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Leukotriene C4 potentiates IL-33 induced ILC2 activation and lung inflammation

Sean J. Lund, M.S.[1], **Alex Portillo, M.S.**[1], **Kellen Cavagnero, B.S.**[1], **Rachel E. Baum, B.S.**[1], **Luay H. Naji**[1], **Jana H. Badrani, B.S.**[1], **Amit Mehta, Ph.D.**[2], **Michael Croft, Ph.D.**[2], **David H. Broide, M.B., Ch.B.**[1], and **Taylor A. Doherty, M.D.**[1]

[1]Department of Medicine, University of California, San Diego, CA

[2]Division of Immune Regulation, La Jolla Institute for Allergy and Immunology, La Jolla, CA

Abstract

Asthma is a complex disease 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) that are present in asthma could further amplify the effects of IL-33 on ILC2 activation and lung inflammation. Here we show that airway challenges with the parent CysLT leukotriene C4 (LTC4) given in combination with Iow dose IL-33 to naïve WT 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 LTE4 receptor P2Y12 mRNAs were increased in ILC2s. The synergistic effect of LTC4 with IL-33 was completely dependent upon CysLT1R as CysLT1R−/−, 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.

Introduction

Asthma is a chronic inflammatory disorder 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 both present in human asthmatic samples and individually promote inflammatory and structural cell responses as well as airway hyperresponsiveness (1–3). IL-33 is an IL-1 family member

Corresponding author: Taylor A. Doherty, M.D., Department of Medicine, University of California San Diego, Biomedical Sciences Building, Room 5090, 9500 Gilman Drive, La Jolla, CA 92093-0635, tdoherty@ucsd.edu tel: (858) 822-7653 fax: (858) 534-2110.

that activates both 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 molecule T1 and ST2 on the surface of mast cells, Th2 cells, and has most recently 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 NFκB signaling as well as activation of MAP kinase/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 cysteinyl leukotrienes include leukotrienes C4, D4, and E4 (LTC4, LTD4, and LTE4) and are generated after arachidonic acid is converted into LTA4 by 5-lipoxygenase and subsequently into LTC4, LTD4 and LTE4 (14, 15). The prototype receptors for the CysLTs include CysLT1R and CysLT2R though 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 as well as immune cells, and stimulation with LTC4 or LTD4 leads to robust airway constriction (1, 17). In mice, LTC4 synthase (LTC4S) deficiency results in reduced influx of eosinophils and airway hyperresponsiveness during allergen challenge (18). Further, LTC4S−/− 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 cysteinyl leukotriene generation (20). Taken together, these studies suggest that cysteinyl leukotrienes contribute to allergic airway responses and promote ILC2 activation. To further elucidate the role of the cysteinyl leukotriene receptors in ILC2 biology, we focus here on the effects of LTC4 on ILC2 responses given the equal affinity to bind both CysLT1R and CysLT2R that are both present in ILC2s (21).

Group 2 innate lymphoid cells are lineage-negative lymphocytes that express both 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 (23). However, unlike IL-33, LTD4 is capable of stimulating ILC2 IL-4 production which may further drive Th2 cell responses (20, 24). Though 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. As 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. We thus 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 week old female C57BL/6 mice were obtained from Jackson Labs (Bar Harbor, ME). RAG2−/− and IL-7R−/− mice were also obtained from Jackson Labs and bred in house (Bar Harbor, ME). CysLT1R−/− and CysLT2R−/− mice were kindly provided by Drs. Yoshihide Kanaoka, Joshua Boyce, Nora Barrett, and Frank Austen at Brigham and Women's Hospital, Boston and bred in house. All animal experiments were approved by the UC San Diego Institutional Animal Care and Use Committee.

In vivo IL-33 and cysteinyl leukotriene challenge models

WT, RAG2−/−. IL-7R−/−, CysLT1R−/−, and CysLT2R−/− mice were challenged with either PBS, 100ng of IL-33, 100ng of LTC4, LTD4, and LTE4 or 100 ng of the combination IL-33 and LTC4, LTD4, and LTE4 daily for three days and then euthanized the day after last challenge. In some experiments, mice were challenged with 50ng of IL-33 or 200ng of LTC4, LTD4, and LTE4, or a combination of IL-33 and the cysteinyl leukotrienes. Bronchoalveolar lavage fluid (BAL) was collected and the lungs were then removed. BAL was then centrifuged at 1500 RPM for 5 minutes 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 (San Diego, CA) lung dissociator according to the manufacturer's protocol.

Alternaria alternata allergen challenge model

WT and CysLT1R−/− mice were challenged with 30 μg of *Alternaria alternata* extract (Greer, Lenoir, NC) on day 0, then 3μg on days 9 and 12. Bronchoalveolar lavage fluid (BAL) was collected and the lungs were then removed. Following, the BAL was then centrifuged at 1500 RPM for 5 minutes 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 (San Diego, CA) lung dissociator according to the manufacturer's protocol.

Flow Cytometry

BAL and lung single cell suspensions were first 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. Eosinophil were identified as CD45+ Siglec-F+ CD11c- cells using anti-CD45.2 conjugated to PerCP (eBioscience), anti-Siglec-F conjugated to PE (BD), anti-CD11c conjugated to FITC (eBioscience), and anti-Gr1 conjugated to APC (eBioscience). Lung ILC2s were stained with a cocktail of lineage antibodies all conjugated to FITC including: a base lineage cocktail (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 APC, and anti-CD45.2 conjugated to PerCP. ILC2s were defined as CD45.2 positive, lineage-negative, Thy1.2 positive lymphocytes. We have previously shown that this population in the mouse lung predominately expresses T1/ST2 (IL-33R) and GATA-3 (25). Intracellular cytokine analysis was performed using anti-IL-5 or anti-IL-13

conjugated to PE (eBioscience, San Diego, CA) after surface staining and permeabilization of whole lung cells that were cultured 16–18 hours with 10ng/mL of IL-2 and IL-7 in RPMI supplemented with Penicillin/Streptomycin, 10% FBS, Glutamine, and 2-mercaptoethanol, and Golgi-Plug (BD). Additionally to assess proliferation in ILC2s, an anti-Thy1.2 conjugated to PE and an anti-Ki67 conjugated to eFluor 660 (eBioscience, San Diego, CA) were used.

ILC2 in vitro studies

ILC2s were sorted from WT mice that were challenged with 3 times with 50μg *Alternaria alternata* on days 0, 3, and 6. On day 7, mice were euthanized and lungs collected. The lungs were digested using the Miltenyi biotec (San Diego, CA) 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 cocktail of lineage antibodies all conjugated to FITC including: a base lineage cocktail (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 APC, and anti-CD45.2 conjugated to PerCP. ILC2s were defined as CD45.2 positive, Lineage negative, Thy1.2 positive 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 hours followed by media changes and functional assays. ILC2s were stimulated with 30ng/mL IL-33, or 10^{-6}M LTC4, LTD4, or LTE4 or a combination of IL-33 and each cysteinyl leukotriene for 6 hours. Cell culture supernatants were collected and stored at −80°C for future ELISA analysis, ILC2s were collected for RNA extraction and qPCR analysis. In other experiments, ILC2s were stimulated for 90 minutes then immediately collected on a slide by Cytospin for immunofluorescent staining.

ELISA

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

Immunofluorescence

Following *in vitro* stimulation, the cells were removed from culture and added to slides by cytospin. Each slide was immediately fixed in cold methanol. After fixation, slides were permeabilized with Triton-X, then blocked with 5% donkey and goat serum. Each slide was stained with AlexaFluor-488 conjugated NFATc1 (NFAT2, Cell Signaling) and an unconjugated goat NFATc2 (NFAT1, Biolegend). An anti-goat AlexaFluor-555 secondary antibody was then added to visualize NFATc2. After washing, the nuclear stain DRAQ5 was added. Slides were then mounted with a Prolong anti-fade reagent and sealed. Images were taken on a Leica TCS confocal microscope at 63× magnification.

qPCR of sorted ILC2s

WT mice received three or four 25μg intranasal *Alternaria* challenges over 9–10 days prior to FACS sorting in order to expand ILC2s. Sorting of ILC2s (CD45+ lineage-negative Thy1.2 positive lymphocytes) was performed using a FACS Aria cell sorter (BD Biosciences). FACS sorted lung ILC2s, CD4+ T1ST2+ and CD4+ T1ST2- cells were lysed using TRIzol (Life technologies) to isolate RNA. An aliquot of total RNA (0.5 μg) was reverse-transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche) following the manufacturer's instructions. Diluted cDNA was subject to real-time PCR using SYBR Green I master (Roche) and the RT-PCR–specific primers. The oligonucleotide primer sequences (5′-3′) were as follows: mIL-5 forward AAAGAGAAGTGTGGCGAGGAGA, mIL-5 reverse CACCAAGGAACTCTTGCAGGTAA, mIL-13 forward GAGCAACATCACACAAGACCAGA, mIL-13 reverse GGCCAGGTCCACACTCCATA CysLT1R forward CAACGAACTATCCACCTTCACC, CysLT1R reverse AGCCTTCTCCTAAAGTTTCCAC, CysLT2R forward TGCTTTTGGAAGAGAGAAGAGTCCA, CysLT2R reverse AATGATACACATCCTTCCCCAGGA, P2Y12 forward CCCGGAGACACTCATATCCTT, P2Y12 reverse GTCCCAGGGGAGAAGGTG, GPR99 (OXGR-1) forward GTGACACCTGGGCTTCTAC, GPR99 (OXGR-1) reverse CTCCACCAGCCTCAGAAACT, L32 forward GAA ACT GGC GGA AAC CCA, and L32 reverse GGATCTGGCCCTTGAACCTT. Data are presented as normalized to housekeeping gene *L32* using Roche LightCycler 480 (Roche Diagnostics, Germany).

Histology

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

Data Analysis

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

Statistics

For all experiments, statistical analysis was performed using the GraphPad Prism software (GraphPad, La Jolla, CA). The Mann-Whitney U test was performed for all experiments unless otherwise specified. Statistical p-values less than 0.05 were considered statistically significant.

Results

LTC4 potentiates IL-33 induced lung inflammation

Previous reports have demonstrated that IL-33 given to naïve mice results in robust type 2 lung inflammation (9, 26). The doses of IL-33 administered in vivo in several studies range from 500ng to 1μg which induces very high levels of airway inflammation and eosinophilia

(9, 26, 27). 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 both CysLT1R and CysLT2R with equal affinity and allowed for testing potential contributions of both receptors during type 2 inflammation. WT C57BL/6 mice were challenged with either PBS, IL-33, LTC4 or the combination of IL-33 and LTC4 on days 0, 1, and 2 and levels of airway eosinophilia, IL-5, IL-13 were analyzed on day 3. We found that IL-33 induced airway eosinophilia even at the lower dose of 100 ng per challenge. However, mice challenged with both IL-33 and LTC4 had nearly double the eosinophils compared with IL-33 alone (Figure 1A). LTC4 also modestly induced an increase in 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 (Supplementary figure S1). Th2 cytokines IL-5 and IL-13 were further increased by the combination of IL-33 and LTC4 versus IL-33 alone (Figure 1B and 1C). Lung tissue sections from mice challenged with IL-33 and LTC4 revealed increased inflammatory cell infiltrate compared with IL-33 or LTC4 alone (Figure 1D). LTC4 alone had little impact on these endpoints. 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 (Supplementary figure S2) and our previous studies with LTD4 (20). Overall, these data suggest that cysteinyl leukotrienes 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 mice that have ILC2s, but lack T and B cells. We also compared levels of eosinophilia and BAL Th2 cytokines in RAG2 knockout (RAG2−/−) mice with IL7R knockout (IL-7R−/−) mice that lack ILC2s (25). IL-7R−/− and RAG2−/− mice underwent airway challenges as in Figures 1A–D. IL-7R−/− mice did not mount an eosinophilic lung response or increase BAL Th2 cytokine production after either IL-33 alone or LTC4 with IL-33 (Figures 1E and F). However, RAG2−/− mice showed similar responses to WT mice with increased eosinophilia and BAL Th2 cytokines after the combination of LTC4 with IL-33 was administered (Figures 1E and F). Together, these results show that potentiation of IL-33-induced inflammation by LTC4 occurs independent of T and B cells (RAG2−/−), but does not occur in mice that lack ILC2s (IL-7R−/−), and thus implicates 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, 28, 29), we assessed lung ILC2 proliferation and cytokine production after mice were exposed to LTC4 with IL-33 or IL-33 alone. Following the protocol in Figure 1, WT mice challenged with both IL-33 and LTC4 had on average a nearly threefold increase in the total number of ILC2s and substantial increases in total numbers of proliferating (Ki-67+) and IL-5 producing ILC2s compared with mice challenged with low dose IL-33 alone (Figures 2A–C). Percentages of ILC2s as well as Ki-67+, IL-5 and IL-13+ ILC2s mirrored the total numbers (Figures 2D–F). Interestingly, LTC4 administered alone did not appear to induce

ILC2 changes at this time point, but when combined with IL-33, led to 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 solely compare the effects of IL-33 alone against LTC4 combined with IL-33 in subsequent experiments. As a control for ILC2 identification, we also analyzed IL-7R−/− mice (Figures 3A–D). RAG2 knockout mice challenged with LTC4 + IL-33 had increased total ILC2 numbers, Ki-67+ ILC2s, and IL-5+ ILC2s compared with IL-33 alone (Figures 3A–C). 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 (Figures 3D–F). Overall, LTC4 increased ILC2 proliferation and cytokine production above IL-33 alone in both WT and RAG2 knockout 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, 24, 30). 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 with the administration of LTC4 in WT and in CysLT2R−/− mice. In contrast, CysLT1R−/− mice did not mount an increase in eosinophilia induced by LTC4 (Figures 4A and 4B). Further, WT and CysLT2R knockout mice developed increased peribronchial cellular infiltration after LTC4 and IL-33 exposure that was not present in sections from CysLT1R−/− mice (Figure 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 (31, 32). To assess levels of CysLT2, P2Y12, and GPR99 and to investigate whether IL-33 increases the sensitivity of ILC2s to CysLTs, we performed qPCR for levels of these receptors on purified lung ILC2s. Levels of IL-5 and IL-13 mRNA were used to confirm IL-33 stimulation (Supplementary figure S3A & B), and as expected, IL-33 rapidly increased both IL-5 and IL-13 mRNAs. Stimulation with IL-33 did not noticeably change the expression of either CysLT1R or CysLT2R at 6 hours; but at 24 hours, stimulated ILC2 had decreased expression of CysLT1R and CysLT2R (Supplementary figure S3C & S3D). P2Y12 receptor expression was increased both at 6 hours and at 24 hours (Supplementary figure S3E) after IL-33 stimulation. GPR99 mRNA was not detected in these experiments (not shown). Thus, lung ILC2s could potentially respond to CysLTs through CysLT1, CysLT2, or P2Y12 receptors.

LTC4 challenged WT and CysLT2R knockout mice showed an increase in percent and total numbers of ILC2s and Ki-67+ ILC2s (Figures 5A–D). In contrast, CysLT1R ILC2s showed no further increase in proliferation after LTC4 exposure. CysLT1R knockout mice also

produced very little airway IL-5 or IL-13 after exposure to LTC4 compared with WT and CysLT2 KO 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−/− mice (Figure 6A). Further, ILC2 IL-5 and IL-13 production in CysLT1R−/− mice remained largely unchanged following addition of LTC4 compared with ILC2s from WT and CysLT2R−/− mice (Figures 6B–D). Interestingly, IL-5+ ILC2s from CysLT2R−/− mice appeared to be somewhat increased (p=0.06, t-test) over WT mice despite other endpoints including lung inflammation and eosinophils not being significantly different. Overall, we determined that CysLT1R was required for ILC2 responses to LTC4 in vivo when co-administered 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 hours with IL-33 alone or IL-33 along with each of the cysteinyl leukotrienes. Supernatants were analyzed by ELISA for IL-5 and IL-13. As expected, stimulation with IL-33 led to increased IL-5 and IL-13 levels compared with media alone. ILC2s stimulated with IL-33 combined with LTC4, LTD4, or LTE4 produced significantly more IL-5 and IL-13 than IL-33 alone (Figure 7A and 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, and phospho-mTOR levels by phospho-flow methods (not shown) in our studies. However, we did identify enhanced nuclear translocation of NFAT1 in leukotriene C4, D4, and E4 stimulated purified ILC2s that also received IL-33 compared with ILC2s stimulated with IL-33 alone (Figure 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 airway challenged WT and CysLT1R−/− mice three times over 12 days with a fungal allergen associated with severe asthma, *Alternaria alternata* (33). 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 (Figure 8A). Notably, CysLT1R−/− mice had significantly reduced lung ILC2 accumulation and proliferation (Figure 8B & 8C), as well as IL-5 and IL-13 production (Figure 8D & 8E). Thus, CysLT1R is critical for ILC2 activation and expansion during exposure 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 studies show that ILC2s also express CysLT2R and candidate LTE4 receptor P2Y12 (31). 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 *Alternaria alternata*. Together, these findings demonstrate that IL-33 and leukotrienes that 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 to CysLT2R (34). LTE4 has low affinity for CysLT1R and CysLT2R and preferentially signals through P2Y12 (31) or GPR99 (32). As we detected high levels of CysLT2 mRNA on ILC2s, we chose to utilize the parent compound LTC4 along with CysLT1 and CysLT2 gene deficient mice to determine to contribution of both receptors in LTC4-induced ILC2 activation and airway inflammation. Though we administered LTC4 in the *in vivo* studies, the possibility exists that endogenous conversion to LTD4 and LTE4 may have contributed to some of the results. Interestingly, we detected elevated levels of P2Y12 on ILC2s and perhaps responsiveness to LTE4 could be in part mediated by this receptor.

IL-33 and the cysteinyl leukotrienes 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, 35–37). IL-33 has also been found to be elevated in patients with severe asthma with fungal sensitization (35). Additionally, a recent report has found that IL-33 and ILC2s to be crucial effectors in maintenance of AHR in a chronic asthma model (36). 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, the likelihood is that several mediators including lipid mediators such as CysLTs and prostaglandin D2 promote overlapping as well as distinct features of asthma. Cysteinyl leukotrienes have long been known to promote airway inflammation, AHR and airway remodeling (1, 2, 38). Recently, we reported increased ILC2s in the nasal mucosa of patients with aspirin exacerbated respiratory disease (AERD) which is characterized by increased CysLTs and PGD2, both of which could recruit and activate ILC2s (39). Further, roles for IL-33 and TSLP in AERD have also been identified, thus supportive of a mediator milieu related to our current studies (40, 41). CysLT receptors CysLT1R or CysLT2R are present on a variety of different immune and structural cell types, including mast cells and T cells as well as smooth muscle cells (34, 42). We first reported that mouse ILC2s respond to the 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 and this likely has implications for disease including asthma and AERD. Synergistic or additive effects of multiple inflammatory mediators likely promotes activation of many airway cell types including ILC2s that may lead to more severe disease.

The potentiated effect we observed by the CysLTs on ILC2 activation may be mediated by NFAT signaling. Nuclear translocation of the NFAT cytoplasmic components is triggered by rapid calcium influx (43) 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 (43, 44). We found that P2Y12 is specifically upregulated following IL-33 stimulation of ILC2 at both 6 and 24 hours. 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, there may be multiple signaling mechanisms that 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 trended to be increased compared to 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 (45, 46). The explanation for the lack of significant differences of ILC2 IL-13 production and lung inflammation endpoints in CysLT2−/− mice versus WT mice is not clear. Possibly, counter regulatory mechanisms exist in CysLT2−/− mice to limit IL-13 production and lung inflammation compared with WT mice. Further studies to address 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 prostaglandin D2, as well as IL-25, IL-33, and TSLP to activate human ILC2s. The CysLTs also promoted ILC2 migration and survival in addition to synergistic IL-13 production (47). The second study utilized a *Nippostronglyus 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 here 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 as multiple mediators including IL-33 and CysLTs are present and may synergistically activate ILC2 responses and worsen disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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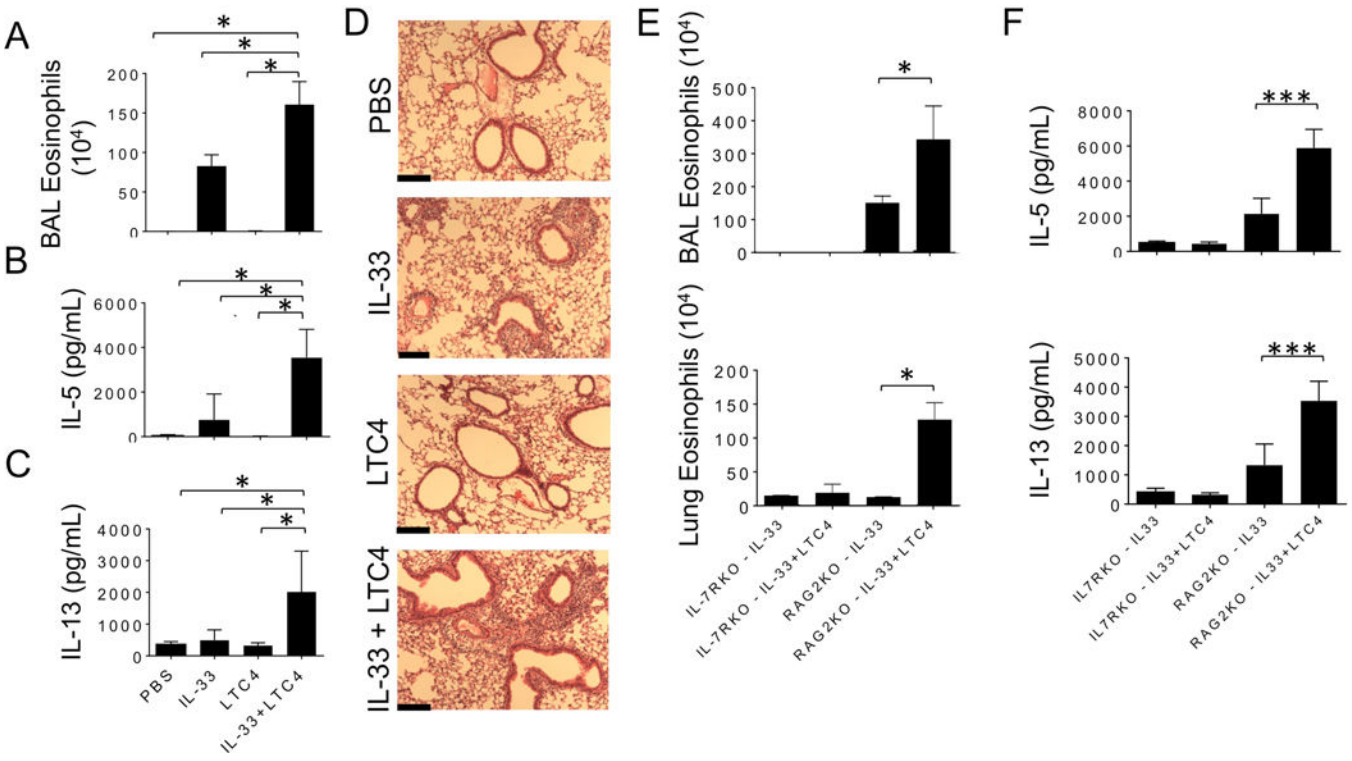

Figure 1.

LTC4 potentiates IL-33 mediated airway inflammation. WT, IL-7R−/− (IL-7RKO), and RAG2−/− (RAG2KO) mice were challenged with either PBS, IL-33, LTC4, or both IL-33 and LTC4 once a day for 3 days. On day 4, the mice were euthanized and BAL and lungs were collected. (A) Total eosinophils in the BAL of wild type mice. (B) IL-5 and (C) IL-13 in the BAL of wild type mice. (D) H&E stained lung sections from PBS, IL-33, LTC4, or both IL-33 and LTC4 challenged wild type mice. Scale bar is 100 μm. (E) Total eosinophils in the BAL (top) and lungs (bottom) of 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 2–5 independent experiments with 4 mice per group. *$p < 0.05$, *** $p < 0.005$, unpaired t test.

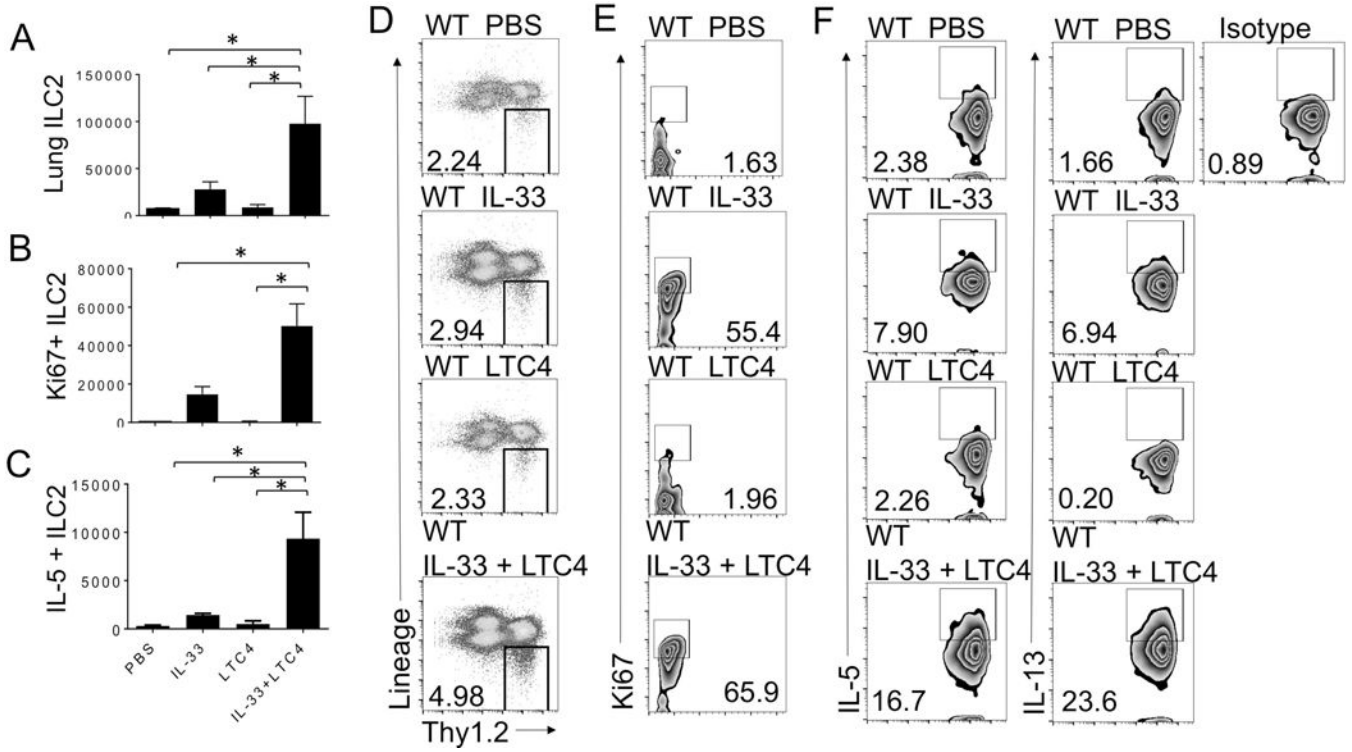

Figure 2.

LTC4 potentiates IL-33-induced ILC2 activation and cytokine production. WT mice were challenged with PBS, IL-33, LTC4, or the combination of IL-33 and LTC4 once a day for 3 days. On day 4, mice were euthanized and BAL and lungs were collected. (A) Total numbers of ILC2s, (B) proliferating Ki67+ ILC2s, and (C) IL-5+ ILC2s in the lungs of wild type mice enumerated. (D) Percentages of lung ILC2s within the lymphocyte gate. (E) FACS plots of ILC2 Ki67 percent and (F) ILC2 IL-5 and IL-13. ILC2s were defined as CD45+ lineage-negative Thy1.2+ lymphocytes. FACS plots in (E) and (F) gated on ILC2s. Data shown are representative of 5 independent experiments, with 4 mice per group. * $p < 0.05$, *** $p < 0.005$, unpaired t test.

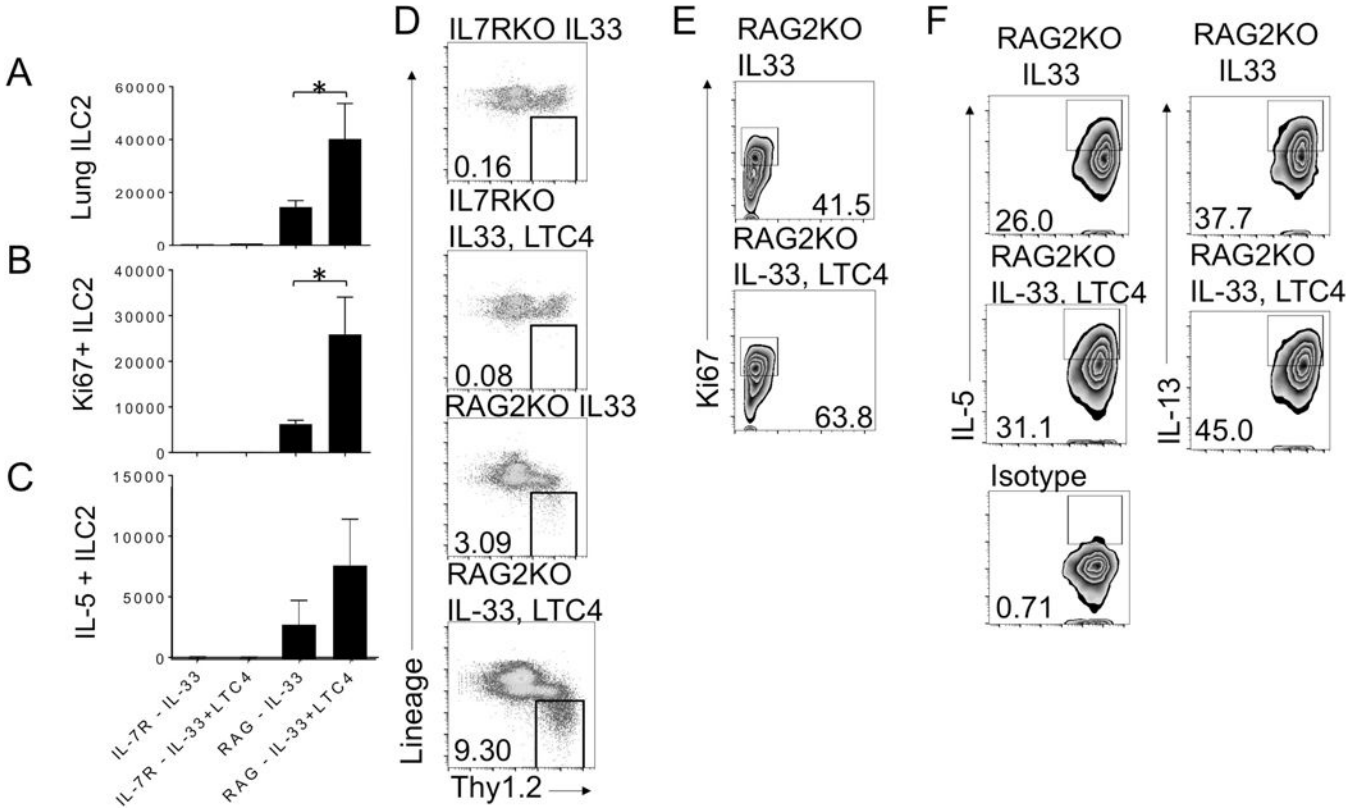

Figure 3.

LTC4 potentiation of IL-33-induced ILC2 responses occurs independent of adaptive immunity. IL-7R−/− (IL-7RKO) and RAG2−/− (RAG2KO) were challenged with either PBS, IL-33, LTC4, or both IL-33 and LTC4 once a day for 3 days. On day 4, mice were euthanized and BAL and lungs were collected. (A) Total numbers of ILC2s, (B) proliferating Ki67+ ILC2s, and (C) IL-5+ ILC2s in the lungs of RAG2−/− enumerated. (D) (E), FACS plots of ILC2 Ki67 in RAG2 knockout mice. (D) Percentages of lung ILC2s within the lymphocyte gate. (E) FACS plots of ILC2 Ki67 percent and (F) ILC2 IL-5 and IL-13 shown from RAG2 KO mice. ILC2s were defined as CD45+ lineage-negative Thy1.2+ lymphocytes. FACS plots in (E) and (F) gated on ILC2s. Data shown are representative of 2–3 independent experiments with 2 mice per group. * $p < 0.05$, *** $p < 0.005$, unpaired t test.

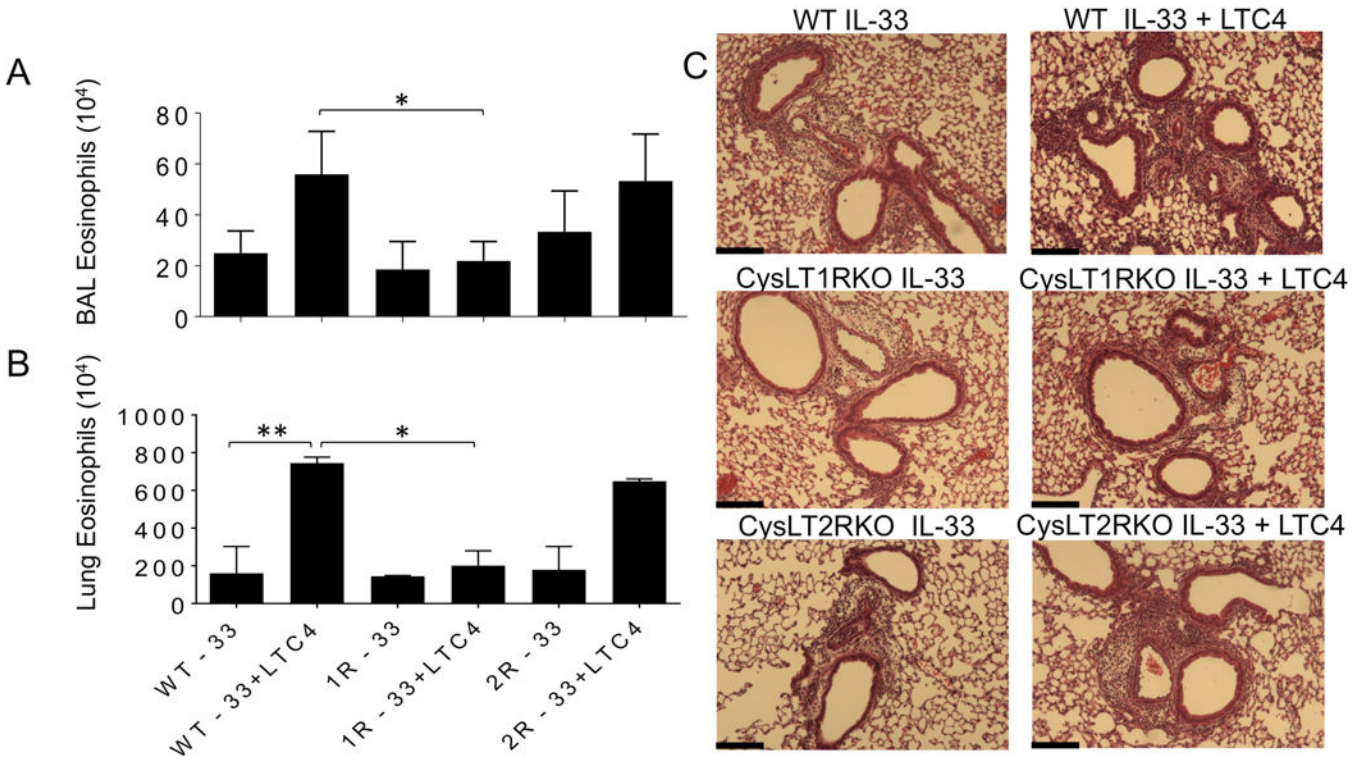

Figure 4.

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 the combination of IL-33 and LTC4 once a day for 3 days. On day 4, mice were euthanized and BAL and lungs were collected. (A) Total eosinophils in the BAL (A) and lungs (B) were enumerated. (C) H&E stained lung sections from from WT, CysLT1R−/− and CysLT2R−/− challenged with IL-33 or IL-33 and LTC4. Images shown are 10×, scale bar is 100 μm. Data shown are representative of 2–4 independent experiments with 2 mice per group. * $p < 0.05$, *** $p < 0.005$, unpaired t test.

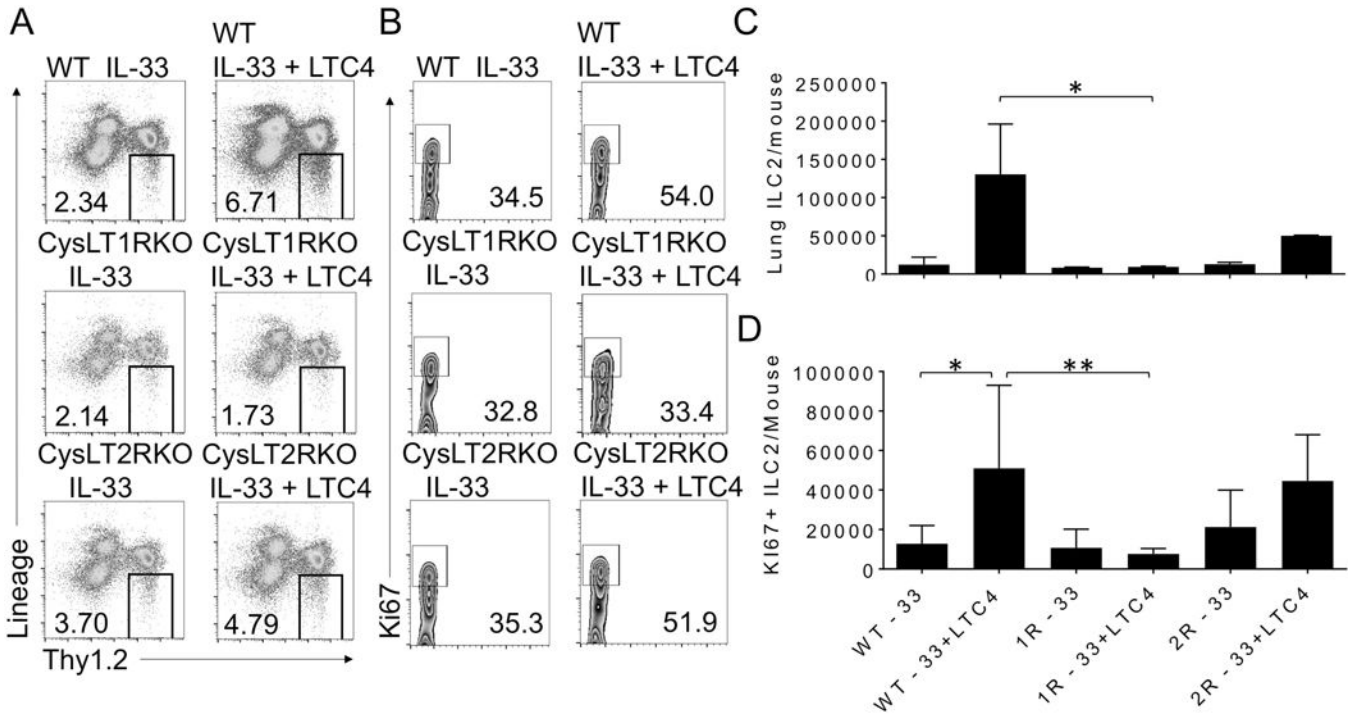

Figure 5.

Potentiation of ILC2 proliferation by LTC4 is dependent on CysLT1R. WT, CysLT1R−/− (1R), and CysLT2R −/− (2R) mice were challenged with IL-33, LTC4, or the combination of IL-33 and LTC4 once a day for 3 days. On day 4, mice were euthanized and BAL and lungs were collected. (A) Percent of ILC2s within lymphocyte gate and (B) Ki-67+ ILCs enumerated. (C) Total numbers of ILC2s and (D) total numbers of Ki67+ ILC2 also shown. Data shown are representative of 2–5 independent experiments with 4 mice per group. * $p < 0.05$, *** $p < 0.005$, unpaired t test.

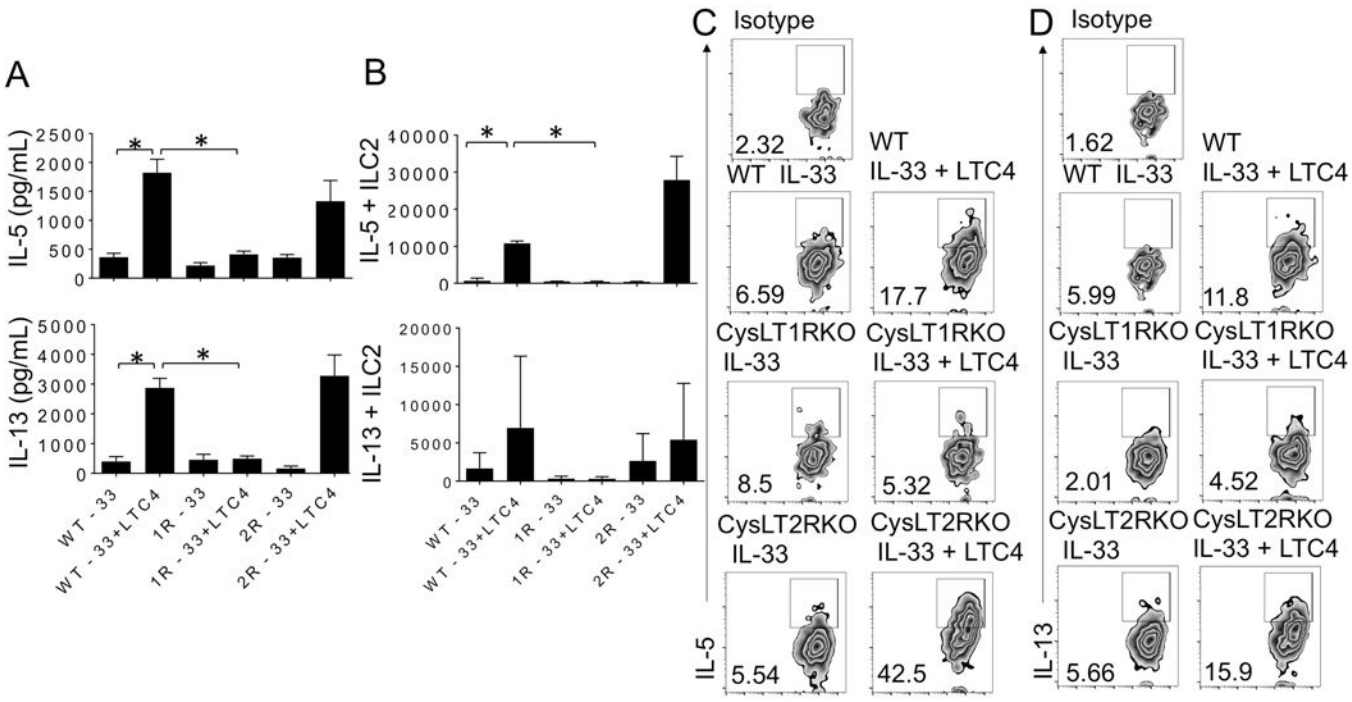

Figure 6.

ILC2 cytokine production potentiation by LTC4 is dependent on CysLT1R. WT, CysLT1R−/− (1R), and CysLT2R−/− (2R) mice were challenged with either PBS, IL-33, LTC4, or both IL-33 and LTC4 once a day for 3 days. On day 4, the mice were euthanized and BAL and lungs were collected. (A) Concentration of IL-5 and IL-13 in the BAL of wild type, CysLT1R−/−, and CysLT2R−/− mice. (B) Total numbers of IL-5+ and IL-13+ ILC2 in wild type, CysLT1R−/−, and CysLT2R−/− mice and (C–D) FACS plots of IL-5 and IL-13. Gated on CD45+ Thy1.2+ lineage-negative lymphocytes. Data shown are representative of 5 independent experiments with 3 mice per group. * $p < 0.05$, *** $p < 0.005$ unpaired t test.

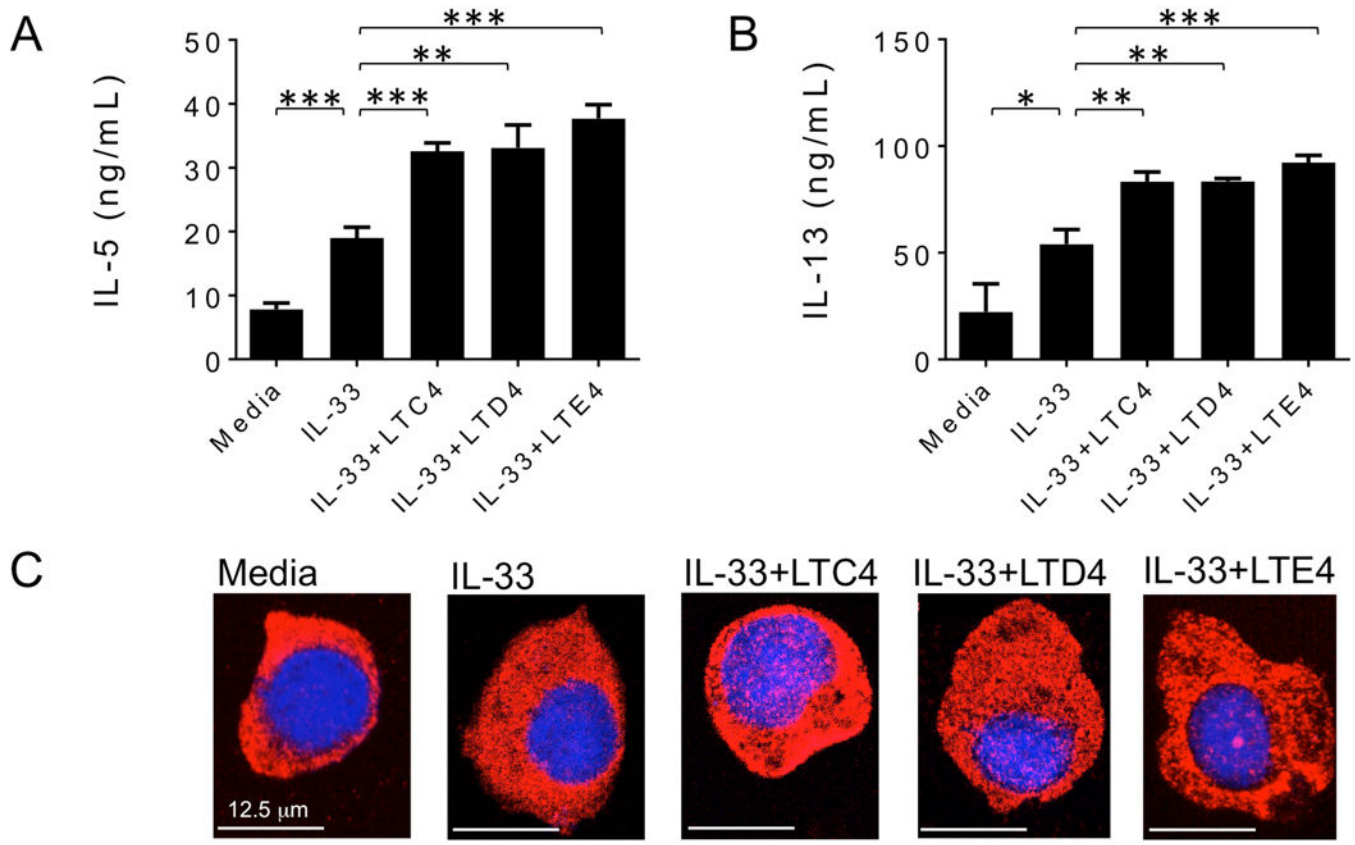

Figure 7.

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 hours. (A) Concentration of IL-5 and (B) IL-13 from culture supernatants. (C) Representative immunofluorescence of ILC2s with NFAT1 staining (red) with nuclear staining (blue). Data shown are representative of 2 independent experiments. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.005$ unpaired t test.

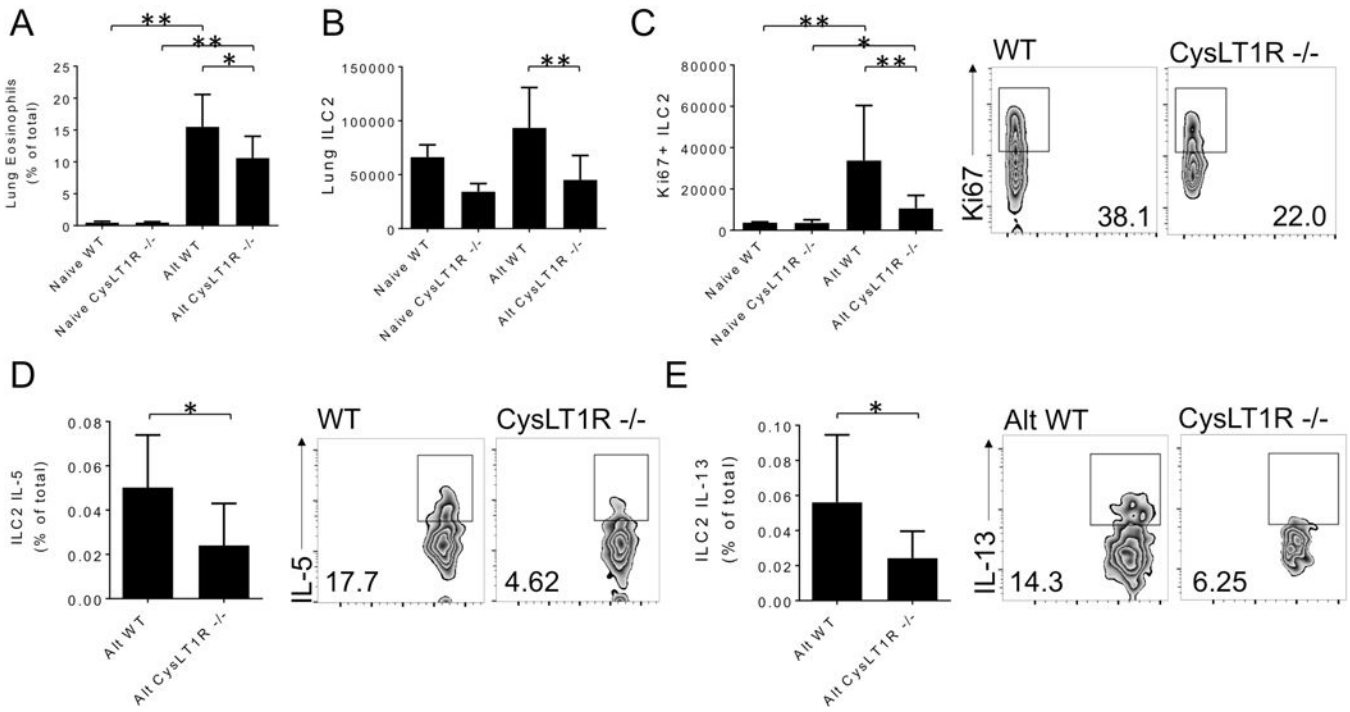

Figure 8.

CysLT1R is required for ILC2 responses during allergen challenge with *Alternaria alternata*. WT and CysLT1R knockout mice were challenged with 30μg *Alternaria alternata* on day 0 followed by 3μg on days 9 and 12. Lung cells were analyzed on D14. (A) Percent of lung eosinophils in naïve and challenged WT and CysLT1R knockout mice. (B) Total number of ILC2s in naïve and challenged WT and CysLT1R knockout mice. (C) Total number of Ki67+ ILC2 in naïve and challenged WT and CysLT1R knockout mice (left) and representative Ki-67% within the ILC2 population from lungs of challenged WT and CysLT1R knockout mice (right). (D) IL-5+ and (E) IL-13+ ILC2s in the lungs of challenged WT and CysLT1R knockout mice (left) and representative ILC2 IL-5% (D) and IL-13% (E) from lungs of challenged WT and CysLT1R knockout mice. Data shown are representative of 2 independent experiments. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.005$ unpaired t test.