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This information is current as of July 7, 2017.

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J Immunol published online 30 June 2017
<http://www.jimmunol.org/content/early/2017/06/29/jimmunol.1601569>

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Leukotriene C4 Potentiates IL-33–Induced Group 2 Innate Lymphoid Cell Activation and Lung Inflammation

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Asthma is a complex disease that is promoted by dysregulated immunity and the presence of many cytokine and lipid mediators. Despite this, there is a paucity of data demonstrating the combined effects of multiple mediators in asthma pathogenesis. Group 2 innate lymphoid cells (ILC2s) have recently been shown to play important roles in the initiation of allergic inflammation; however, it is unclear whether lipid mediators, such as cysteinyl leukotrienes (CysLTs), which are present in asthma, could further amplify the effects of IL-33 on ILC2 activation and lung inflammation. In this article, we show that airway challenges with the parent CysLT, leukotriene C4 (LTC4), given in combination with low-dose IL-33 to naive wild-type mice, led to synergistic increases in airway Th2 cytokines, eosinophilia, and peribronchial inflammation compared with IL-33 alone. Further, the numbers of proliferating and cytokine-producing lung ILC2s were increased after challenge with both LTC4 and IL-33. Levels of CysLT1R, CysLT2R, and candidate leukotriene E4 receptor P2Y12 mRNAs were increased in ILC2s. The synergistic effect of LTC4 with IL-33 was completely dependent upon CysLT1R, because CysLT1R^{-/-} mice, but not CysLT2R^{-/-} mice, had abrogated responses. Further, CysLTs directly potentiated IL-5 and IL-13 production from purified ILC2s stimulated with IL-33 and resulted in NFAT1 nuclear translocation. Finally, CysLT1R^{-/-} mice had reduced lung eosinophils and ILC2 responses after exposure to the fungal allergen *Alternaria alternata*. Thus, CysLT1R promotes LTC4- and *Alternaria*-induced ILC2 activation and lung inflammation. These findings suggest that multiple pathways likely exist in asthma to activate ILC2s and propagate inflammatory responses. *The Journal of Immunology*, 2017, 199: 000–000.

Asthma is a chronic inflammatory disorder that is characterized by a complex interplay between many cell types and mediators leading to airway inflammation, remodeling, and hyperresponsiveness. The cytokine IL-33 and the cysteinyl leukotrienes (CysLTs) are present in human asthmatic samples and individually promote inflammatory and structural cell responses, as well as airway hyperresponsiveness (AHR) (1–3). IL-33 is an IL-1 family member that activates innate and adaptive immune cells and is considered an “alarmin,” with high expression in the airway epithelium and other cells, including smooth muscle and macrophages (4–7). IL-33 interacts with a heterodimeric complex of IL-1 family receptor accessory molecules T1 and ST2 on the surface of mast cells and Th2 cells and has most recently been shown to be a critical activator of group 2 innate lymphoid cells (ILC2s) (6, 8, 9). Interaction between IL-33 and T1/ST2 leads to MyD88-dependent NF- κ B signaling, as well as activation of

MAPK/p38 pathways (10–12). In human asthmatics, a recent study demonstrated that levels of IL-33 are correlated with asthma severity, as well as thickness of the lung airway epithelium (13).

The CysLTs include leukotriene C4 (LTC4), leukotriene D4 (LTD4), and leukotriene E4 (LTE4) and are generated after arachidonic acid is converted into leukotriene A4 by 5-lipoxygenase and subsequently into LTC4, LTD4, and LTE4 (14, 15). The prototype receptors for CysLTs include CysLT1R and CysLT2R, although other receptors, including GPR99 and P2Y12, are also involved in LTE4 responses (16). The parent CysLT, LTC4, binds equally to CysLT1R and CysLT2R, whereas LTD4 binds preferentially to CysLT1R. In humans, CysLT1R is expressed on airway smooth muscle cells and immune cells, and stimulation with LTC4 or LTD4 leads to robust airway constriction (1, 17). In mice, LTC4 synthase deficiency results in reduced influx of eosinophils and AHR during allergen challenge (18). Further, LTC4 synthase-deficient and CysLT1R^{-/-} mice fail to develop a Th2 response to house dust mite, suggesting critical innate contributions of CysLTs (19). Our group has previously shown that LTD4 is a potent activator of mouse ILC2s and that challenge with the fungal allergen *Alternaria alternata* induced rapid airway CysLT generation (20). Taken together, these studies suggest that CysLTs contribute to allergic airway responses and promote ILC2 activation. To further elucidate the role of CysLT receptors in ILC2 biology, we focus, in this study, on the effects of LTC4 on ILC2 responses given the equal affinity to bind CysLT1R and CysLT2R that are present in ILC2s (21).

ILC2s are lineage-negative lymphocytes that express IL-33R (T1/ST2) and CysLT1R and are increasingly recognized contributors to type 2 asthma pathogenesis from studies in mice and humans (22). IL-33 is a quintessential activator of ILC2s that promotes rapid and robust ILC2 IL-5 and IL-13 production (8). However, unlike IL-33, LTD4 is capable of stimulating ILC2 IL-4 production, which may further drive Th2 cell responses (20, 23).

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Received for publication September 8, 2016. Accepted for publication June 6, 2017.

This work was supported by National Institutes of Health Grants AI114585 and AI070535 (to T.A.D.), and AI107779, AI038425, AI070535, and AI242236 (to D.H.B.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AERD, aspirin-exacerbated respiratory disease; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage fluid; CysLT, cysteinyl leukotriene; ILC2, group 2 innate lymphoid cell; KO, knockout; LTC4, leukotriene C4; LTD4, leukotriene D4; LTE4, leukotriene E4; qPCR, quantitative PCR; WT, wild-type.

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Although significant progress has been made regarding activation of ILC2s by individual cytokines *in vivo*, there is a paucity of data regarding the combined effects of lipid mediators and IL-33 on ILC2 activation and lung inflammation. Further, the role of CysLT1R in ILC2 responses of allergen-challenged mice is unknown. Because combinations of mediators are present in human asthma, the possibility of synergistic effects on ILC2 activation could lead to amplified responses and detrimental outcomes in patients. Thus, we hypothesized that LTC4 may potentiate IL-33-induced ILC2 activation and proliferation, as well as lung inflammation.

Materials and Methods

Mice

Wild-type (WT) 6–8-wk-old female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). RAG2^{-/-} and IL-7R^{-/-} mice were also obtained from The Jackson Laboratory and bred in-house. CysLT1R^{-/-} and CysLT2R^{-/-} mice were kindly provided by Dr. Y. Kanaoka, Dr. J. Boyce, Dr. N. Barrett, and Dr. F. Austen (Brigham and Women's Hospital, Boston, MA) and bred in-house. All animal experiments were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

In vivo IL-33 and CysLT challenge models

WT, RAG2^{-/-}, IL-7R^{-/-}, CysLT1R^{-/-}, and CysLT2R^{-/-} mice were challenged with PBS, 100 ng of IL-33, 100 ng of LTC4, LTD4, or LTE4, or 100 ng of IL-33 + LTC4, LTD4, or LTE4 daily for 3 d and euthanized the day after last challenge. In some experiments, mice were challenged with 50 ng of IL-33, 200 ng of LTC4, LTD4, or LTE4, or IL-33 + CysLTs. Bronchoalveolar lavage fluid (BAL) was collected, and the lungs were removed. BAL was centrifuged at 1500 rpm for 5 min at 4°C, and the supernatant was kept and stored at -80°C for ELISA of cytokines. BAL cells were analyzed by flow cytometry. The lungs were digested using a Miltenyi Biotec (San Diego, CA) lung dissociator, according to the manufacturer's protocol.

A. alternata allergen challenge model

WT and CysLT1R^{-/-} mice were challenged with 30 µg of *A. alternata* extract (Greer, Lenoir, NC) on day 0, then 3 µg on days 9 and 12. BAL was collected and the lungs were then removed. Following, the BAL was then centrifuged at 1500 RPM for 5 min at 4°C and the supernatant was kept and stored at -80°C for ELISA of cytokines. BAL cells were analyzed by flow cytometry. The lungs were digested using the Miltenyi Biotec lung dissociator according to the manufacturer's protocol.

Flow cytometry

BAL and lung single-cell suspensions were counted using an Accuri C6 Flow Cytometer (BD) and then prepared for staining by washing with a PBS solution of 10% FBS and 0.01% sodium azide. Eosinophils were identified as CD45⁺ Siglec-F⁺ CD11c⁻ cells using anti-CD45.2 conjugated to PerCP (eBioscience, San Diego, CA), anti-Siglec-F conjugated to PE (BD), anti-CD11c conjugated to FITC (eBioscience), and anti-Gr1 conjugated to allophycocyanin (eBioscience). Lung ILC2s were stained with a mixture of lineage Abs all conjugated to FITC, including a base lineage mixture (BioLegend, San Diego, CA), anti-CD4, anti-CD8, anti-CD11c, anti-CD19, anti-Nk1.1, anti-FcεR1, and anti-TCRγδ, along with anti-Thy1.2 conjugated to allophycocyanin and anti-CD45.2 conjugated to PerCP. ILC2s were defined as CD45.2⁺ lineage⁻ Thy1.2⁺ lymphocytes. We have previously shown that this population in the mouse lung predominantly expresses T1/ST2 (IL-33R) and GATA-3 (24). Intracellular cytokine analysis was performed using anti-IL-5 or anti-IL-13 conjugated to PE (eBioscience) after surface staining and permeabilization of whole lung cells that were cultured for 16–18 h with 10 ng/ml IL-2 and IL-7 in RPMI 1640 supplemented with penicillin/streptomycin, 10% FBS, glutamine, 2-ME, and Golgi-Plug (BD). Additionally, to assess proliferation in ILC2s, an anti-Thy1.2 Ab conjugated to PE and an anti-Ki67 Ab conjugated to eFluor 660 (eBioscience) were used.

ILC2 *in vitro* studies

ILC2s were sorted from WT mice that were challenged three times with 50 µg of *A. alternata* on days 0, 3, and 6. On day 7, mice were euthanized, and lungs were collected. The lungs were digested using a Miltenyi Biotec

lung dissociator, according to the manufacturer's protocol. Cells were prepared for staining by washing with a PBS solution of 10% FBS and 0.01% sodium azide. Lung ILC2s were stained with a mixture of lineage Abs all conjugated to FITC, including a base lineage mixture (BioLegend), anti-CD4, anti-CD8, anti-CD11c, anti-CD19, anti-Nk1.1, anti-FcεR1, and anti-TCRγδ, along with anti-Thy1.2 conjugated to allophycocyanin and anti-CD45.2 conjugated to PerCP. ILC2s were defined as CD45.2⁺ lineage⁻ Thy1.2⁺ lymphocytes. Cells were sorted using a BD FACS Aria II cell sorter. ILC2s from sorts reached a purity of 97–99%.

Purified ILC2s were cultured with 10 ng/ml IL-2 and IL-7 and rested for 48 h, followed by media changes and functional assays. ILC2s were stimulated with 30 ng/ml IL-33, 10⁻⁶ M LTC4, LTD4, or LTE4, or a combination of IL-33 and each CysLT for 6 h. Cell culture supernatants were collected and stored at -80°C for future ELISA analysis, and ILC2s were collected for RNA extraction and quantitative PCR (qPCR) analysis. In other experiments, ILC2s were stimulated for 90 min and then collected immediately on a slide using cytospin for immunofluorescent staining.

ELISA

ELISAs for IL-5 and IL-13 (R&D Systems, Minneapolis, MN; eBioscience) were performed on BAL and *in vitro* culture supernatants, according to the manufacturers' protocol. ELISA plates were read on a model 680 microplate reader (Bio-Rad, Hercules, CA) at 450 nm with correction at 550 nm.

Immunofluorescence

Following *in vitro* stimulation, the cells were removed from culture and added to slides using cytospin. Each slide was immediately fixed in cold methanol. After fixation, slides were permeabilized with Triton-X and blocked with 5% donkey and goat serum. Each slide was stained with Alexa Fluor 488-conjugated NFATc1 (NFAT2; Cell Signaling) and an unconjugated goat NFATc2 (NFAT1; BioLegend). An anti-goat Alexa Fluor 555 secondary Ab was added to visualize NFATc2. After washing, the nuclear stain DRAQ5 was added. Slides were mounted with ProLong Gold Antifade Mountant and sealed. Images were taken using a Leica TCS confocal microscope at 630× magnification.

qPCR of sorted ILC2s

WT mice received three or four 25-µg intranasal *Alternaria* challenges over 9–10 d prior to FACS sorting to expand ILC2s. Sorting of ILC2s (CD45⁺ lineage⁻ Thy1.2⁺) was performed using a FACS Aria cell sorter (BD Biosciences). FACS-sorted lung ILC2s, CD4⁺ T1ST2⁺ cells, and CD4⁺ T1ST2⁻ cells were lysed using TRIzol Reagent (Life Technologies) to isolate RNA. An aliquot of total RNA (0.5 µg) was reverse transcribed to cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche), following the manufacturer's instructions. Diluted cDNA was subjected to RT-PCR using SYBR Green I Master (Roche) and RT-PCR-specific primers. The oligonucleotide primer sequences (5'–3') were as follows: mIL-5 forward AAGAGAAGTGTGGCGAGGAGA; mIL-5 reverse CACCAAGGAACCTCTTGCAGGTAA; mIL-13 forward GAGCAACATCACAC-AAGACCAGA; mIL-13 reverse GCCAGGTCCACACTCCATA; CysLT1R forward AACGAACATATCCACCTTCACC; CysLT1R reverse AGCCTTC-TCTAAAGTTTCCAC; CysLT2R forward GCTTTTGGAAGAGAGAA-GAGTCCA; CysLT2R reverse AATGATACACATCCTTCCCCAGGA; P2Y12 forward CCCGGAGACACTCATCTCTT; P2Y12 reverse GTCCAGGG-GAGAAGGTG; GPR99 (OXGR-1) forward GTGACACCTGGGCTTCTAC; GPR99 (OXGR-1) reverse CTCCACAGCCTCAGAAACT; L32 forward G-AAACTGGCGGAAACCCA; and L32 reverse GGATCTGGCCCTTGAAC-CTT. Data are presented as normalized to housekeeping gene *L32* using Roche LightCycler 480 (Roche Diagnostics).

Histology

For selected experiments, the left side of the lung was fixed by perfusion with 4% paraformaldehyde prior to sectioning and staining. H&E staining was performed at the Histology Core at the University of California, San Diego Moores Cancer Center (La Jolla, CA).

Data analysis

Flow cytometry data plots were analyzed using FlowJo version 7.8 (TreeStar). ELISA data were analyzed using Microsoft Excel.

Statistics

For all experiments, statistical analysis was performed using GraphPad Prism software (GraphPad, La Jolla, CA). An unpaired *t* test was performed for all experiments unless otherwise specified. The *p* values <0.05 were considered statistically significant.

Results

LTC4 potentiates IL-33-induced lung inflammation

Previous reports have demonstrated that IL-33 given to naive mice results in robust type 2 lung inflammation (9, 25). The doses of IL-33 administered in vivo in several studies range from 500 ng to 1 μ g, which induces very high levels of airway inflammation and eosinophilia (9, 25, 26). To assess for possible synergistic responses to the parent leukotriene, LTC4, we used a lower dose of IL-33 (100 ng), alone or in combination with LTC4. We chose to focus on the effects of LTC4 versus other CysLTs because LTC4 binds to CysLT1R and CysLT2R with equal affinity and allowed for the testing of potential contributions of both receptors during type 2 inflammation. WT C57BL/6 mice were challenged with PBS, IL-33, LTC4, or IL-33 + LTC4 on days 0, 1, and 2, and levels of airway eosinophilia, IL-5, and IL-13 were analyzed on day 3. We found that IL-33 induced airway eosinophilia even at the lower dose (100 ng per challenge). However, mice challenged with IL-33 + LTC4 had nearly double the eosinophils compared with those challenged with IL-33 alone (Fig. 1A). LTC4 also modestly increased neutrophils and lymphocytes in the airways of mice challenged with LTC4 and IL-33; however, these differences were minor compared with the level of eosinophil accumulation (Supplemental Fig. 1). The Th2 cytokines IL-5 and IL-13 were further increased by the combination of IL-33 + LTC4 versus IL-33 alone (Fig. 1B, 1C). Lung tissue sections from mice challenged with IL-33 + LTC4 exhibited increased inflammatory cell infiltrate compared with those challenged with IL-33 or LTC4 (Fig. 1D). LTC4 alone had little impact on these end points. Additionally, we administered LTD4 and LTE4 to WT mice receiving IL-33 and found enhanced eosinophilia and ILC2 activation, consistent with our results with LTC4 (Supplemental Fig. 2)

and our previous studies with LTD4 (20). Overall, these data suggest that CysLTs, including LTC4, potentiate IL-33-induced lung inflammation.

To assess whether the changes induced by LTC4 and IL-33 were independent of adaptive immunity, we analyzed airway and lung inflammation in RAG2-knockout (KO) mice that have ILC2s but lack T and B cells. We also compared levels of eosinophilia and BAL Th2 cytokines in RAG2KO (RAG2^{-/-}) mice with those from IL7RKO (IL-7R^{-/-}) mice that lack ILC2s (24). IL-7R^{-/-} and RAG2^{-/-} mice underwent airway challenges as shown in Fig. 1A–D. IL-7R^{-/-} mice did not mount an eosinophilic lung response or increase BAL Th2 cytokine production after challenge with IL-33 alone or LTC4 + IL-33 (Fig. 1E, 1F). However, RAG2^{-/-} mice showed similar responses to WT mice, with increased eosinophilia and BAL Th2 cytokines after LTC4 + IL-33 was administered (Fig. 1E, 1F). Together, these results show that potentiation of IL-33-induced inflammation by LTC4 occurs independently of T and B cells (RAG2^{-/-}) but does not occur in mice that lack ILC2s (IL-7R^{-/-}), which suggests that ILC2s may be contributing to the inflammatory phenotype.

LTC4 potentiates IL-33-induced ILC2 proliferation and cytokine production

Given that ILC2s promote innate type 2 responses after IL-33 administration in vivo (9, 27, 28), we assessed lung ILC2 proliferation and cytokine production after mice were exposed to LTC4 + IL-33 or IL-33 alone. Following the protocol in Fig. 1, WT mice challenged with IL-33 + LTC4 had, on average, a nearly 3-fold increase in the total number of ILC2s and substantial increases in the total numbers of proliferating (Ki-67⁺) and IL-5-producing ILC2s compared with mice challenged with low-dose IL-33 alone (Fig. 2A–C). The percentages of ILC2s, as well as Ki-67⁺, IL-5⁺,

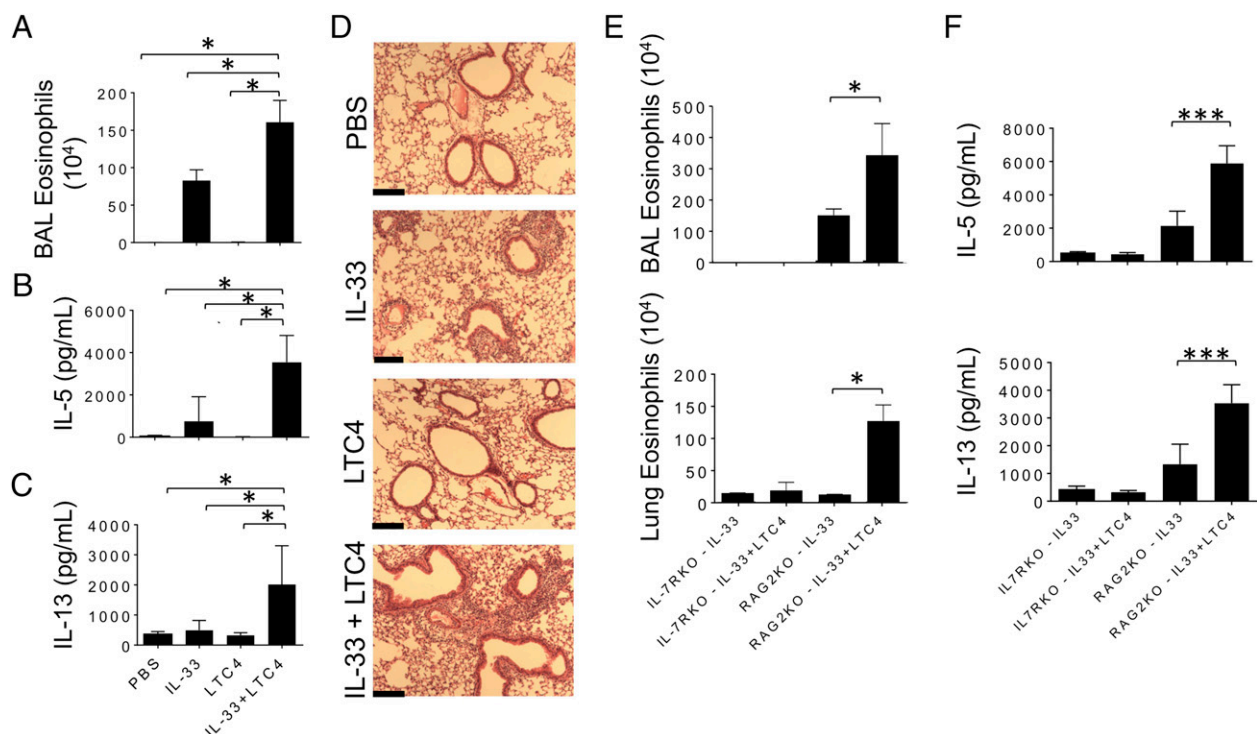


FIGURE 1. LTC4 potentiates IL-33-mediated airway inflammation. WT, IL-7R^{-/-} (IL-7RKO), and RAG2^{-/-} (RAG2KO) mice were challenged with PBS, IL-33, LTC4, or IL-33 + LTC4 once a day for 3 d. On day 4, the mice were euthanized, and BAL and lungs were collected. (A) Total eosinophils in BAL from WT mice. IL-5 (B) and IL-13 (C) in BAL from WT mice. (D) H&E-stained lung sections from WT mice challenged with PBS, IL-33, LTC4, or IL-33 + LTC4. Scale bar, 100 μ m. (E) Total eosinophils in BAL (upper panel) and lungs (lower panel) from challenged IL-7R^{-/-} and RAG2^{-/-} mice. (F) BAL IL-5 and IL-13 in challenged IL-7R^{-/-} and RAG2^{-/-} mice. Data shown are representative of two to five independent experiments with four mice per group. * p < 0.05, *** p < 0.005, unpaired t test.

and IL-13⁺ ILC2s, mirrored the total numbers (Fig. 2D–F). Interestingly, LTC4 administered alone did not appear to induce ILC2 changes at this time point; however, when combined with IL-33, LTC4 induced a robust potentiation of ILC2 activation and proliferation.

We next determined the effect of LTC4 and IL-33 on ILC2 responses from RAG2^{-/-} mice. Given the relatively mild effect of LTC4 alone, we chose to compare the effects of IL-33 alone against LTC4 + IL-33 in subsequent experiments. As a control for ILC2 identification, we also analyzed IL-7R^{-/-} mice (Fig. 3A–D). RAG2^{-/-} mice challenged with LTC4 + IL-33 had increased total ILC2 numbers, Ki-67⁺ ILC2s, and IL-5⁺ ILC2s compared with mice challenged with IL-33 alone (Fig. 3A–C). The percentages of ILC2s, as well as Ki-67⁺ and cytokine-producing ILC2s, were also increased with the addition of LTC4 but less so compared with WT mice (Fig. 3D–F). Overall, LTC4 increased ILC2 proliferation and cytokine production above IL-33 alone in WT and RAG2^{-/-} mice.

LTC4 potentiation of IL-33-induced inflammation is dependent on CysLT1R but not CysLT2R

CysLTs have been shown to induce lung inflammation and activate ILC2s (20, 23, 29). To determine the overall roles of CysLT1R and CysLT2R in LTC4-enhanced lung inflammation, we administered the same protocol in Fig. 1 to WT, CysLT1R^{-/-}, and CysLT2R^{-/-} mice. BAL and lung eosinophils accumulated upon challenge with IL-33 and were potentiated by LTC4 administration in WT and in CysLT2R^{-/-} mice. In contrast, CysLT1R^{-/-} mice did not mount an increase in eosinophilia induced by LTC4 (Fig. 4A, 4B). Further, WT and CysLT2RKO mice developed increased peribronchial cellular infiltration after LTC4 and IL-33 exposure that was not present in sections from CysLT1R^{-/-} mice (Fig. 4C).

LTC4 potentiation of ILC2 activation is dependent on CysLT1R but not CysLT2R

We have previously shown that ILC2s express CysLT1R that is activated by LTD4 to induce Th2 cytokine production (20). The CysLT2 receptor has only very recently been shown to be present on ILC2s (21). Further, the terminal CysLT, LTE4, has been shown to induce inflammatory responses through P2Y12 and GPR99, suggesting that CysLTs may activate ILC2s through a number of receptors (30, 31). To assess levels of CysLT2, P2Y12, and GPR99, as well as to investigate whether IL-33 increases the sensitivity of ILC2s to CysLTs, we performed qPCR to determine the levels of these receptors on purified lung ILC2s. Levels of IL-5 and IL-13 mRNA were used to confirm IL-33 stimulation (Supplemental Fig. 3A, 3B), and, as expected, IL-33 rapidly increased IL-5 and IL-13 mRNAs. Stimulation with IL-33 did not noticeably change the expression of CysLT1R or CysLT2R at 6 h; however, at 24 h, stimulated ILC2s exhibited decreased expression of CysLT1R and CysLT2R (Supplemental Fig. 3C, 3D). P2Y12 receptor expression was increased at 6 and 24 h (Supplemental Fig. 3E) after IL-33 stimulation. GPR99 mRNA was not detected in these experiments (data not shown). Thus, lung ILC2s could potentially respond to CysLTs through CysLT1, CysLT2, or P2Y12 receptors.

LTC4-challenged WT and CysLT2RKO mice showed an increase in the percentages and total numbers of ILC2s and Ki-67⁺ ILC2s (Fig. 5). In contrast, CysLT1R ILC2s showed no further increase in proliferation after LTC4 exposure. CysLT1RKO mice also produced very little airway IL-5 or IL-13 after exposure to LTC4 compared with WT and CysLT2RKO mice, suggesting that CysLT1R, not CysLT2R, regulates LTC4-mediated ILC2 proliferation. BAL Th2 cytokine responses to LTC4 revealed increased IL-5 and IL-13 in WT and CysLT2R^{-/-} mice, but not in CysLT1R^{-/-}

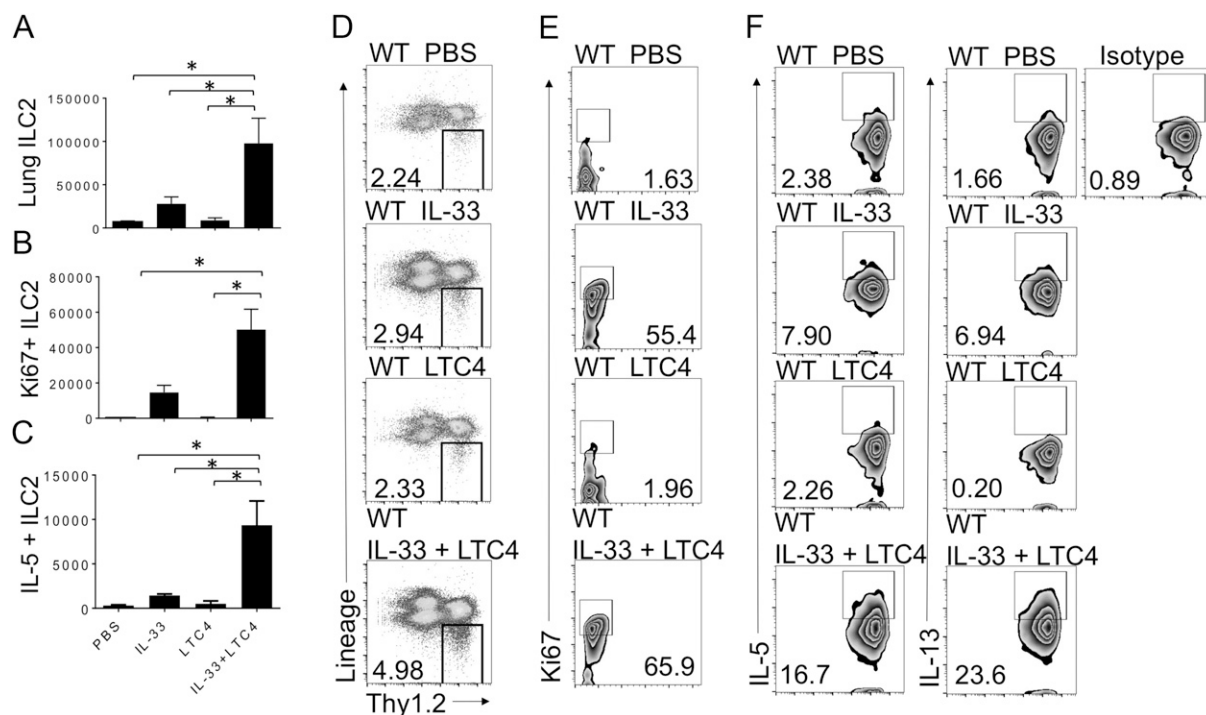


FIGURE 2. LTC4 potentiates IL-33-induced ILC2 activation and cytokine production. WT mice were challenged with PBS, IL-33, LTC4, or IL-33 + LTC4 once a day for 3 d. On day 4, mice were euthanized, and BAL and lungs were collected. Total numbers of ILC2s (A), proliferating Ki67⁺ ILC2s (B), and IL-5⁺ ILC2s (C) in the lungs of WT mice. (D) Percentages of lung ILC2s within the lymphocyte gate. FACS plots of ILC2 Ki67 (E) and ILC2 IL-5 and IL-13 (F). ILC2s were defined as CD45⁺ lineage⁻ Thy1.2⁺ lymphocytes. FACS plots in (E) and (F) were gated on ILC2s. Data shown are representative of five independent experiments, with four mice per group. **p* < 0.05, unpaired *t* test.

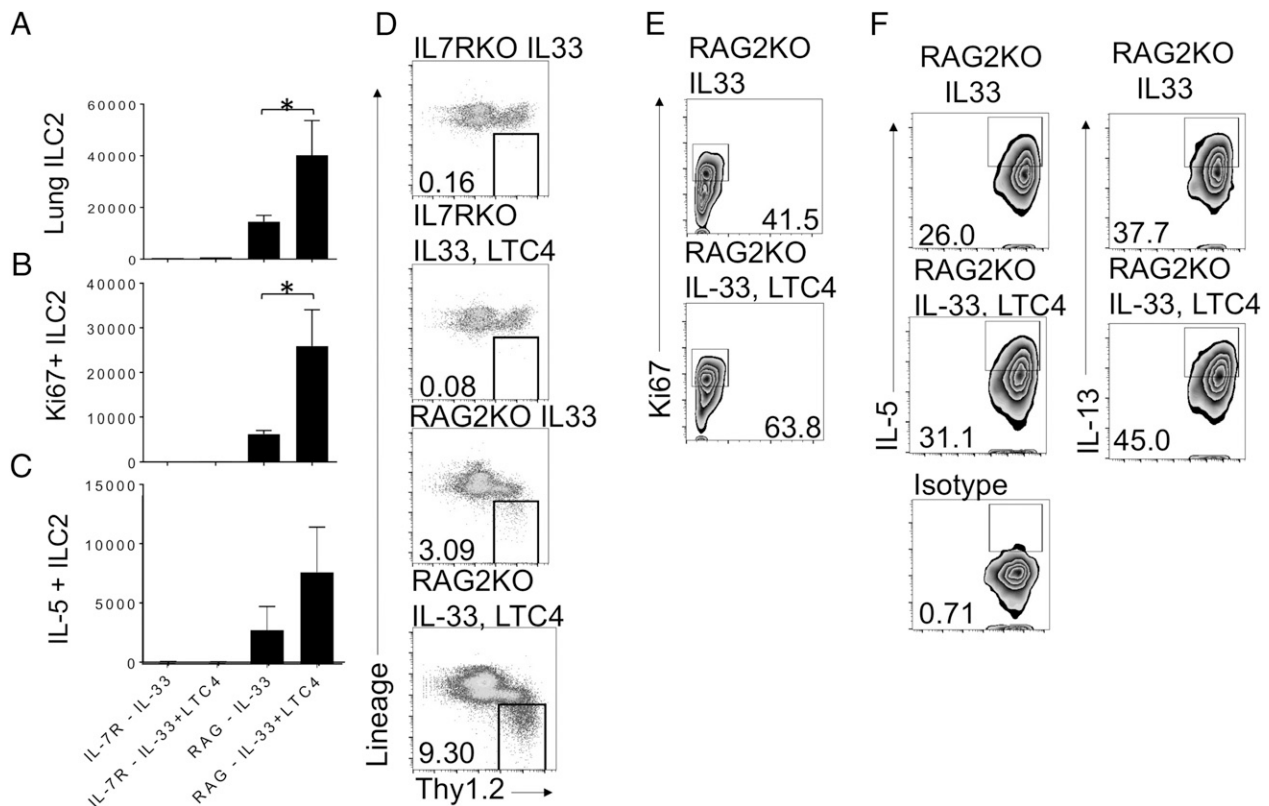


FIGURE 3. LTC4 potentiation of IL-33-induced ILC2 responses occurs independently of adaptive immunity. IL-7R^{-/-} (IL7RKO) and RAG2^{-/-} (RAG2KO) mice were challenged with PBS, IL-33, LTC4, or IL-33 + LTC4 once a day for 3 d. On day 4, mice were euthanized, and BAL and lungs were collected. Total numbers of ILC2s (**A**), proliferating Ki67⁺ ILC2s (**B**), and IL-5⁺ ILC2s (**C**) in the lungs of RAG2^{-/-} mice. (**D** and **E**) FACS plots of ILC2 Ki67 in RAG2KO mice. (**D**) Percentages of lung ILC2s within the lymphocyte gate. FACS plots of ILC2 Ki67 (**E**) and ILC2 IL-5 and IL-13 (**F**) from RAG2KO mice. ILC2s were defined as CD45⁺ lineage⁻ Thy1.2⁺ lymphocytes. FACS plots in (**E**) and (**F**) were gated on ILC2s. Data shown are representative of two or three independent experiments with two mice per group. **p* < 0.05, unpaired *t* test.

mice (Fig. 6A). Further, the production of IL-5 and IL-13 by ILC2s in CysLT1R^{-/-} mice remained largely unchanged following the addition of LTC4 compared with ILC2s from WT and CysLT2R^{-/-} mice (Fig. 6B–D). Interestingly, IL-5⁺ ILC2s from CysLT2R^{-/-} mice appeared to be increased (*p* = 0.06, *t* test) over WT mice, despite the fact that other end points, including lung inflammation and eosinophils, were not significantly different. Overall, we determined that CysLT1R was required for ILC2 responses to LTC4 in vivo when coadministered with IL-33.

CysLTs directly enhance IL-33-stimulated ILC2 activation and NFAT nuclear translocation

To determine whether CysLTs could directly potentiate ILC2 activation by IL-33 in vitro, we purified lung ILC2s and stimulated them in vitro for 6 h with IL-33 alone or IL-33 along with each of the CysLTs. Supernatants were analyzed for IL-5 and IL-13 by ELISA. As expected, stimulation with IL-33 led to increased IL-5 and IL-13 levels compared with media alone. ILC2s stimulated with IL-33 + LTC4, LTD4, or LTE4 produced significantly more IL-5 and IL-13 than did stimulation with IL-33 alone (Fig. 7A, 7B). Thus, similar to our in vivo results, CysLTs potentiated IL-33-induced ILC2 activation in vitro.

We further assessed for activation of signaling molecules that might mediate CysLT activation of ILC2s. We did not identify changes in ILC2 phospho-ERK, phospho-p38, or phospho-mTOR levels by phospho-flow methods (data not shown) in our studies. However, we did identify enhanced nuclear translocation of NFAT1 in LTC4-, LTD4-, and LTE4-stimulated purified ILC2s that also received IL-33 compared with ILC2s stimulated with IL-33 alone

(Fig. 7C). We detected minimal NFAT2 staining in any condition (data not shown).

CysLT1R promotes ILC2 activation and proliferation during allergen challenge

To determine the role of CysLT1R in ILC2 responses during allergen-induced lung inflammation, we challenged WT and CysLT1R^{-/-} mice three times over 12 d with a fungal allergen associated with severe asthma, *A. alternata* (32). Lungs were collected on day 14 for analysis of eosinophilia and ILC2 responses. After *Alternaria* challenge, CysLT1R^{-/-} mice had reduced lung eosinophilia compared with WT mice (Fig. 8A). Notably, CysLT1R^{-/-} mice had significantly reduced lung ILC2 accumulation and proliferation (Fig. 8B, 8C), as well as IL-5 and IL-13 production (Fig. 8D, 8E). Thus, CysLT1R is critical for ILC2 activation and expansion during exposure to the clinically relevant allergen *Alternaria*.

Discussion

Our work demonstrates that LTC4 promotes IL-33-induced lung inflammation, as well as ILC2 cytokine production and proliferation, that is dependent on CysLT1R. We previously showed that ILC2s express CysLT1R (20), and the current study shows that ILC2s also express CysLT2R and candidate LTE4 receptor P2Y12 (30). Further, CysLT stimulation of purified lung ILC2s in the presence of IL-33 resulted in enhanced IL-5 and IL-13 production, as well as induction of NFAT1 nuclear translocation. Importantly, CysLT1R was required for ILC2 activation during lung inflammation induced by the clinically relevant fungal allergen *A. alternata*. Together, these findings demonstrate that IL-33 and leukotrienes that

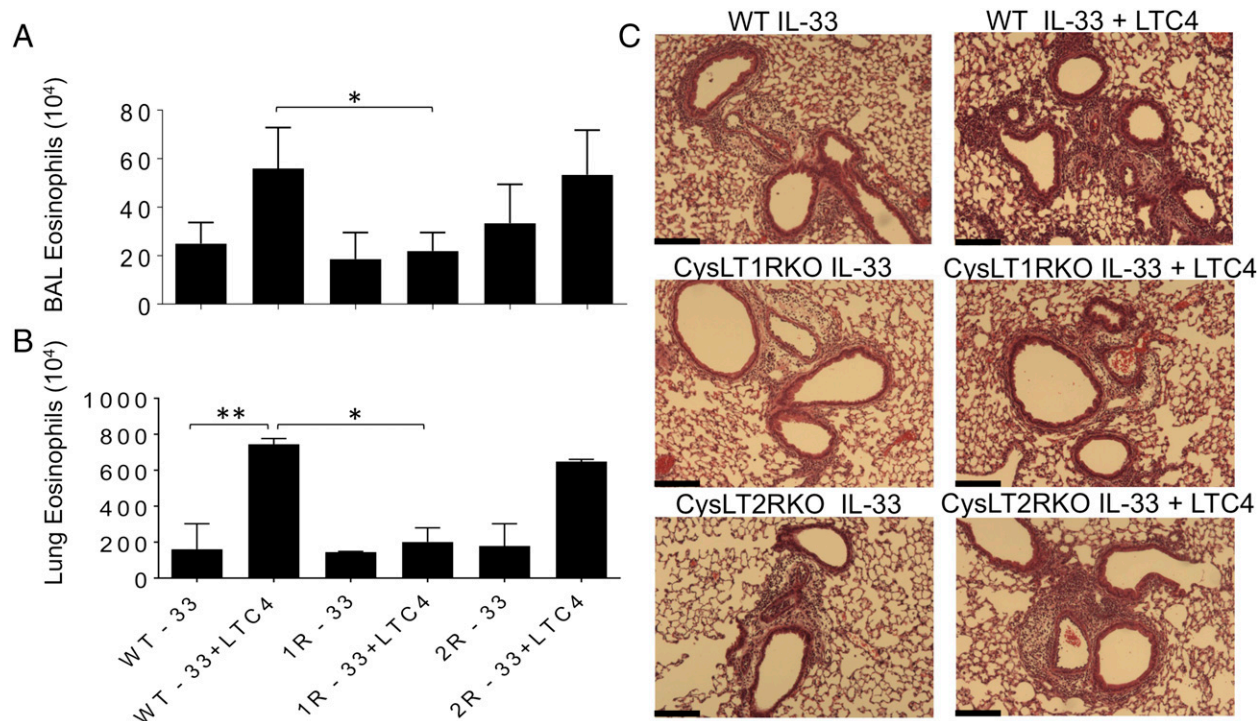


FIGURE 4. LTC4 potentiation of IL-33-induced airway inflammation is dependent on CysLT1R. WT, CysLT1R^{-/-} (1R), and CysLT2R^{-/-} (2R) mice were challenged with IL-33, LTC4, or IL-33 + LTC4 once a day for 3 d. On day 4, mice were euthanized, and BAL and lungs were collected. Total eosinophils in BAL (A) and lungs (B). (C) H&E-stained lung sections from WT, CysLT1R^{-/-}, and CysLT2R^{-/-} mice challenged with IL-33 or IL-33 + LTC4. Scale bars, 100 μ m. Data shown are representative of two to four independent experiments with two mice per group. * p < 0.05, ** p < 0.01, unpaired t test.

are present in asthma may synergistically exacerbate allergic airway inflammation through ILC2 activation.

We found that LTC4 enhancement of ILC2 activation and airway inflammation was dependent on CysLT1R. LTD4 binds with high affinity to CysLT1R, and LTC4 binds with equal affinity to CysLT1R

and CysLT2R (33). LTE4 has low affinity for CysLT1R and CysLT2R and preferentially signals through P2Y12 (30) or GPR99 (31). Because we detected high levels of CysLT2 mRNA on ILC2s, we chose to use the parent compound, LTC4, along with CysLT1 and CysLT2 gene-deficient mice to determine the contribution of

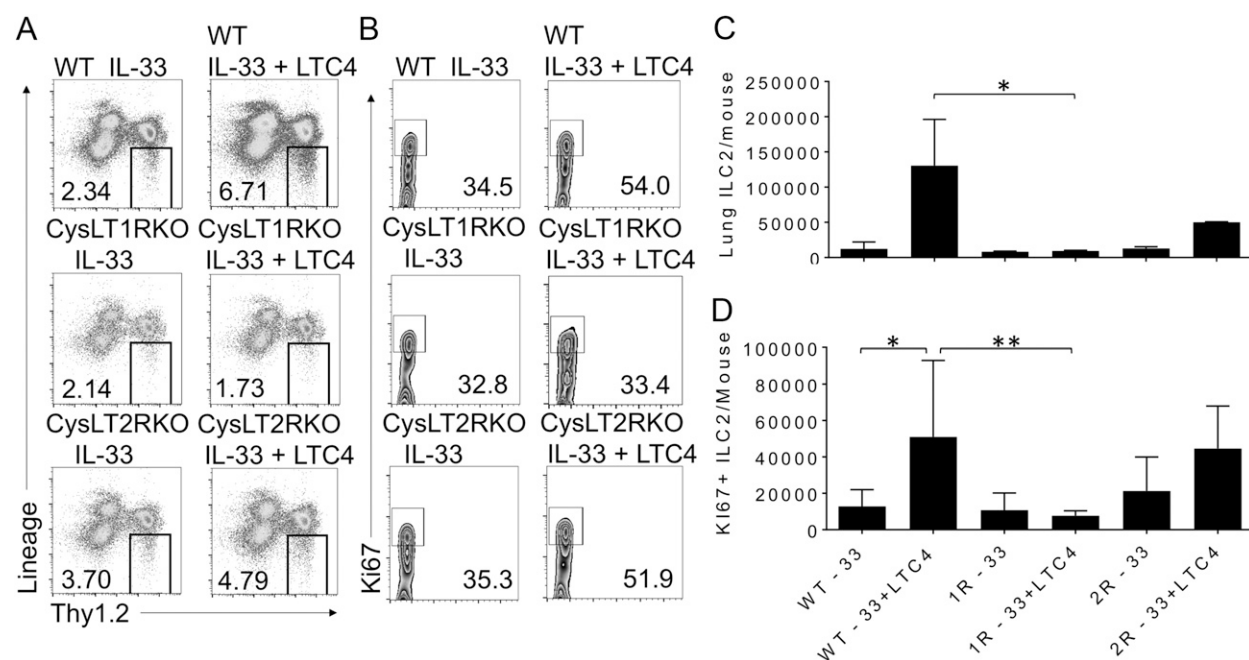


FIGURE 5. Potentiation of ILC2 proliferation by LTC4 is dependent on CysLT1R. WT, CysLT1R^{-/-} (1R), and CysLT2R^{-/-} (2R) mice were challenged with IL-33, LTC4, or both once a day for 3 d. On day 4, mice were euthanized, and BAL and lungs were collected. Percentages of ILC2s within lymphocyte gate (A) and Ki67⁺ ILCs (B). Total numbers of ILC2s (C) and Ki67⁺ ILC2s (D). Data shown are representative of two to five independent experiments with four mice per group. * p < 0.05, ** p < 0.01, unpaired t test.

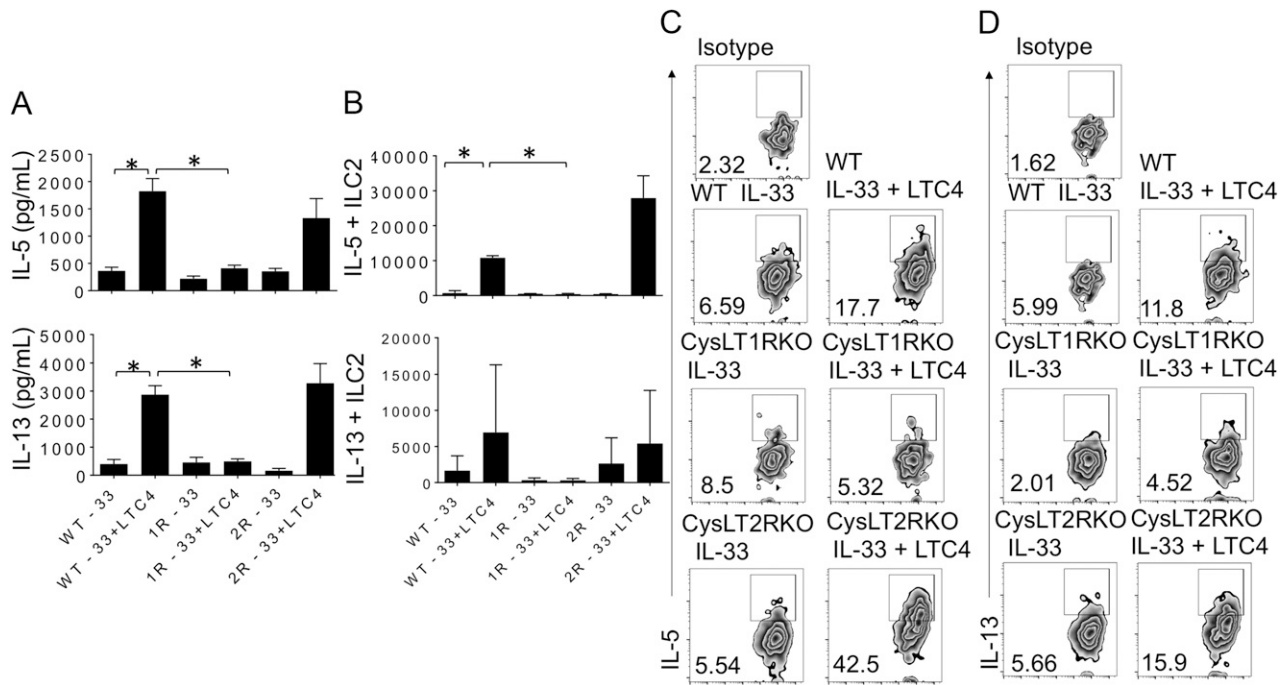


FIGURE 6. Potentiation of ILC2 cytokine production by LTC4 is dependent on CysLT1R. WT, CysLT1R^{-/-} (1R), and CysLT2R^{-/-} (2R) mice were challenged with PBS, IL-33, LTC4, or IL-33 + LTC4 once a day for 3 d. On day 4, the mice were euthanized, and BAL and lungs were collected. (A) Concentration of IL-5 and IL-13 in BAL from WT, CysLT1R^{-/-}, and CysLT2R^{-/-} mice. (B) Total number of IL-5⁺ ILC2s and IL-13⁺ ILC2s in WT, CysLT1R^{-/-}, and CysLT2R^{-/-} mice. FACS plots of IL-5 (C) and IL-13 (D). Gated on CD45⁺ Thy1.2⁺ lineage⁻ lymphocytes. Data shown are representative of five independent experiments with three mice per group. **p* < 0.05, unpaired *t* test.

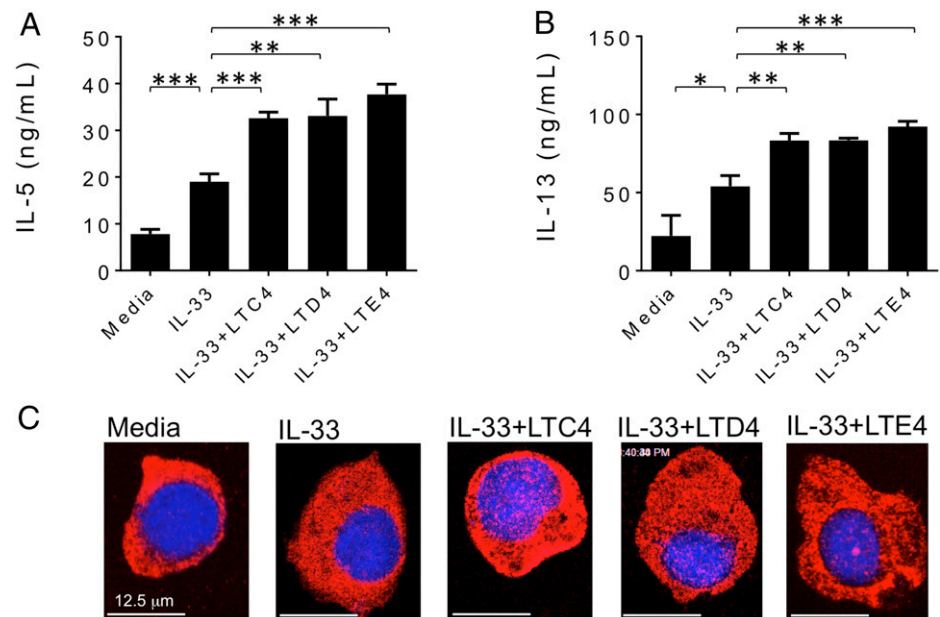
both receptors to LTC4-induced ILC2 activation and airway inflammation. Although we administered LTC4 in the *in vivo* studies, the possibility exists that endogenous conversion to LTD4 and LTE4 contributed to some of the results. Interestingly, we detected elevated levels of P2Y12 on ILC2s; perhaps, responsiveness to LTE4 is mediated, in part, by this receptor.

IL-33 and CysLTs are thought to play important roles in asthma pathogenesis and type 2 inflammation. Several studies have found correlations between the presence of IL-33 and the severity of asthma and atopic dermatitis in human patients (13, 34–36). IL-33 has also been found to be elevated in patients with severe asthma with fungal sensitization (34). Additionally, a recent study has

found that IL-33 and ILC2s are crucial effectors in the maintenance of AHR in a chronic asthma model (35). These studies suggest that IL-33 may be an important mediator of lung inflammation and AHR in humans with asthma, including those with severe disease.

In addition to cytokines, such as IL-33, it is likely that several mediators, including lipid mediators (e.g., CysLTs and PGD2), promote overlapping, as well as distinct, features of asthma. CysLTs have long been known to promote airway inflammation, AHR, and airway remodeling (1, 2, 37). Recently, we reported increased ILC2s in the nasal mucosa of patients with aspirin-exacerbated respiratory disease (AERD), which is characterized

FIGURE 7. CysLTs directly potentiate ILC2 activation and NFAT1 translocation. Lung ILC2s were purified and then stimulated with media, IL-33, or IL-33 in combination with each of the leukotrienes for 6 h. Concentration of IL-5 (A) and IL-13 (B) from culture supernatants. (C) Representative immunofluorescence of ILC2s, with NFAT1 staining (red) and nuclear staining (blue). Data shown are representative of two independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.005, unpaired *t* test.



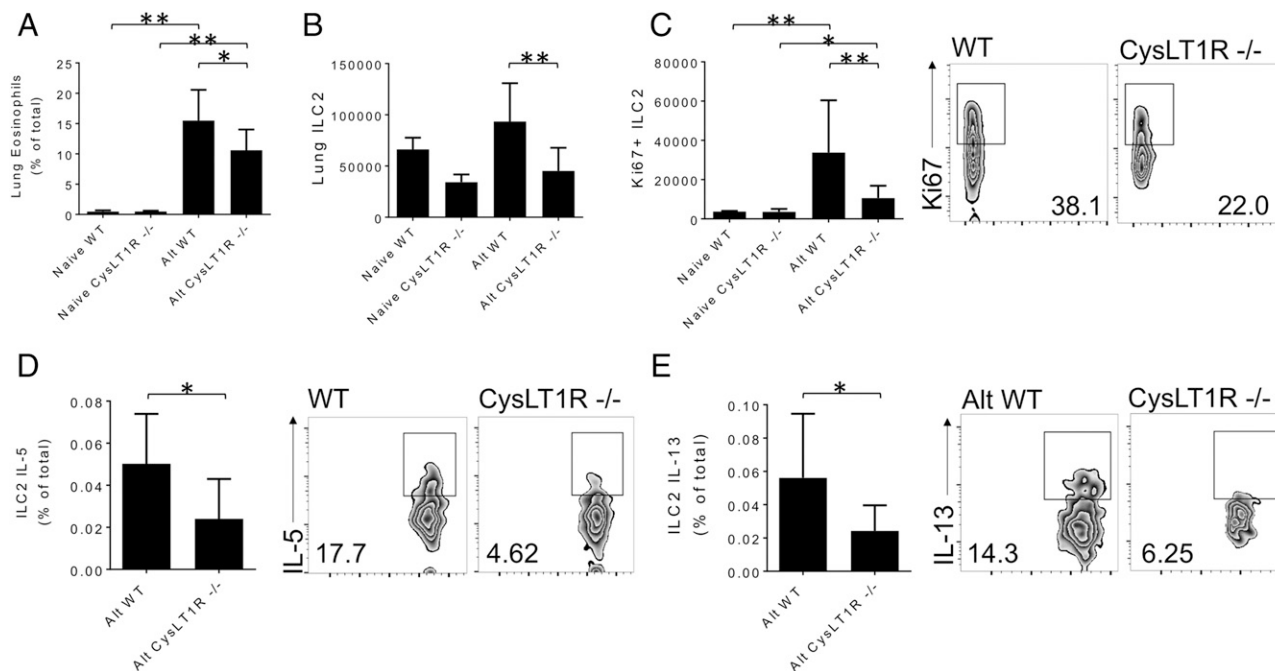


FIGURE 8. CysLT1R is required for ILC2 responses during allergen challenge with *A. alternata*. WT and CysLT1R^{-/-} mice were challenged with 30 μ g of *A. alternata* on day 0, followed by 3 μ g on days 9 and 12. Lung cells were analyzed on day 14. **(A)** Percentage of lung eosinophils in naive and challenged WT and CysLT1R^{-/-} mice. **(B)** Total number of ILC2s in naive and challenged WT and CysLT1R^{-/-} mice. **(C)** Total number of Ki67⁺ ILC2s in naive and challenged WT and CysLT1R^{-/-} mice (left panel) and representative Ki67⁺ ILC2 population from the lungs of challenged WT and CysLT1R^{-/-} mice (right panel). IL-5⁺ ILC2s **(D)** and IL-13⁺ ILC2s **(E)** in the lungs of challenged WT and CysLT1R^{-/-} mice (left panel) and representative ILC2 IL-5 **(D)** and IL-13 **(E)** from the lungs of challenged WT and CysLT1R^{-/-} mice (right panel). Data shown are representative of two independent experiments. * p < 0.05, ** p < 0.01, unpaired t test.

by increased CysLTs and PGD₂, both of which could recruit and activate ILC2s (38). Further, roles for IL-33 and TSLP in AERD have also been identified, thus supporting a mediator milieu related to our current studies (39, 40). The CysLT receptors CysLT1R and CysLT2R are present on a variety of immune and structural cell types, including mast cells and T cells, as well as smooth muscle cells (33, 41). We first reported that mouse ILC2s respond to CysLTs with increased Th2 cytokine production and proliferation (20). However, our previous work did not address whether CysLTs could regulate ILC2 activation in the presence of another ILC2 activator, such as IL-33. The current work shows that the parent CysLT, LTC₄, can indeed promote ILC2 responses in concert with IL-33; this likely has implications for disease, including asthma and AERD. Synergistic or additive effects of multiple inflammatory mediators likely promote the activation of many airway cell types, including ILC2s, that may lead to more severe disease.

The effect potentiated by CysLTs on ILC2 activation that we observed may be mediated by NFAT signaling. Nuclear translocation of NFAT cytoplasmic components is triggered by rapid calcium influx (42), and we previously showed that LTD₄ elicited a rapid and robust calcium influx in ILC2s (20). Further, a very recent study showed that leukotriene-induced ILC2 cytokine production was abrogated in the presence of the NFAT pathway inhibitor cyclosporine (21). Interestingly, studies have also shown that purinergic receptors can signal through NFAT in various cell types (42, 43). We found that P2Y₁₂ is specifically upregulated following IL-33 stimulation of ILC2s at 6 and 24 h. This finding, coupled with the fact that P2Y₁₂ has been shown to mediate LTE₄-induced responses, might explain why LTE₄ can activate ILC2s, despite CysLT1R inhibition (20). Thus, multiple signaling mechanisms may contribute to synergistic CysLT- and IL-33-induced ILC2 responses, and further work will be needed to precisely identify these pathways.

Interestingly, we observed that IL-5-producing ILC2s from CysLT2R^{-/-} mice tended to be increased compared with those from WT mice, despite lung inflammation and eosinophils not being different. This effect could be related to the established negative-signaling role that CysLT2R has in other cell types, including dendritic cells and mast cells (44, 45). The explanation for the lack of significant differences in ILC2 IL-13 production and lung inflammation end points in CysLT2^{-/-} mice and WT mice is not clear; possibly, counterregulatory mechanisms exist in CysLT2^{-/-} mice to limit IL-13 production and lung inflammation. Further studies on the effects of CysLT2R on CysLT1R signaling in ILC2s would be important to address this point.

Since the initial submission of this work, two studies have been published that demonstrate activation of ILC2s by CysLTs in the presence of other mediators. In the first report, LTE₄ was shown to synergize with PGD₂, as well as with IL-25, IL-33, and TSLP, to activate human ILC2s. CysLTs also promoted ILC2 migration and survival, in addition to synergistic IL-13 production (46). The second study used a *Nippostrongylus brasiliensis* parasite model to show that CysLT1R was required for ILC2 responses and type 2 inflammation. Further, they found that CysLTs induced NFAT activation, which was required for ILC2 cytokine production (21). Our work supports and extends the results of these studies and further demonstrates the novel finding that CysLT1R regulates ILC2 responses during allergen challenge with *Alternaria*, a clinically relevant allergen associated with severe asthma.

In summary, we show that LTC₄ potentiates IL-33-induced ILC2 activation and proliferation in vivo that is dependent on CysLT1R. Further, all three CysLTs activated purified ILC2s in vitro above IL-33 alone and induced NFAT nuclear localization. Finally, ILC2 responses in allergen-challenged mice were dependent on CysLT1R. These studies have important implications for asthma, because multiple mediators, including IL-33 and CysLTs,

are present and may synergistically activate ILC2 responses and worsen disease.

Disclosures

The authors have no financial conflicts of interest.

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Figure S1

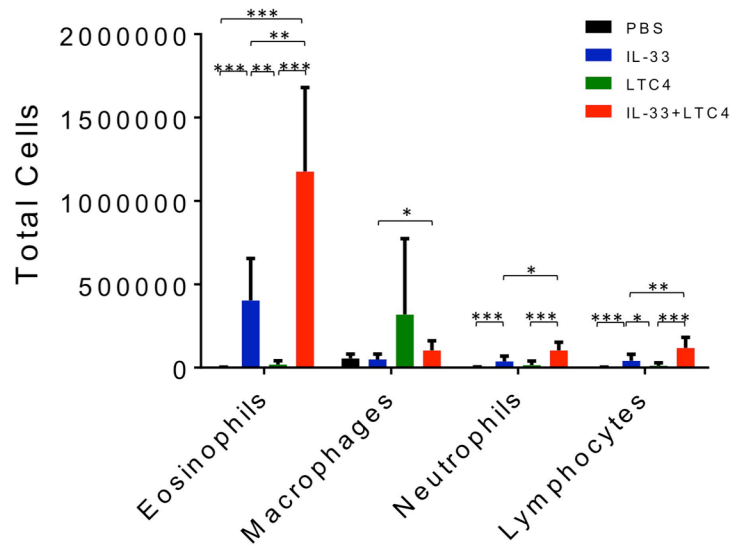


Figure S1. BAL cell count differential from WT mice challenged with PBS, IL-33, LTC4, or IL-33 and LTC4. Data shown are from 5 independent experiments. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.005$ Mann-Whitney test.

Figure S2

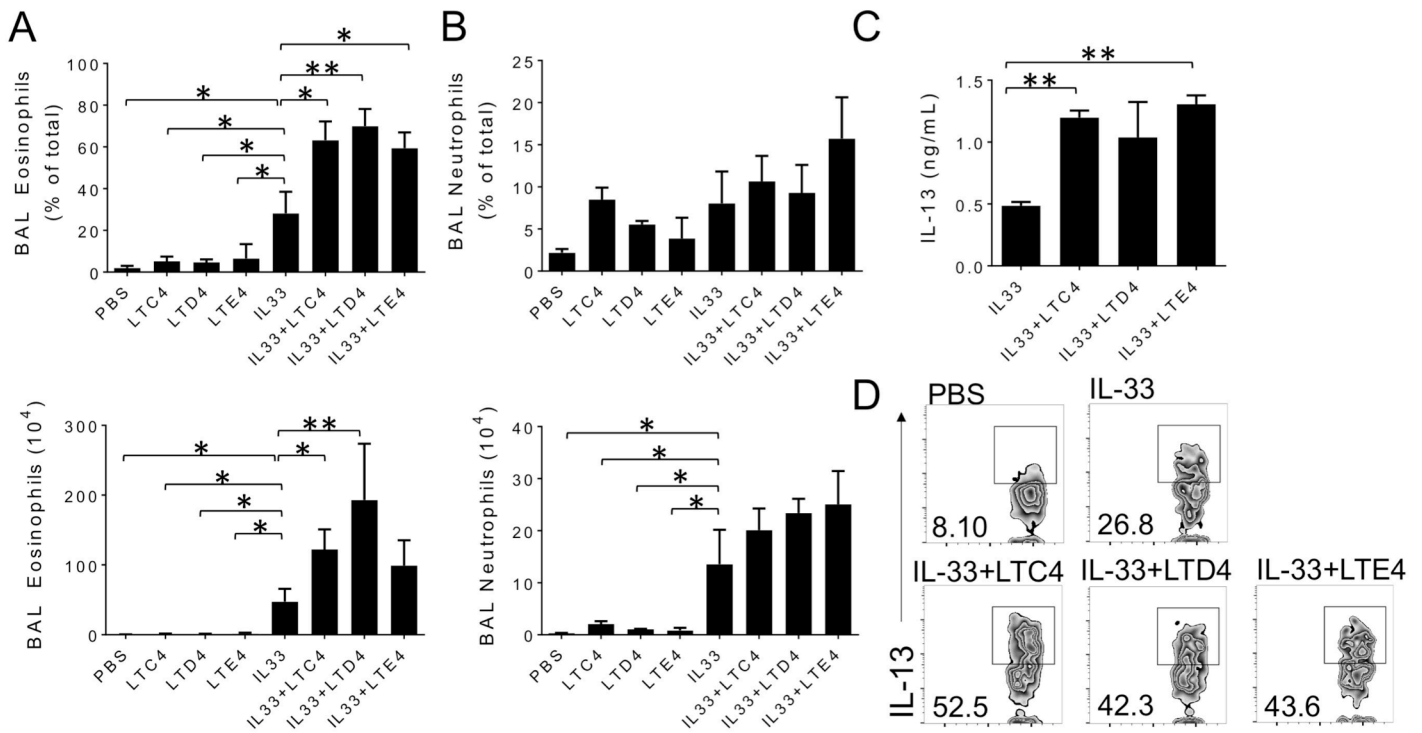


Figure S2. LTC₄, LTD₄, and LTE₄ potentiate the IL-33-driven airway type 2 response and ILC2 activation. WT mice were challenged with PBS, LTC₄, LTD₄, LTE₄, IL-33, or the combination of IL-33 and each CysLT once per day for 3 days. On day 4, mice were euthanized and BAL and lungs were collected. (A) Percentage (top) and total numbers (bottom) of eosinophils in the airway. (B) Percentage (top) and total numbers (bottom) of neutrophils in the airway. (C) Concentration of IL-13 in the airway. (D) Representative FACS plots of ILC2 IL-13. Data shown are representative of 3 different experiments. *p < 0.05, ** p < 0.01, *** p < 0.005, unpaired t test.

Figure S3

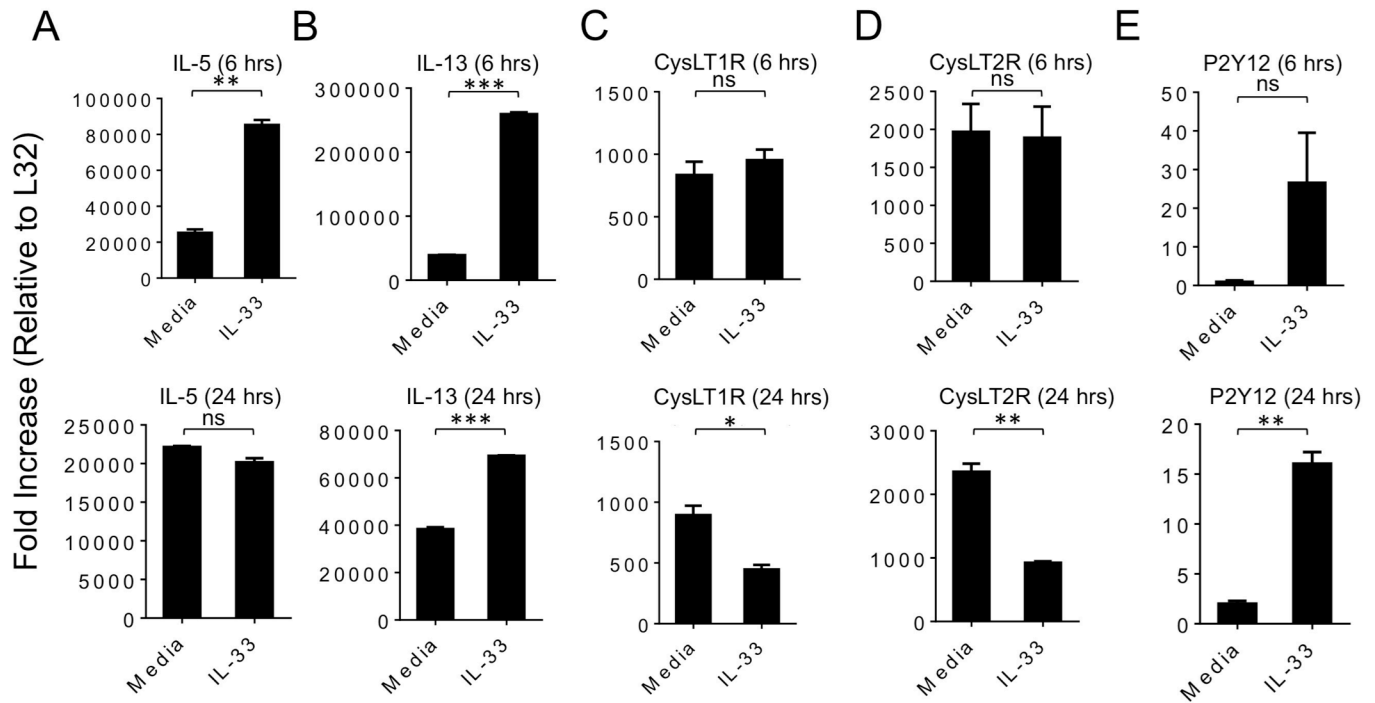


Figure S3. CysLT1R, CysLT2R and P2Y12 mRNA are expressed by ILC2s and P2Y12 is upregulated after IL-33 exposure. ILC2 were sorted from *Alternaria* challenged mice and then stimulated with media or IL-33 for 6 or 24 hours and cells processed for mRNA levels. (A) Expression of IL-5 at 6 (top) or 24 (bottom) hours. (B) Expression of IL-13 at 6 (top) or 24 (bottom) hours. (C) Expression of CysLT1R at 6 (top) or 24 (bottom) hours. (D) Expression of CysLT2R at 6 (top) or 24 (bottom) hours. (E) Expression of P2Y12 at 6 (top) or 24 (bottom) hours. Data shown are representative of 2 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ unpaired t test.