophilia (55, 56) and strong expression of IL-33 in the respiratory tract (40). Our previous studies demonstrated that endogenous cysLTs were necessary to drive the robust *Df*-induced lung eosinophilia and high level steady-state IL-33 expression in the lungs of AERD-like *Ptges*$^{-/-}$ mice (40). We therefore undertook this study to determine the ligand, receptor, and cellular requirements responsible for cysLT-driven IL-33 expression, and to determine its contribution to cysLT-mediated pathobiology, including ILC2 homeostasis.

To study the role of each receptor in a context where endogenous cysLTs play a major pathogenic role, we introduced the *Cysltr1*, *Cysltr2*, and *Cysltr3* null alleles into *Ptges*$^{-/-}$ mice. CysLTs play a dominant role in driving *Df*-induced immunopathology in this strain (40, 41). All three receptor deletions decreased the induction of pulmonary eosinophilia in *Ptges*$^{-/-}$ mice (Fig. 1A), but had minimal effects in the PGE_2-sufficient controls (Supplemental Fig. 2). The *Ptges/Cysltr2*$^{-/-}$ DKO strain showed the greatest reduction, equaling the efficacy of *Ltc4s* deletion. All three receptor null strains (and the *Ptges/Ltc4s*$^{-/-}$ strain) showed similarly impaired ILC2 expansion relative to the *Ptges*$^{-/-}$ strain (Fig. 1B). The blunted ILC2 expansion observed in the *Ptges/Cysltr1*$^{-/-}$ mice (Fig. 1B), the ILC2 expansion induced by the preferred $CysLT_1R$ ligand LTD_4 in sensitized WT mice (Fig. 2B), and the reduced LTC_4-induced ILC2 expansion observed in *Cysltr1*$^{-/-}$ mice (Fig. 5B) are each consistent with mitogenic effects mediated by $CysLT_1R$ on ILC2s reported elsewhere (28, 30). Nonetheless, the lack of eosinophilia induced by exogenous LTD_4 (Fig. 2A), its lack of effect on whole lung IL-5/IL-13 levels (Fig. 2C)), and the inability of *Cysltr1* deletion to impair *Df*-induced IL-13 expression in *Ptges*$^{-/-}$ mice (Supplemental Fig. 1)

suggest that direct $CysLT_1R$-mediated ILC2 expansion is insufficient alone to drive cysLT-induced type 2 immunopathology. In contrast, both exogenous LTC_4 and N-methyl LTC_4 elicited lung eosinophilia (Fig. 3A), ILC2 expansion (Fig. 3B) and IL-5/IL-13 production (Fig. 3C), all of which were markedly diminished in *Cysltr2*$^{-/-}$ mice. The unexpectedly marked effects of *Cysltr2* deletion on both eosinophilic inflammation and ILC2 expansion in both models (Fig. 1, Fig. 3) prompted a focus on potential mechanisms by which $CysLT_2R$ signaling might promote ILC2 effector function indirectly.

We focused our attention to IL-33 for several reasons. First, it potently activates resident ST2+ ILC2s and mast cells (57, 58) to generate IL-5, IL-13, and other type 2 cytokines. Second, it synergizes with cysLTs (acting at $CysLT_1R$) to promote type 2 cytokine generation by ILC2s ex vivo (30). Third, IL-33 expression in the lungs of *Df*-primed *Ptges*$^{-/-}$ mice is upregulated by a pathway requiring endogenous cysLTs (40). Studies in the receptor DKO strains revealed that endogenous cysLT-driven expression of IL-33 in *Df*-primed *Ptges*$^{-/-}$ mice depended entirely on $CysLT_2R$ (Fig. 4A). Pharmacologic blockade of $CysLT_2R$ yielded similar results (Fig. 4B, 4C), indicating that the effects of $CysLT_2R$ deletion on IL-33 expression were not due to epistasis. Moreover, full induction of IL-33 expression by exogenous LTC_4 and N-methyl LTC_4 required $CysLT_2R$ in PGE_2-sufficient mice as well (Fig. 4G, Fig. 5C). Conversely, studies in both *Ptges*/*Cysltr1* DKO mice (Fig. 4A), in WT mice treated with LTD_4 (Fig. 4F), and in LTC_4-challenged *Cysltr1*$^{-/-}$ mice (Fig. 5C) verify that $CysLT_1R$ plays no direct role in inducing IL-33 expression, contrasting with its role in expansion of ILC2s (Fig. 1B, Fig. 2B, Fig. 5B).

Although deletion of the epithelially-dominant $CysLT_3R$ also reduced eosinophilia and ILC2 accumulation (Fig. 1), it did not suppress IL-33 induction by *Df* (Fig. 4A), suggesting the involvement of an additional to-be characterized type 2 immune pathway. This is further supported by the fact that eosinophilia, ILC2 expansion, and IL-5 generation induced by exogenous LTE_4 was abrogated in *Cysltr3*$^{-/-}$ mice, even though the modest increment in IL-33 remained largely intact. Thus, cysLTs control ILC2 homeostasis by both direct ($CysLT_1R$-dependent) and indirect ($CysLT_2R$ and possibly $CysLT_3R$-mediated) effects. These effects at anatomically distinct targets may synergistically promote type 2 immunopathology in contexts where cysLT production is increased. Moreover, $CysLT_2R$ and $CysLT_3R$ in the lung show sharp respective preferences for LTC_4 and LTE_4, respectively.

$CysLT_2R$-dependent IL-33 expression in our study was restricted primarily to SPC+ AT2 cells (Fig. 4D), which are alveolar stem cells that constitutively express IL-33 and participate in epithelial regeneration (49, 59). Both the studies using gene deleted mice and pharmacologic blockade indicate that $CysLT_2R$ controls both the total number of IL-33+AT2 cells and the amount of IL-33 protein per cell. Although AT2 cells from rat lung were previously shown to express $CysLT_2R$ in preference to $CysLT_1R$ (60), cysLTs failed to induce IL-33 expression directly by A549 cells (an AT2 cell line). While this may reflect a limitation of the cell line used, AT2 cells could be the target of additional inductive factors that either synergize with or result from $CysLT_2R$ signaling to upregulate IL-33 expression in vivo. Because $CysLT_2R$ is expressed broadly by hematopoietic (platelets, macrophages, eosinophils) and non-hematopoietic (fibroblasts, endothelium), such cell-cell interactions seem plausible.

While IL-33 elicits cytokine generation by both ILC2s and CD4+ effector Th2 cells, only ILC2s can drive an IL-13-mediated epithelial feed-forward system that sustains innate type 2 inflammation through IL-33 (14). Blocking experiments supported the importance of IL-33 for full LTC_4-induced eosinophilic inflammation (Fig. 6A), ILC2 expansion (Fig. 6B), and IL-5 and IL-13 production (Fig. 6C). Depletion of either ILC2s (with anti-CD90.2) or conventional T cells (with anti-CD4) both dramatically suppressed the increase in total BAL fluid cell counts and the induction of eosinophilia by LTC_4 (Fig. 6D), whereas anti-NK1.1 was inactive. Although anti-CD90.2 was highly effective for depletion of ILC2s (Fig. 6E), its effects on eosinophilia could well reflect depletion of one or more additional relevant populations (including T cells) that also express this marker. However, the marked reductions in lung levels of IL-5, IL-13 and IL-33 protein levels induced by this depletion was not replicated by T cell or NK cell-depleting antibodies (Fig. 6F,6G). It is likely that LTC_4, by both directly targeting ILC2s (via $CysLT_1R$) and inducing IL-33 expression by AT2 cells (via $CysLT_2R$), facilitates a bilateral feed-forward pathway between IL-33 and ILC2s noted in other models (14). The suppression of eosinophilia by anti-CD4 likely reflects a requirement for allergen-specific sensitization to OVA in this protocol to induce maximal eosinophilia, and could also reflect synergy between conventional T cells and ILC2s recently reported in other models (61, 62).

CysLT-driven reactions to the ingestion of nonselective COX inhibitors are pathognomonic of AERD. Such reactions putatively result from depletion of PGE_2, which “unbrakes” 5-LO in granulocytes and other cells in the respiratory tract. Whereas selective $CysLT_1R$ antagonists can attenuate the decline in lung function occurring in these reactions (63), the 5-LO inhibitor zileuton prevents additional sinonasal, gastrointestinal, and cutaneous manifestations (37), suggesting that receptors other than $CysLT_1R$ may contribute prominently to the pathophysiology of reactions. Curiously, 5-LO inhibition also blocks the cryptic, pharmacologically-mediated activation of MCs that occurs during such reactions in both humans (38) and *Ptges*$^{-/-}$ mice (41), indicating that 5-LO products (likely cysLTs) are essential to this activation mechanism. We previously verified that changes in R_L induced by Lys-ASA challenges of *Df*-primed *Ptges*$^{-/-}$ mice recapitulated the cysLT-dependent pharmacology of reactions in humans, including blockade of changes in lung function with $CysLT_1R$ antagonists (41). We also demonstrated that 5-LO inhibition prevented the activation of MCs (41), and that the latter depended on cysLT-driven IL-33 release(40). Our current study demonstrates that $CysLT_2R$ signaling is essential for the downstream pathophysiologic consequences of incremental cysLT generation, activation of MCs (and potentially basophils), and increases in R_L (Fig. 7A–E). Moreover, endogenous cysLTs act through $CysLT_2R$ to amplify their own synthesis (Fig. 7B) (likely via downstream IL-33-mediated mast cell activation) when the PGE_2-dependent brake is removed. Given that other cells (e.g., endothelium, granulocytes) also express $CysLT_2R$, cysLTs could well have additional relevant targets in these reactions that further amplify their severity and/or contribute to their resolution. Notably, the effects of CD90.2 depletion (Fig. 7F–J) support the likely interposition of cysLT-activated ILC2s (via IL-13 or other cytokines) in maintaining IL-33 necessary for Lys-ASA-induced MC activation. The cysLTs that initiate the reactions may derive from platelets, as suggested by our previous studies (41).

Our findings illustrate distinct and previously unrecognized functions for $CysLT_2R$ in type 2 immunopathology. These functions promote a feed-forward system between ILC2s and IL-33. CysLTs released in response to allergen challenges in atopic subjects elicit bronchoconstriction primarily through $CysLT_1R$ (64). Although no studies have directly contrasted the effects of $CysLT_1R$ antagonists with those of 5-LO inhibition, a recent study demonstrated that a dual $CysLT_1R$/$CysLT_2R$ antagonist, but not a $CysLT_1R$-selective antagonist, prevented the increase in sputum eosinophils occurring in atopic asthmatic subjects following allergen inhalation, even though both drugs blocked the attendant changes in lung function (65). The markedly dysregulated cysLT synthesis, strong IL-33 expression, and high levels of tissue eosinophilia observed in AERD may reflect, in part, immunopathologic contributions from $CysLT_2R$. The findings support the potential therapeutic utility of drugs with the capacity to block $CysLT_2R$, alone or in combination with the other cysLT receptors, in AERD and other conditions characterized by robust type 2 immunopathology and cysLT production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AERD	aspirin exacerbated respiratory disease
AT2	type 2 alveolar cell
COX	cyclooxygenase
CysLTs	cysteinyl LTs
$CysLT_1R$	type 1 cysLT receptor
$CysLT_2R$	type 2 cysLT receptor
$CysLT_3R$	type 3 cysLT receptor
Df	extract from *Dermatophagoides farinae*
DKO	double knockout
ILC2s	group 2 innate lymphoid cells
LT	leukotriene
Lys-ASA	lysine aspirin
LTC_4S	leukotriene C_4 synthase
MC	mast cell

MMCP	mouse MC protease
PGE_2	prostaglandin E_2
SPC	surfactant protein C
ST2	suppressor of tumorogenicity 2
TXA_2	thromboxane A_2
5-LO	5-lipoxygenase

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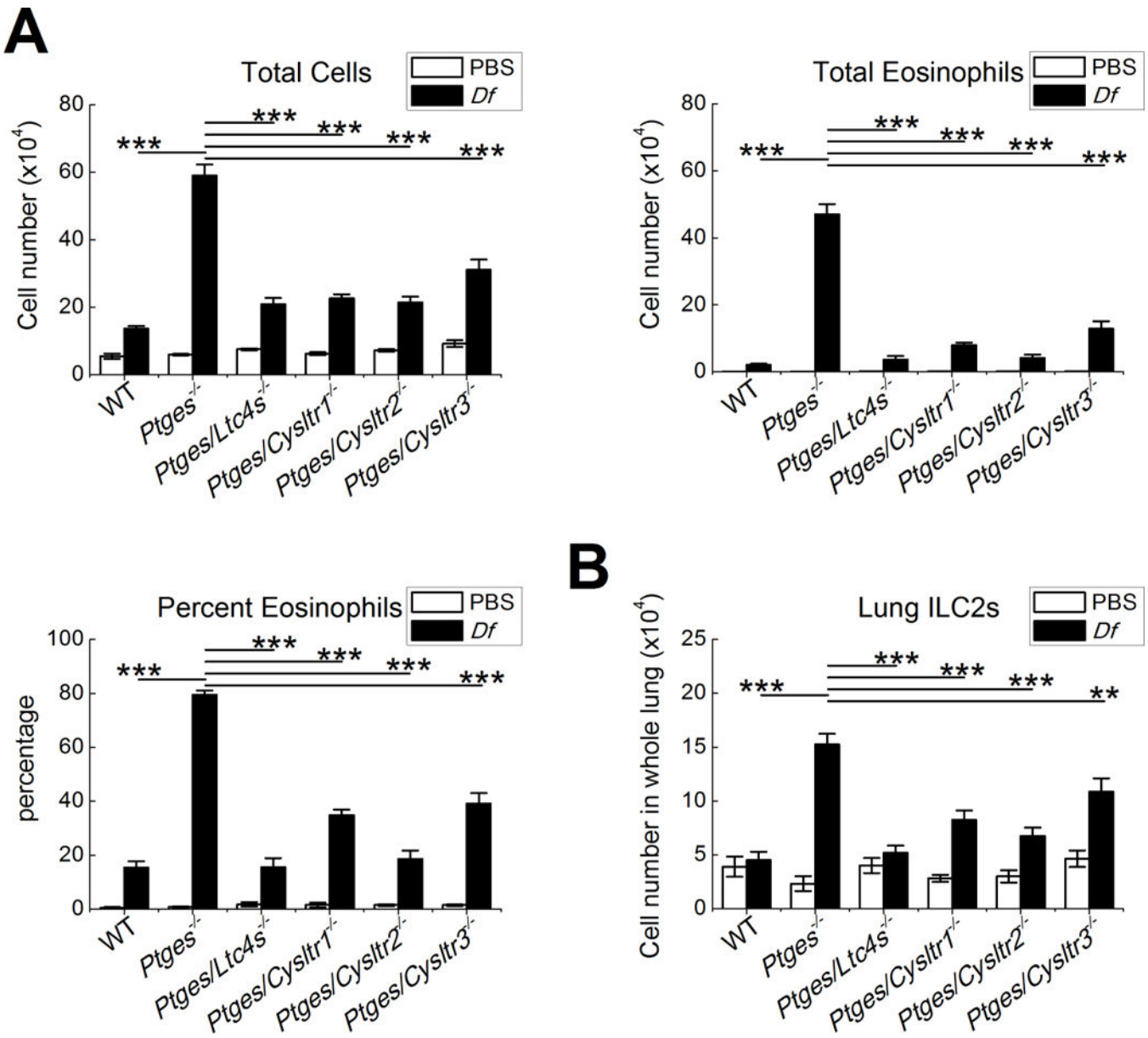

Figure 1. Contributions of individual cysLT receptors and ligands to features of type 2 immunopathology

Mice of the indicated genotypes were treated on six occasions with *Df* (3 μg) or PBS over a 17-day period. Twenty-four hours after the last dose, mice were euthanized for studies. **A.** BAL fluid total cell counts (left), eosinophil counts (right), and percentages of eosinophils (bottom). **B.** Numbers of ILC2s (Lin-CD45+,CD44+,CD278+,CD90.2+ cells) in the lungs of the indicated strains. Results from 10 mice/group. *** $P < 0.001$, ** $P < 0.01$.

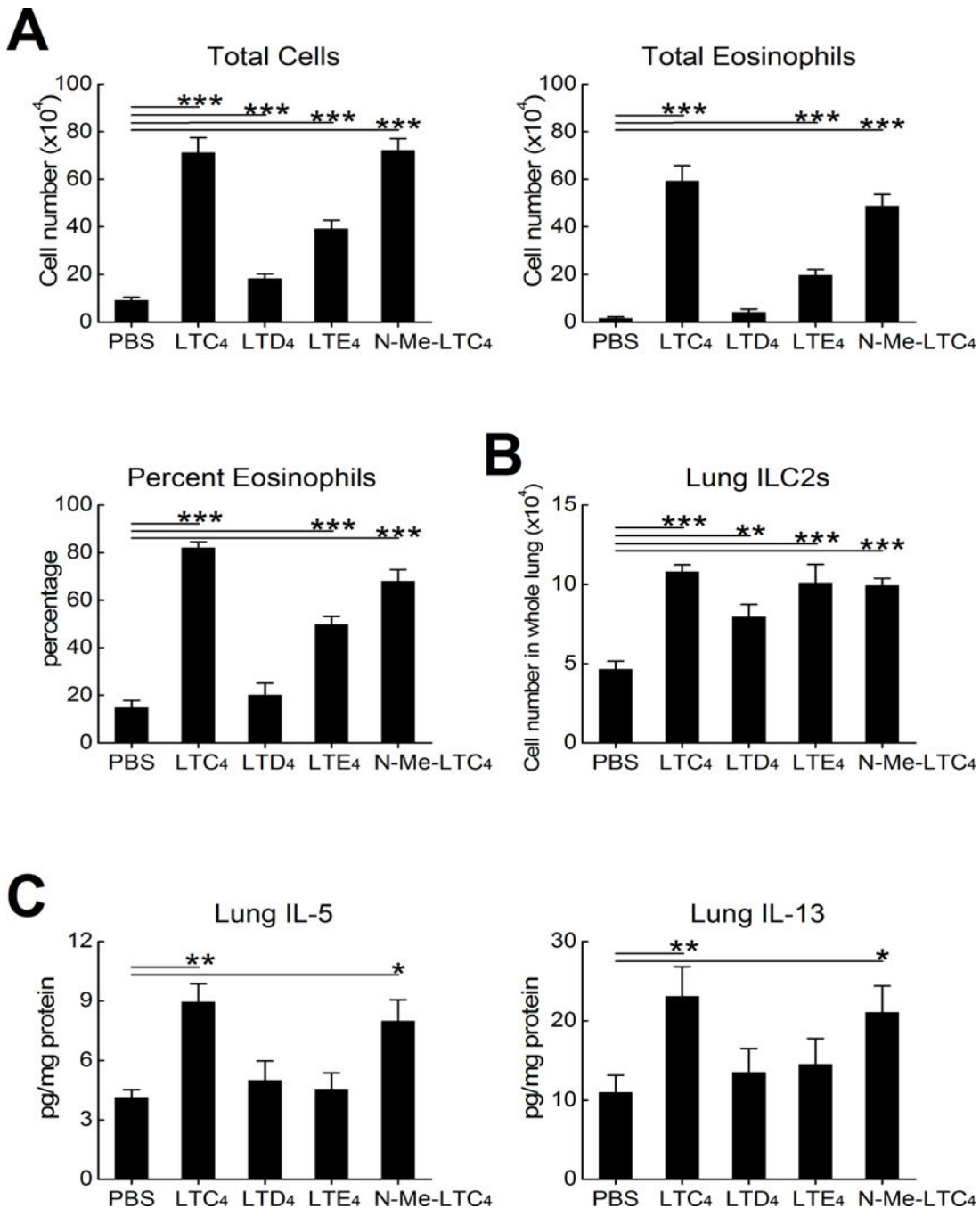

Figure 2. Exogenous cysLTs induce type 2 immunopathology in PGE_2-sufficient mice

OVA sensitized WT C57BL/6 mice received challenges with 0.1% OVA on three successive days. The indicated groups of mice received 2.2 nmol of the indicated cysLTs, N-methyl LTC_4, or a vehicle control intranasally 12 h prior to each OVA challenge. **A.** BAL fluid total cell counts (left), percentage eosinophils (right) and total eosinophil counts (below) from mice euthanized 24 h after the last challenge. **B.** Numbers of ILC2s in the lungs of the indicated groups. **C.** Levels of IL-5 and IL-13 proteins in lysates of the lungs from the same mice as in **B**. Results are from 10 mice/group. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

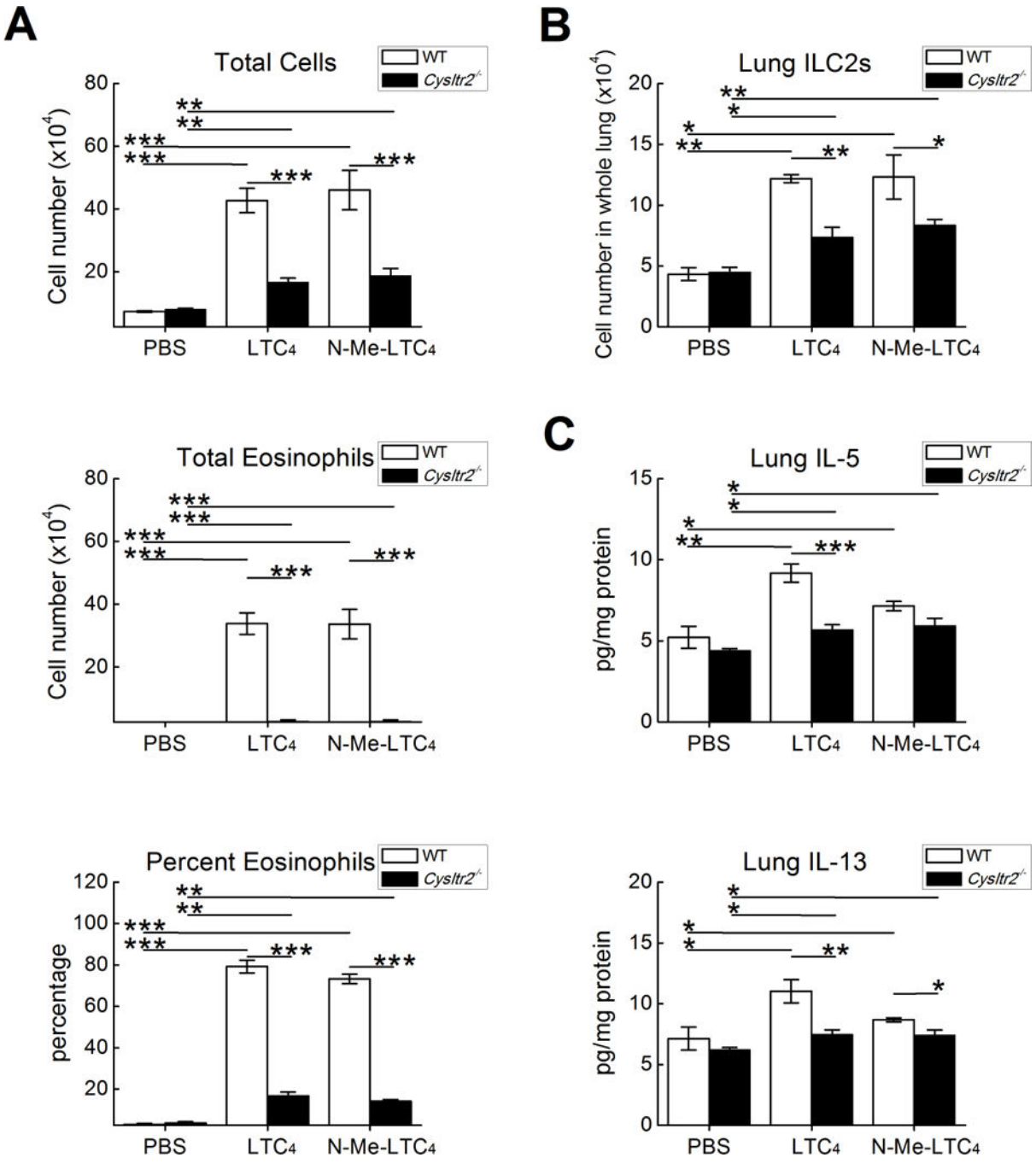

Figure 3. CysLT$_2$R mediates responses to exogenous LTC$_4$

OVA sensitized WT C57BL/6 mice received challenges with 0.1% OVA on three successive days. The indicated groups of mice received 2.2 nmol of LTC_4, N-methyl LTC_4, or a vehicle control intranasally 12 h prior to each OVA challenge. **A.** BAL fluid total cell counts (top), percentage eosinophils (middle) and total eosinophil counts (bottom) from mice euthanized 24 h after the last challenge. **B.** Numbers of ILC2s in the lungs of the indicated groups. **C.** Levels of IL-5 (top) and IL-13 (bottom) proteins in lysates of the lungs from the same mice as in **B**. Results are from 10 mice/group. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

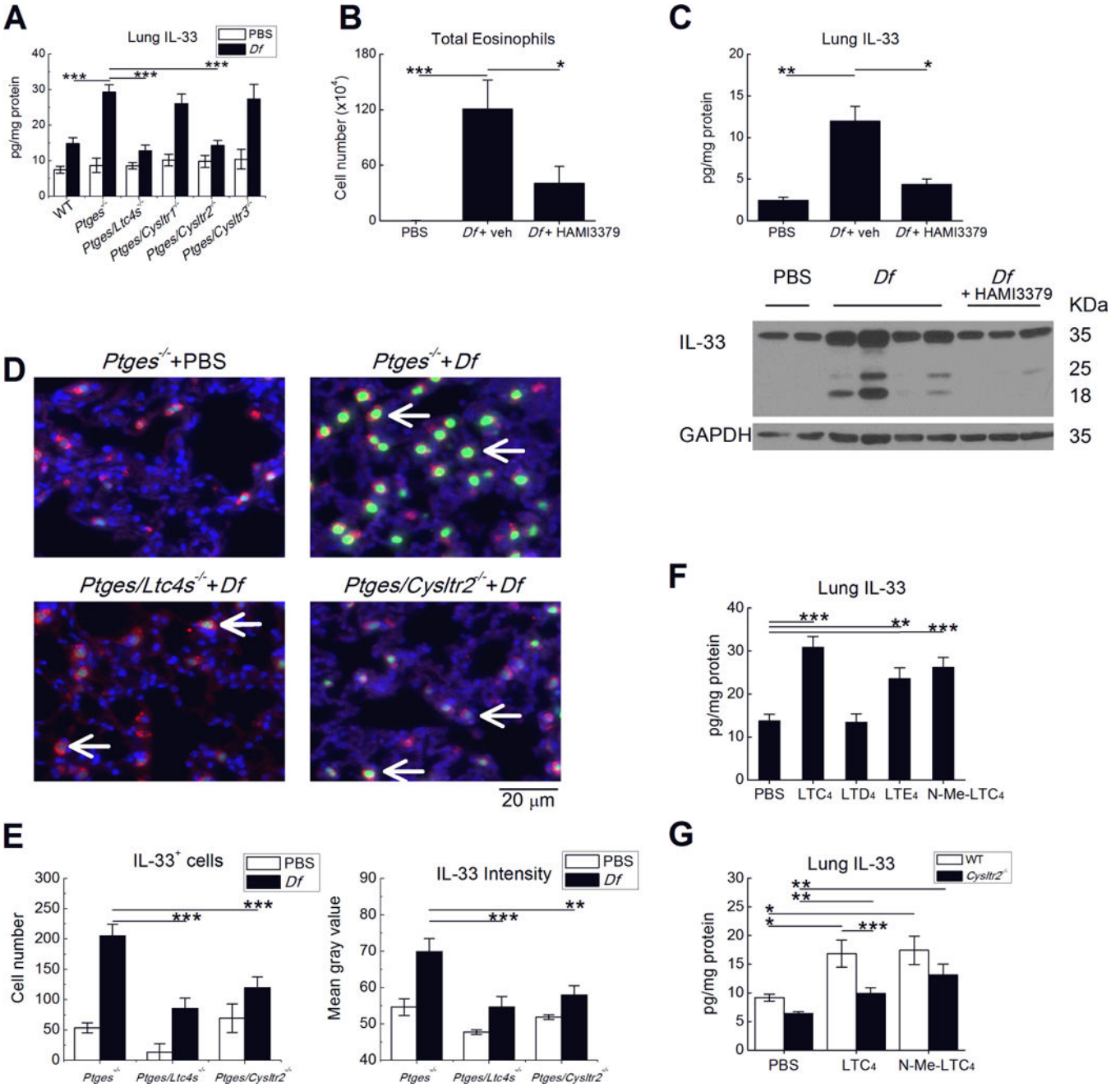

Figure 4. CysLT$_2$R is necessary for IL-33 expression induced by endogenous or exogenous cysLTs

A. Levels of IL-33 protein detected in the lysates of lungs from the indicated strains of mice treated on six occasions with *Df* (3 μg) intranasally, or with PBS. **B.** Total BAL fluid eosinophils and **C.** Lung IL-33 levels (top) in *Ptges*$^{-/-}$ mice treated daily with the CysLT$_2$R-selective antagonist HAMI-3379 during priming with *Df*. Western blot (bottom) showing full length (~35 kDa) and cleaved forms of IL-33 in whole lung lysates from *Ptges*$^{-/-}$ mice of the indicated treatment groups. **D.** Immunofluorescent stains for IL-33 (green) localizing to the nuclei (blue) of SPC (red)-expressing lung AT2 cells from representative mice of the

indicated strains and treatment groups. **E.** Quantitative analysis of numbers (left) and per cell intensity (right) of IL-33 staining in AT2 cells. **F.** Effects of exogenous cysLTs on lung IL-33 protein in OVA-sensitized and challenged PGE_2 sufficient mice. **G.** Effect of $CysLT_2R$ deletion on lung IL-33 expression induced by the indicated exogenous cysLTs. Results in **A-C** and **E-G** are from 10 mice/group. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

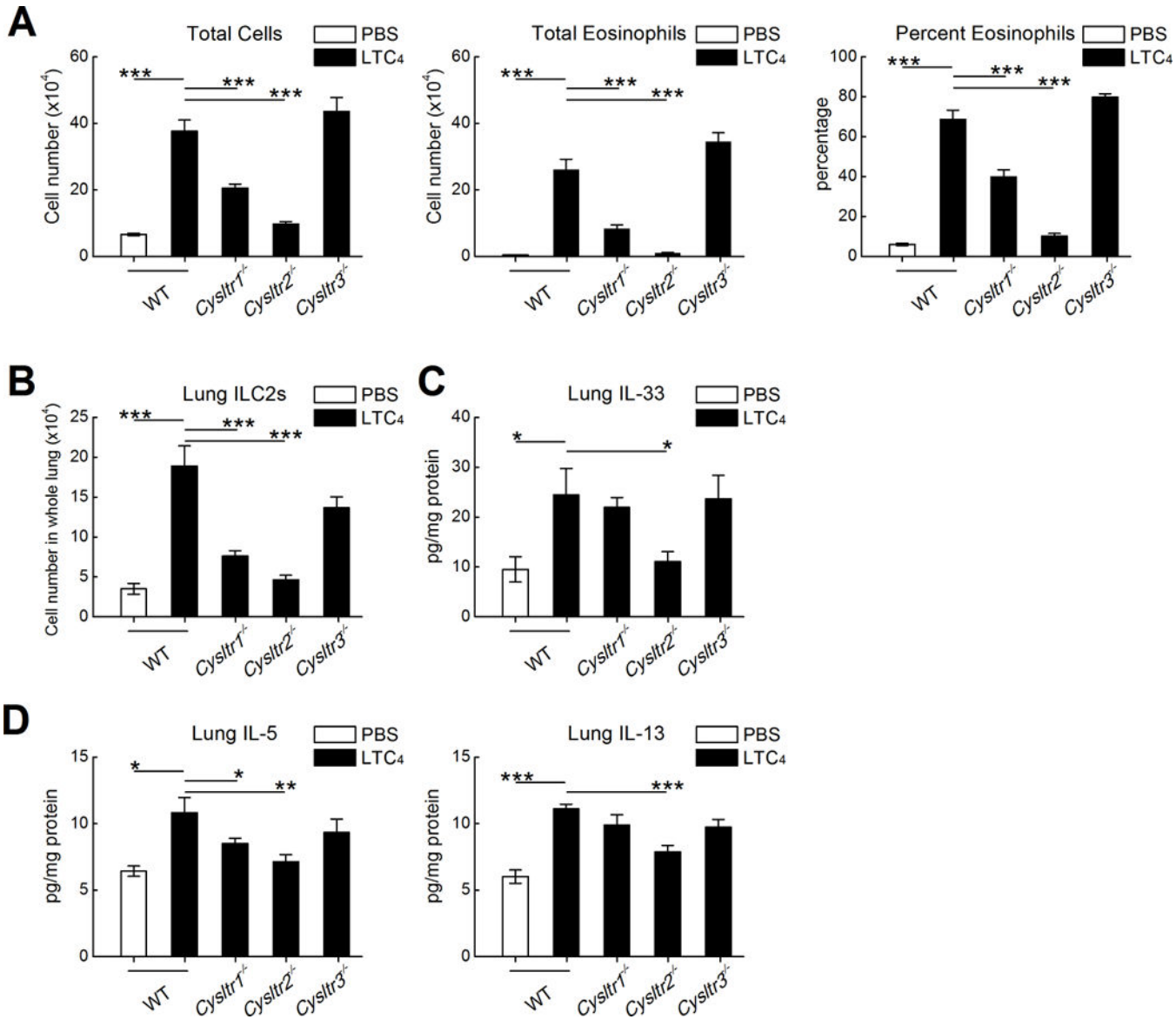

Figure 5. CysLT$_1$R and CysLT$_2$R contribute separately to LTC$_4$-induced ILC2 expansion

OVA sensitized mice of the indicated genotypes received challenges with 0.1% OVA on three successive days. The indicated groups of mice received 2.2 nmol of LTC$_4$ or control. **A.** Total BAL fluid cell counts (left), eosinophil counts (middle) and percentages of eosinophils (right). **B.** Total numbers of lung ILC2s from the same mice as in **A. C.** Levels of IL-33 detected in whole lung lysates from the indicated mice. **D.** Levels of IL-5 (left) and IL-13 (right) proteins in the same samples as in **C.** Results are from 10 mice/group. *** P < 0.001, ** P < 0.01, * P <0.05.

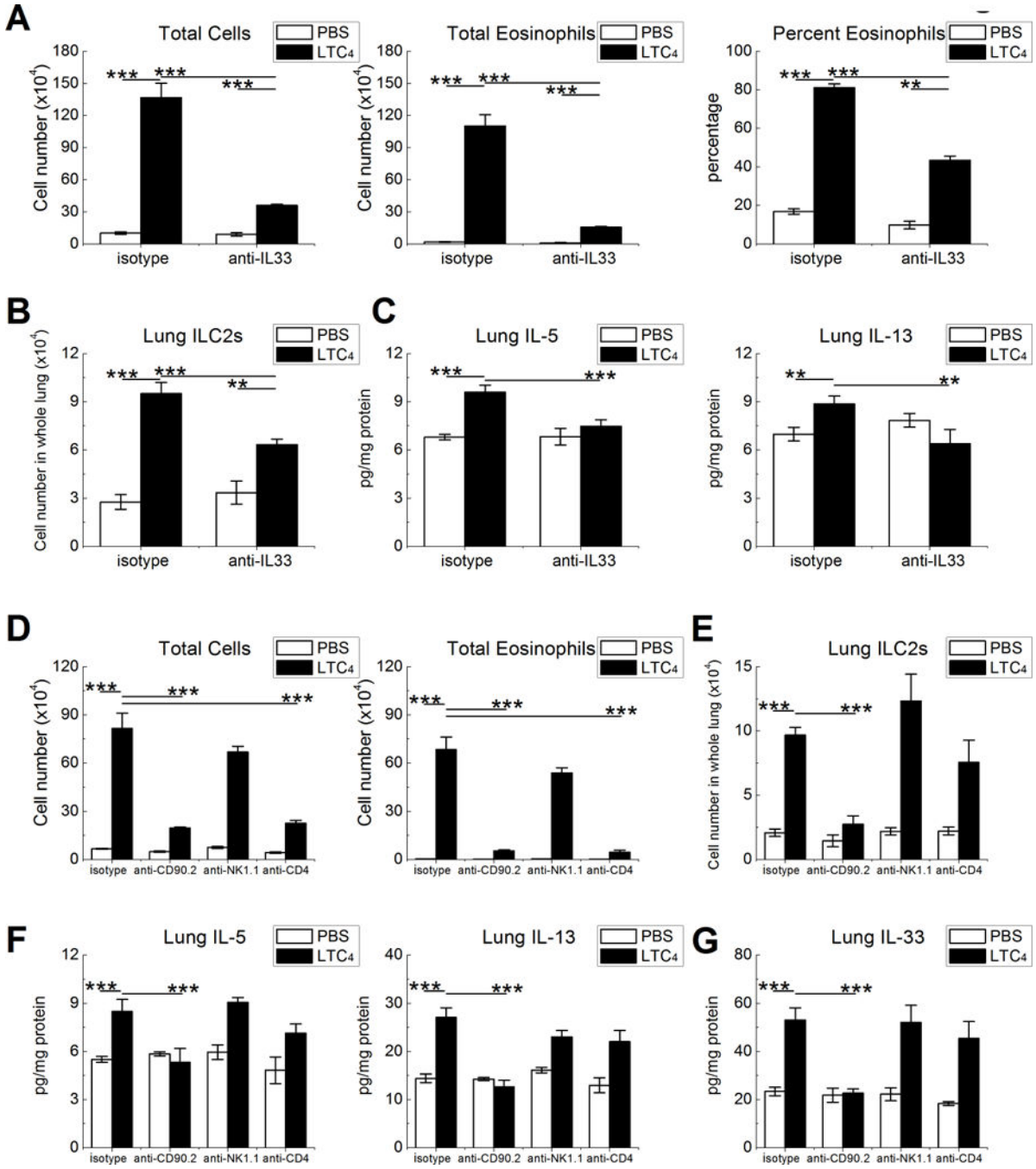

Figure 6. LTC_4 elicits a bilateral feed-forward system between IL-33 and CD90.2+ cells

OVA sensitized CD57/BL6 mice were challenged three times with LTC_4 prior to OVA inhalation. Abs against the indicated proteins or isotype controls were administered to cohorts of mice before the 2nd and 3rd challenges. **A.** Effect of treatment with a neutralizing anti-IL-33 Ab on BAL fluid total cell counts (left) and eosinophil counts (middle) and percentages (right). **B.** Effect IL-33 neutralization on lung ILC2 numbers. **C.** Effect of IL-33 neutralization on IL-5 and IL-13 levels in lung lysates. **D.** BAL fluid total cell (left) and total eosinophil (right) counts in mice treated with the Abs against the indicated cell surface

markers. **E.** Lung ILC2 counts from the same mice as in **D**. **F.** Levels of IL-5 and IL-13 detected by ELISA of lung lysates. **G.** IL-33 protein levels from the same samples as in **E.** Results are from at least 10 mice per group. *** $P < 0.001$, ** $P < 0.01$.

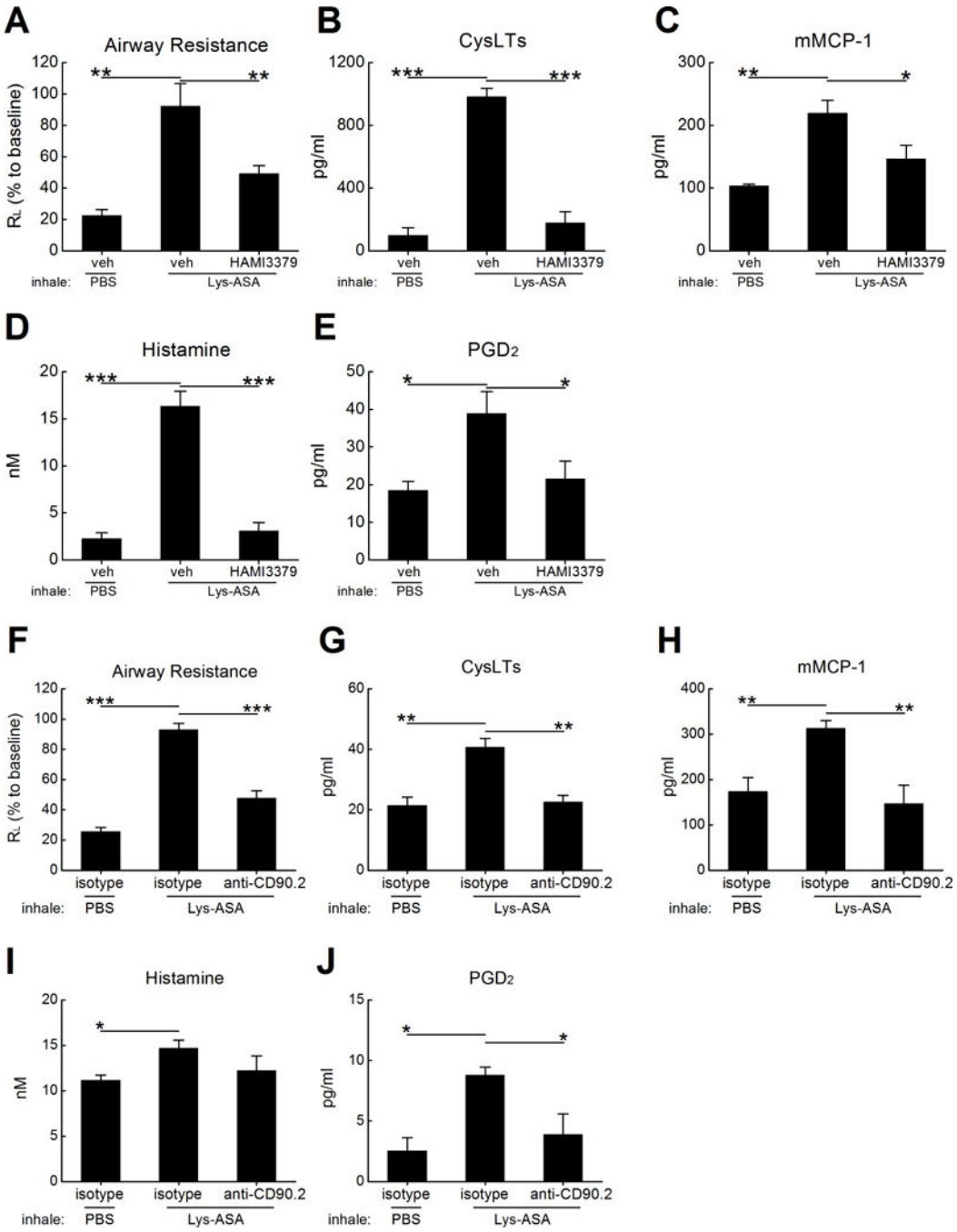

Figure 7. CysLT$_2$R blockade prevents physiologic responses to aspirin challenge in AERD-like mice

Df-primed *Ptges*$^{-/-}$ mice were treated overnight with IP doses of HAMI-3379 or vehicle control. Sedated and mechanically ventilated mice were challenged by inhalation with Lys-ASA or PBS. **A.** Peak changes in R_L monitored over a 45-min period during the challenge. **B.** BAL fluid levels of cysLTs in the same mice. **C.** BAL fluid levels of mMCP-1, **D.** histamine and **E.** PGD_2 from the same mice. **F.** Effects of depleting CD90.2+ cells on R_L. **G.** BAL fluid levels of cysLTs, **H.** mMCP-1, **I.** histamine, and **J.** PGD_2 from the same mice as

in **F**. Results in **A-E** are from 10 mice/group, and those in **F-J** are from five per group. *** P < 0.001, ** P < 0.01, * P < .05.