

Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates T_H2 cytokine production

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Background: Cysteinyl leukotrienes (CysLTs) contribute to asthma pathogenesis, in part through cysteinyl leukotriene receptor 1 (CysLT1R). Recently discovered lineage-negative type 2 innate lymphoid cells (ILC2s) potentially produce IL-5 and IL-13.

Objectives: We hypothesized that lung ILC2s might be activated by leukotrienes through CysLT1R.

Methods: ILC2s (Thy1.2⁺ lineage-negative lymphocytes) and CysLT1R were detected in the lungs of wild-type, signal transducer and activator of transcription 6-deficient (STAT6^{-/-}), and recombination-activating gene 2-deficient (RAG2^{-/-}) mice by means of flow cytometry. T_H2 cytokine levels were measured in purified lung ILC2s stimulated with leukotriene D₄ (LTD₄) in the presence or absence of the CysLT1R antagonist montelukast. Calcium influx was measured by using Fluo-4 intensity. Intranasal leukotriene C₄, D₄, and E₄ were administered to naive mice, and levels of ILC2 IL-5 production were determined. Finally, LTD₄ was coadministered with *Alternaria* species repetitively to RAG2^{-/-} mice (with ILC2s) and IL-7 receptor-deficient mice (lack ILC2s), and total ILC2 numbers, proliferation (Ki-67⁺), and bronchoalveolar lavage fluid eosinophil numbers were measured.

Results: CysLT1R was expressed on lung ILC2s from wild-type, RAG2^{-/-}, and STAT6^{-/-} naive and *Alternaria* species-challenged mice. *In vitro* LTD₄ induced ILC2s to rapidly generate high levels of IL-5 and IL-13 within 6 hours of stimulation. Interestingly, LTD₄, but not IL-33, induced high levels of IL-4 by ILC2s. LTD₄ administered *in vivo* rapidly induced ILC2 IL-5 production that was significantly reduced by montelukast before treatment. Finally, LTD₄ potentiated *Alternaria* species-induced eosinophilia, as well as ILC2 accumulation and proliferation.

Conclusions: We present novel data that CysLT1R is expressed on ILC2s and LTD₄ potentially induces CysLT1R-dependent ILC2 production of IL-4, IL-5, and IL-13. Additionally, LTD₄ potentiates *Alternaria* species-induced eosinophilia and ILC2 proliferation and accumulation. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Asthma, type 2 innate lymphoid cells, cysteinyl leukotrienes, CysLT1R

Type 2 innate lymphoid cells (ILC2s) have recently been discovered and encompass the originally named natural helper cells, nuocytes, and innate type 2 helper cells.¹⁻⁴ ILC2s do not express known lineage markers (lineage-negative) and produce large amounts of IL-5 and IL-13 in response to the cytokines IL-33, IL-25, or both.¹⁻³ In mice studies have demonstrated the presence of ILC2s in the gastrointestinal tract, mesenteric fat and lymph nodes, spleen, liver, bone marrow, and lung,^{1-3,5-8} whereas studies in human subjects have detected ILC2s in the gastrointestinal tract, lungs, bronchoalveolar lavage (BAL) fluid, and nasal polyps.^{7,9} Importantly, ILC2s have been shown to contribute to airway hyperresponsiveness and type 2 lung inflammatory responses in mice infected with influenza virus and after challenge with multiple allergens, including *Alternaria* species, papain, house dust mite, and ovalbumin.^{8,10-14} Thus, these reports suggest a role for ILC2s in asthma pathogenesis.

In addition to T_H2 cytokines, cysteinyl leukotrienes (CysLTs) play an established role as mediators in human asthma,¹⁵⁻¹⁸ as well as contributing to lung inflammation, airway hyperresponsiveness, and airway remodeling in mice.¹⁹⁻²² CysLTs are lipid mediators derived from arachidonic acid and are generated by mast cells, eosinophils, macrophages, and dendritic cells.²³ Cysteinyl leukotriene receptor 1 (CysLT1R) is the high-affinity receptor for leukotriene D₄ (LTD₄) and is expressed on structural cells, such as airway smooth muscle cells and immune cells, including eosinophils, mast cells, macrophages, and dendritic cells.²⁴⁻²⁶ There is more than 80% sequence homology between human and mouse CysLT1R, allowing for studies to be performed in mice by using pharmacologic blockade with CysLT1R antagonists, such as montelukast.^{20,21}

T_H2 cytokines, including IL-4 and IL-13, have been shown to upregulate CysLT1R on various cell types in human subjects, and direct administration of IL-13 to airways of mice increases CysLT levels.²⁶⁻²⁸ Furthermore, human T_H2 cells were recently shown to selectively express CysLT1R when compared with T_H1 cells, and stimulation of T_H2 cells with CysLTs induced calcium influx, chemotaxis, and IL-13 production that was dependent on CysLT1R.^{29,30} Collectively, these reports strongly suggest that T_H2 cytokines and CysLTs/CysLT1R regulate each other reciprocally. Evidence is mounting that ILC2s, similar to T_H2 cells, might

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Abbreviations used

APC:	Allophycocyanin
BAL:	Bronchoalveolar lavage
CysLT:	Cysteinyl leukotriene
CysLT1R:	Cysteinyl leukotriene receptor 1
FACS:	Fluorescence-activated cell sorting
FITC:	Fluorescein isothiocyanate
ILC2:	Type 2 innate lymphoid cell
IL-7R:	IL-7 receptor
LTC ₄ :	Leukotriene C ₄
LTD ₄ :	Leukotriene D ₄
LTE ₄ :	Leukotriene E ₄
PE:	Phycoerythrin
RAG2:	Recombination-activating gene 2
STAT6:	Signal transducer and activator of transcription 6
TCR:	T-cell receptor
TSLP:	Thymic stromal lymphopoietin
WT:	Wild-type

contribute significantly to type 2 lung inflammation and hyperresponsiveness through IL-5 and IL-13 production,^{8,10-14} and we hypothesized that ILC2s can be activated by CysLTs and promote cytokine production or proliferative responses.

Previous studies have demonstrated that IL-33, IL-25, or both are the primary inducers of ILC2 expansion and cytokine production.¹⁻³ These reports used reporter mice stimulated with IL-25 or IL-33 to identify ILC2 populations and further showed that *in vitro* stimulation of purified ILC2s with IL-25, IL-33, or both led to dramatic increases in IL-5 and IL-13 production but very little IL-4.^{1-3,31} More recently, a report showed that ILC2s obtained from human nasal polyp tissue stimulated with thymic stromal lymphopoietin (TSLP) led to induction of T_H2 cytokine production, including IL-4.³² Apart from IL-33, IL-25, and possibly TSLP, additional mediators that regulate ILC2 cytokine production and expansion are not known and might be important in asthma pathogenesis.

Here we report the novel findings that CysLT1R is expressed on lung ILC2s in mice and that LTD₄ potently induces CysLT1R-dependent ILC2 production of IL-4, IL-5, and IL-13. Additionally, LTD₄ potentiates *Alternaria* species-induced eosinophilia and ILC2 proliferation and accumulation. Thus, these studies are the first to provide a link between ILC2 responses and leukotriene mediators known to contribute to asthma pathogenesis.

METHODS**Mice, airway challenges, and pharmacologic interventions**

Six- to 8-week-old male and female wild-type (WT) C57BL/6, recombination-activating gene 2-deficient (RAG2^{-/-}), IL-7 receptor-deficient (IL-7R^{-/-}) and signal transducer and activator of transcription 6-deficient (STAT6^{-/-}) mice were purchased from Jackson Laboratories (Bar Harbor, Me) and bred in house. Mice were challenged with either a single intranasal dose (25 or 100 µg) of *Alternaria alternata* extract (lot nos. 177372 and 161037) and killed 12 hours, 24 hours, or 3 days later or received 4 challenges over 10 days, as we have previously reported.¹⁴ In some experiments naive WT mice received intranasal leukotriene C₄ (LTC₄), LTD₄, or leukotriene E₄ (LTE₄; 100 ng in 2.5% ethanol; Cayman Chemical, Ann Arbor, Mich) and were killed 3 hours later. Mice were treated with intragastric montelukast (0.2 mg in 1% dimethyl sulfoxide, Cayman Chemical) or 1% dimethyl sulfoxide in PBS administered daily for 3 days before LTD₄ or LTE₄ challenge. In other

experiments RAG2^{-/-} mice received intranasal *Alternaria* species (25 µg) with or without LTD₄ (100 ng, Cayman Chemical). All studies were approved by the University of California San Diego Institutional Animal Care and Use Committee.

Lung and bone marrow processing

BAL fluid was obtained by means of intratracheal insertion of a catheter and 5 lavages with 0.7 mL of 2% BSA (Sigma, St Louis, Mo).¹⁴ Lungs were placed in RPMI, and single-cell suspensions were obtained by using a gentleMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol. Live total BAL fluid and lung cells were counted with a flow cytometer (Accuri C6; BD Biosciences, San Jose, Calif). Bone marrow was isolated from both tibias and fibulas, as we have previously described.³³

Flow cytometry

Lung single-cell suspensions or BAL fluid cells were first incubated with an mAb to CD16/CD32 (24G.2) for 10 minutes to block Fc receptors. BAL fluid cells were stained with peridinin-chlorophyll-protein complex-conjugated CD45.2, phycoerythrin (PE)-conjugated Siglec-F, allophycocyanin (APC)-conjugated Gr-1, and fluorescein isothiocyanate (FITC)-conjugated CD11c (eBioscience, San Diego, Calif). BAL fluid eosinophils were identified as Siglec F-positive CD11c-negative cells. To identify lung ILC2s, lung cells were stained with peridinin-chlorophyll-protein complex-conjugated CD45.2 (eBioscience), and lineage staining was performed only with FITC antibodies that included FITC-conjugated lineage cocktail (BioLegend, San Diego, Calif), CD11c (eBioscience), NK1.1 (eBioscience), CD4 (eBioscience), CD5 (BD Biosciences), CD8 (BD Biosciences), FcεRI (BioLegend), T-cell receptor (TCR) β (BD Biosciences), TCRγδ (BioLegend), and CD19 (BioLegend). Lung cells were also stained with APC-conjugated Thy1.2 (eBioscience), and ILC2s were identified as lineage-negative Thy1.2⁺ lymphocytes.¹⁴ Lung T_H2 and non-T_H2 cells were identified by T1/ST2⁺ (MD Biosciences, St Paul, Minn) or T1/ST2⁻ CD4⁺ lymphocytes, respectively. To identify ILC2 CysLT1R expression, lung cells were stained with polyclonal rabbit to the C-terminus portion of CysLT1R (Cayman Chemical) after permeabilization or with polyclonal rabbit to the EC3 extracellular portion of CysLT1R (clone RB34, kindly provided by Dr Joshua Boyce, Harvard, Boston, Mass)³⁴ without permeabilization, followed by PE-conjugated F(ab')₂ donkey anti-rabbit IgG (eBioscience). In some experiments CysLT1R blocking peptide (Cayman Chemical) and C-terminal CysLT1R antibody in a 10:1 ratio were incubated for 3 hours before staining of lung cells by using permeabilized methods. Intracellular IL-5 staining was performed after 10⁴ lung cells per well were incubated in a 96-well plate for 16 to 18 hours with 10 ng/mL IL-2 (R&D Systems, Minneapolis, Minn), 10 ng/mL IL-7 (R&D Systems), and Golgi-Plug (BD Biosciences), followed by fixation and permeabilization (BD Biosciences) and staining with PE-conjugated IL-5 or isotype control (eBioscience). Ki-67 staining was performed with a forkhead box protein 3 staining kit (eBioscience), followed by APC-conjugated Ki-67 (eBioscience).¹⁴ Flow cytometry was performed with an Accuri C6 cytometer (BD Biosciences), and sample data were further analyzed with FlowJo software (Tree Star, Ashland, Ore).

ILC2 cell sorting and culture

WT mice received three or four 25-µg intranasal *Alternaria* species challenges over 9 to 10 days before fluorescence-activated cell sorting (FACS) to expand ILC2s. Sorting of ILC2s (CD45⁺ lineage-negative Thy1.2⁺ lymphocytes) was performed with a FACSaria cell sorter (BD Biosciences). Purified ILC2s (>99%) were rested for 40 hours in 10 ng/mL IL-2 and IL-7 (R&D Systems) in a 96-well flat bottom plate (40,000 cells per well). Before stimulation, the media was changed, and ILC2s were cultured for 6 hours with IL-33 (30 ng/mL, R&D Systems) or LTD₄ (10⁻⁶ or 10⁻⁸ mol/L in 0.5% ethanol, Cayman Chemical). In some wells ILC2s were pretreated with 1 µmol/L montelukast in 0.5% ethanol (Cayman Chemical) for 2 hours before stimulation with LTD₄. After 6 hours of stimulation, supernatants were collected for cytokine analysis by using ELISA.

Calcium signaling

Purified ILC2s were cultured with 10 ng/mL each of IL-2 and IL-7 and rested for at least 3 days before calcium-signaling experiments. Calcium signaling was detected with a Fluo-4 Direct Calcium Assay Kit (Invitrogen, Carlsbad, Calif), according to the manufacturer's protocol. Calcium influx was recorded in the FL2 channel of an Accuri C4 flow cytometer, as previously reported,³⁵ and 10^{-6} mol/L LTD₄ was added after 60 seconds of recording. In some experiments ILC2s were incubated at 37°C with 1 μ M of montelukast before performing calcium tracer studies.

ELISA for BAL fluid cytokines and CysLT1R

ELISAs of cell-culture supernatants for IL-4, IL-5, and IL-13 (all R&D Systems) and BAL CysLT Enzyme Immunoassay (Cayman Chemical) were performed according to the manufacturer's instructions and read with a microplate reader (model 680; Bio-Rad Laboratories, Hercules, Calif).

RT-PCR and real-time PCR

FACS-sorted lung ILC2s and CD4⁺T1ST2⁺ and CD4⁺T1ST2⁻ cells were lysed by using TRIzol (Life Technologies) to isolate RNA. An aliquot of total RNA (0.5 μ g) was reverse transcribed to cDNA by using the Transcriptor First Strand cDNA Synthesis kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. Diluted cDNA was subject to real-time PCR with SYBR Green I master (Roche) and the RT-PCR-specific primers. The oligonucleotide primer sequences were as follows: CysLT1R forward, 5'-CAACGAATATC-CACCTTCACC-3'; CysLT1R reverse, 5'-AGCCTTCTCCTAAAGTTTC-CAC-3'; L32 forward, 5'-GAA ACT GGC GGA AAC CCA-3'; and L32 reverse, 5'-GGA TCT GGC CCT TGA ACC TT-3'. Data are presented as normalized to the housekeeping gene *L32* by using the Roche LightCycler 480 (Roche Diagnostics).

Statistical analyses

Statistical analysis was performed with GraphPad Prism software (GraphPad Software, La Jolla, Calif). The Mann-Whitney test or unpaired *t* test was used, where indicated. A *P* value of less than .05 was considered statistically significant.

RESULTS

CysLT1R is expressed on lung and bone marrow ILC2s

We have previously reported the presence of ILC2s in the lungs of mice defined as CD45⁺ lineage-negative Thy1.2⁺ lymphocytes.¹⁴ ILC2s lack surface marker expression of CD3, CD4, CD8, TCR β , TCR δ , CD5, CD19, B220, NK1.1, Ter119, Gr-1, CD11c and Fc ϵ RI.¹⁻³ This combination excludes B, T, natural killer, and natural killer T cells, as well as mast cells, basophils, granulocytes, dendritic cells, and macrophages. Lung ILC2s express T1/ST2 (IL-33 receptor), Sca-1, c-kit, IL-7R, Thy 1.2, CD25, and CD44¹⁴ and have a phenotype most consistent with previously described natural helper cells.¹ We will now refer to these cells as ILC2s, as is standard convention based on consensus.⁴

To determine whether ILC2s express CysLT1R, we stained single-cell suspensions from the lungs of naive WT mice for FACS analysis. We used both permeabilized and nonpermeabilized protocols for staining because CysLT1R can be expressed both on the cell surface and nuclear membrane.³⁴ Lineage-negative Thy1.2⁺ lymphocytes showed strong CysLT1R expression with permeabilization using the C-terminus antibody (Fig 1, A). To determine whether the CysLT1R antibody we used was specific for CysLT1R, we incubated some samples

with CysLT1R-specific blocking peptide (amino acids 318-337). Samples incubated with CysLT1R blocking peptide had complete abrogation of CysLT1R staining, demonstrating antibody specificity for CysLT1R. To confirm the presence of CysLT1R on ILC2s, we also performed staining with an antibody specific to the extracellular EC3 region (RB34) of CysLT1R in the absence of permeabilization (Fig 1, B).³⁴ Staining with the RB34 antibody further demonstrated expression of CysLT1R on ILC2s. Additionally, we assessed CysLT1R mRNA levels by means of quantitative PCR in FACS-purified ILC2s from the lungs of *Alternaria* species-challenged mice compared with levels from sorted lung T_H2 cells (CD4⁺T1/ST2⁺ cells) and non-T_H2 cells (CD4⁺T1/ST2⁻). We detected increased CysLT1R mRNA levels in ILC2s compared with T_H2 cells, which are known to express CysLT1R,³⁶ and very little expression in non-T_H2 cells (Fig 1, C). To determine whether CysLT1R was expressed on bone marrow ILC2s, we performed similar staining with bone marrow from naive mice (Fig 1, D) and found that ILC2s in the bone marrow express CysLT1R similar to lung ILC2s. Thus lung and bone marrow ILC2s from WT naive mice express CysLT1R, suggesting that ILC2s might be primed to respond to CysLTs.

CysLT1R is expressed on lung ILC2s independent of STAT6 and RAG2

Previous reports have suggested that IL-4 and IL-13 can upregulate CysLT1R expression on different cell types.^{27,37} Because downstream IL-4 and IL-13 signaling is primarily mediated through STAT6,³⁸ we sought to determine whether STAT6 was required for ILC2 CysLT1R expression. Lung ILC2s from naive STAT6^{-/-} mice displayed similar surface levels of CysLT1R compared with ILC2s from WT mice (Fig 1, E). RAG2^{-/-} mice lack B and T cells but have innate lymphoid cell populations, including lung ILC2s.^{1,7,8,14} ILC2s from lungs of RAG2^{-/-} mice displayed expression of CysLT1R (Fig 1, F). Thus lung ILC2 expression of CysLT1R is constitutive and independent of STAT6 or the presence of adaptive immune cells.

ILC2 expression of CysLT1R is stable after allergen challenge

We next determined whether ILC2 expression of CysLT1R was further upregulated after mice were challenged with intranasal allergen. We have previously demonstrated that mice receiving a single intranasal challenge with the fungal allergen *Alternaria* species have innate eosinophilia, increased BAL fluid IL-5 and IL-13 levels, and ILC2 activation.^{14,33} Lung ILC2s from mice that received 1 challenge of *Alternaria* species maintained similar CysLT1R expression compared with that seen in PBS control-challenged mice when measured at 24 hours and 3 days after challenge. ILC2s from mice that received 4 challenges with *Alternaria* species over 10 days, a protocol that induces a strong adaptive T_H2 response and increased IgE levels,¹⁴ also had similar CysLT1R expression compared with PBS-challenged mice (Fig 2). Additionally, STAT6^{-/-} (Fig 2, A) and RAG2^{-/-} (Fig 2, B) ILC2s maintained expression of CysLT1R after single and multiple challenges with *Alternaria* species, although we observed slightly decreased expression on ILC2s from RAG2^{-/-} mice

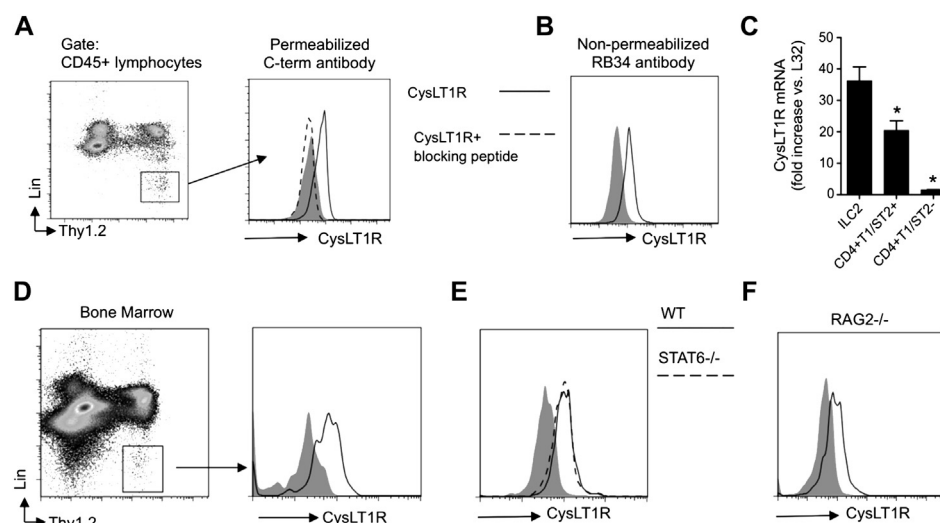


FIG 1. CysLT1R is expressed on lung and bone marrow ILC2s. Single-cell suspensions from lungs of naive mice were stained with CD45, lineage (CD3, CD4, CD5, CD8, CD19, Gr-1, CD11b, CD11c, B220, Ter-119, NK1.1, FcεRI, TCRβ, and TCRγδ), and Thy1.2. **A**, ILC2 gated on CD45⁺ lineage-negative Thy1.2⁺ lymphocytes (left). Cells were then stained with a C-terminal antibody for CysLT1R with and without blocking peptide by using permeabilized methods (right). **B**, ILC2s from naive WT mice were stained with an antibody to the extracellular portion of CysLT1R (RB34) by using nonpermeabilized methods. **C**, CysLT1R mRNA levels from FACS-purified lung ILC2s, CD4⁺T1/ST2⁺ cells, and CD4⁺T1/ST2⁻ cells from WT mice receiving 4 challenges with *Alternaria* species. Data are shown from triplicate samples: $P < .05$, Mann-Whitney test. **D**, Bone marrow was taken from naive WT mice and processed for FACS analysis of ILC2 CysLT1R expression by using the RB34 antibody. **E** and **F**, Lung ILC2 CysLT1R expression from WT and STAT6^{-/-} mice (Fig 1, **E**) and RAG2^{-/-} (Fig 1, **F**) mice after staining with the RB34 antibody. Plots are representative of at least 2 independent experiments, and control antibody staining is shown in solid gray.

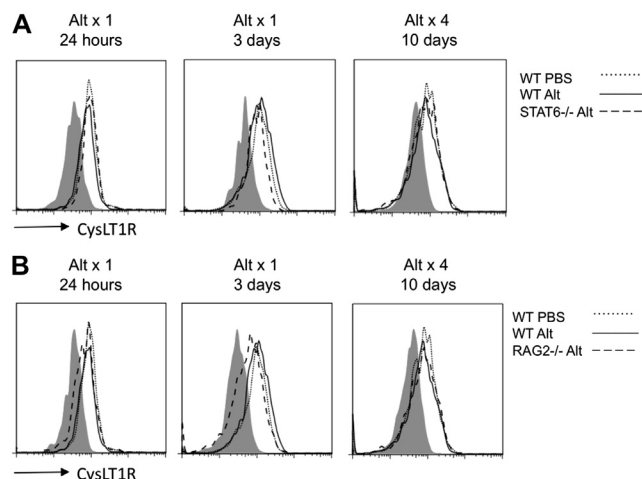


FIG 2. ILC2 expression of CysLT1R is stable after allergen challenges and independent of STAT6 or RAG2. WT, STAT6^{-/-}, and RAG2^{-/-} mice were administered a single intranasal challenge with *Alternaria* species allergen once and lung cells obtained at 24 hours and 3 days or administered in 4 challenges over 10 days. Single suspensions of lungs were stained for ILC2s and surface CysLT1R by using the RB34 antibody or control antibody (gray). Histograms gated on lung ILC2s (CD45⁺ Lineage-negative Thy1.2⁺ lymphocytes). **A**, WT and STAT6^{-/-} ILC2 CysLT1R expression in *Alternaria* species-challenged mice at 24 hours, 3 days, or 10 days. **B**, WT and RAG2^{-/-} ILC2 CysLT1R expression in *Alternaria* species-challenged mice at 24 hours, 3 days, or 10 days. Plots are representative of 2 independent experiments at each time point, and control antibody staining is shown in solid gray.

compared with WT mice at baseline (Fig 1, **F**), 24 hours, and 3 days after challenge. Overall, this suggests that ILC2 expression of CysLT1R is stable after allergen challenge and

is not significantly affected by the absence of STAT6 or adaptive immune cells.

LTD₄ potently induces ILC2 T_H2 cytokine production through CysLT1R

To determine whether CysLTs can activate lung ILC2s, we performed FACS of lung ILC2s from mice that received 3 to 4 *Alternaria* species intranasal challenges *in vivo*. Lung ILC2s expand from approximately 10,000 per mouse to more than 100,000 per mouse after 3 to 4 *Alternaria* species challenges, thus facilitating *in vitro* studies with a greater number of purified cells. After sorting, we obtained a greater than 99% pure ILC2 population (Fig 3, **A**) and then rested the cells for 2 days, with media changes before stimulation studies. Rested ILC2s were cultured with IL-33 and LTD₄ with and without the CysLT1R antagonist montelukast for 6 hours before collecting culture supernatants for ELISA. ILC2s stimulated with IL-33 (30 ng/mL) produced large amounts of IL-5 and IL-13 (>60 ng/mL IL-5 and 150 ng/mL IL-13; Fig 3, **B**), as previously reported.^{1-3,31} Interestingly, LTD₄ (10⁻⁶ mol/L) induced similar levels of IL-5 and only slightly lower levels of IL-13 compared with IL-33. Pretreatment with montelukast abrogated the LTD₄-induced IL-5 and IL-13 production. Previous *in vitro* studies have shown that ILC2s produce large amounts of IL-5 and IL-13 but very little IL-4 and only after phorbol 12-myristate 13-acetate plus ionomycin stimulation.^{1-3,31} Strikingly, lung ILC2s (40,000 cells/well) stimulated with 10⁻⁶ mol/L LTD₄ produced more than 6 ng/mL IL-4 (Fig 3, **C**). In contrast, IL-33-stimulated ILC2s produced no significant increase in IL-4 levels compared with media, as is consistent with previous

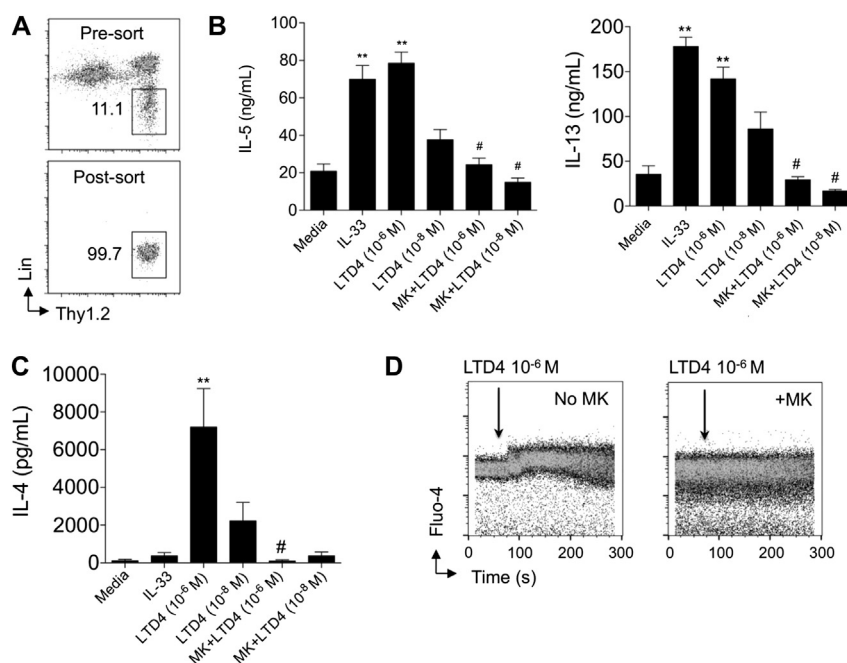


FIG 3. LTD₄ induces ILC2 TH2 cytokine production through CysLT1R. **A**, Lung ILC2s were expanded *in vivo* after 3 *Alternaria* species challenges, followed by FACS and resting for 40 hours before *in vitro* studies. Presort (top) and postsort (bottom) ILC2 percentages are shown. **B** and **C**, IL-5 and IL-13 (Fig 3, B) and IL-4 (Fig 3, C) levels determined by using ELISA from supernatants of purified ILC2s stimulated with IL-33 or LTD₄ (10⁻⁶ and 10⁻⁸ mol/L) for 6 hours with and without 1 μmol/L montelukast before treatment for 2 hours. **D**, ILC2 Fluo-4 intensity over time in seconds after 10⁻⁶ mol/L LTD₄ was added at 60 seconds without montelukast (left) and after pretreatment with montelukast (right). Data in Fig 3, A and D, are representative of 2 independent experiments, and data in Fig 3, B and C, are combined from triplicate wells of each condition of 2 independent experiments. MK, Montelukast. ***P* < .005 compared with media and #*P* < .01 compared with the same dose of LTD₄ alone, Mann-Whitney test.

reports. Furthermore, cells that were cultured for 2 hours with montelukast before LTD₄ stimulation produced very low IL-4 levels that were comparable with those in media. Taken together, LTD₄ induces a dose-dependent increase in IL-4, IL-5, and IL-13 levels that is mediated by CysLT1R. In contrast to LTD₄, the established ILC2 activator IL-33 did not induce IL-4 production.

CysLT1R is a G protein-coupled receptor that rapidly promotes calcium influx when activated. To determine whether LTD₄ could induce ILC2 calcium influx directly, we cultured sorted ILC2s (Fig 3, A) from the lungs of mice with the calcium tracer Fluo-4 and used flow cytometry to measure changes in calcium influx.³⁵ LTD₄ was added to purified ILC2s at 60 seconds, and Fluo-4 intensity was measured (Fig 3, D). Some ILC2s were pretreated with the CysLT1R antagonist montelukast. ILC2s that received LTD₄ without pretreatment had a rapid and significant increase in calcium influx after LTD₄ was added. In contrast, calcium influx was abrogated in ILC2s that received montelukast before the addition of LTD₄. Thus LTD₄ directly and rapidly induces lung ILC2 calcium influx dependent on CysLT1R.

CysLTs are rapidly induced by *Alternaria* species challenge in a STAT6-dependent manner

We have previously reported that ILC2s are activated within hours after a single airway exposure to *Alternaria* species, a clinically relevant aeroallergen associated with severe asthma.¹⁴ To determine whether CysLT levels are increased after *Alternaria* species challenge and thus might be available to activate ILC2s,

we performed ELISA for CysLTs in the BAL fluid of challenged and unchallenged mice. BAL fluid from WT mice showed an increase in CysLT levels 12 hours after a single *Alternaria* species challenge compared with those seen in PBS-challenged mice (Fig 4, A). A previous report showed that BAL fluid CysLT levels are increased after direct administration of IL-13 to the airways of mice.²⁸ Because IL-13 downstream signaling occurs through STAT6, we assessed levels of BAL fluid CysLTs in STAT6^{-/-} mice after *Alternaria* species challenge. Impressively, the increased BAL fluid level of CysLTs in WT mice was completely abrogated in STAT6^{-/-} mice receiving *Alternaria* species challenge. Thus rapid production of CysLTs occurs in the airway after exposure to a clinically relevant allergen and is dependent on STAT6.

LTD₄ increases IL-5-producing ILC2 numbers *in vivo* dependent on CysLT1R

To determine whether LTD₄ can activate ILC2s *in vivo*, we administered a single intranasal challenge of LTD₄ to WT mice and performed FACS analysis for ILC2 intracellular IL-5 (Fig 4, B). Single-cell lung suspensions were obtained 3 hours after LTC₄, LTD₄, or LTE₄ challenge and cultured overnight with a protein transport inhibitor and stained for ILC2 intracellular IL-5 the following day. Some groups of mice received 3 daily doses of montelukast by means of oral gavage before LTC₄, LTD₄, or LTE₄ challenge. Mice receiving LTC₄, LTD₄, or LTE₄ had a significantly increased percentage of IL-5-producing

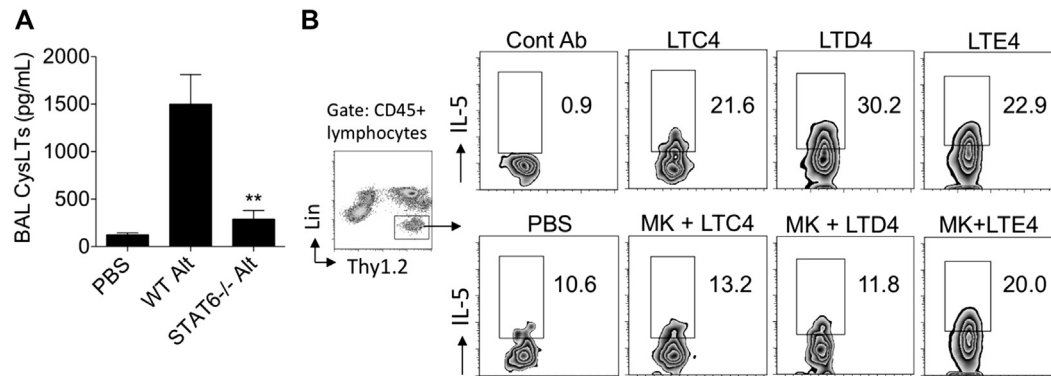


FIG 4. STAT6-dependent CysLTs are induced by *Alternaria* species and promote ILC2 IL-5 production *in vivo*. **A**, BAL fluid CysLT levels measured from naive WT and STAT6^{-/-} mice 12 hours after a single intranasal challenge with 100 μ g of *Alternaria* species. Nine mice per group were challenged with *Alternaria* species, and 3 mice per group were challenged with PBS. ** $P < .005$, Mann-Whitney test. **B**, WT naive mice received a single intranasal challenge with LTC₄, LTD₄, LTE₄, or PBS, and some mice were administered 3 doses of intragastric montelukast before challenge. Lung cells obtained 3 hours after challenge were cultured overnight with protein transport inhibitor and stained for ILC2 intracellular IL-5. Lung ILC2s (left) were gated, and intracellular IL-5 levels from mice receiving LTC₄, LTD₄, and LTD₄ with (bottom row) and without (top row) montelukast were measured. Control antibody staining and ILC2 IL-5 from mice receiving PBS challenge was as shown. MK, Montelukast. Data shown are representative of 2 to 3 independent experiments.

ILC2s (21.6%, 30.2%, and 22.0%, respectively) compared with PBS-challenged mice (10.6%). Mice receiving montelukast before LTC₄ and LTD₄ administration had significantly reduced IL-5-producing ILC2s (13.2% and 11.8%, respectively) compared with those receiving LTC₄ or LTD₄ alone. However, montelukast pretreatment did not significantly affect the percentage of LTE₄-induced IL-5-producing ILC2s (20.0%). Thus all 3 CysLTs induce IL-5 production by lung ILC2s *in vivo*, although only LTC₄- and LTD₄-induced IL-5 production was inhibited by an antagonist of CysLT1R.

LTD₄ potentiates *Alternaria* species-induced eosinophilia and ILC2 accumulation and proliferation

To investigate whether LTD₄ has a potentiating effect on ILC2s during repetitive allergen challenges, we administered 4 intranasal challenges of *Alternaria* species alone or with LTD₄ to RAG2^{-/-} and IL-7R^{-/-} mice. RAG2^{-/-} mice possess lung ILC2s (Fig 5, A) but lack B and T cells and allowed for determination of the effects of LTD₄ on innate non-B/non-T-cell populations. In contrast, IL-7R^{-/-} mice lack ILC2s (Fig 5, A) and have severely reduced B- and T-cell compartments. RAG2^{-/-} mice that received 4 intranasal challenges with *Alternaria* species have increased ILC2 percentages compared with naive RAG2^{-/-} mice (Fig 5, A). Furthermore, mice that received LTD₄ and *Alternaria* species had an increased percentage (Fig 5, A) and approximately 3-fold increase in total numbers of lung ILC2s (Fig 5, B) compared with mice that received *Alternaria* species only. We have previously reported that lung ILC2s proliferate, as identified by using Ki-67⁺ staining, and accumulate in the lungs after mice are exposed to *Alternaria* species.¹⁴ Thus we assessed whether LTD₄ increased the proliferation of lung ILC2s and detected significantly increased numbers and percentages of Ki-67⁺ ILC2s in mice receiving *Alternaria* species and LTD₄ compared with mice receiving *Alternaria* species only (Fig 5, B). We then determined numbers of IL-5-producing ILC2s in the lungs of

both groups of mice and found increased IL-5⁺ ILC2 numbers in mice administered *Alternaria* species and LTD₄ compared with those seen in mice given *Alternaria* species only (Fig 5, C). We also assessed levels of BAL fluid eosinophils in both RAG2^{-/-} and IL-7R^{-/-} mice and detected increased eosinophil numbers in mice receiving LTD₄ in RAG2^{-/-} mice (Fig 5, C). In contrast, IL-7R^{-/-} mice that do not have ILC2s did not mount eosinophilia after administration of *Alternaria* species with or without LTD₄. These studies demonstrate that LTD₄ potentiates lung ILC2 proliferation, accumulation, and airway eosinophilia independent of adaptive immunity.

DISCUSSION

Our studies demonstrate several novel findings. First, we show that CysLT1R is expressed on lung and bone marrow ILC2s in unchallenged mice and remains expressed on lung ILC2s after allergen challenges independent of STAT6 and B and T cells. Second, stimulation of purified ILC2s with LTD₄, the dominant ligand of CysLT1R, results in robust IL-4, IL-5, and IL-13 production dependent on CysLT1R. Third, LTD₄ administered to airways of mice regulates ILC2 IL-5 production and proliferation and potentiates allergen-induced airway eosinophilia independent of adaptive immunity. Our results have significant potential implications for type 2 diseases, including allergic asthma, in which CysLT levels are increased and available to potentially activate lung ILC2s.

We demonstrated that CysLT1R is expressed on lung ILC2s constitutively and remains expressed after *Alternaria* species challenges. CysLT1R has been detected on immune cells in the airways of human asthmatic patients compared with healthy control subjects, and its levels increase during severe asthma exacerbations.³⁹ CysLT1R has also been found to be upregulated after administration of IL-13 to the airways of mice and on various human cell types *in vitro* after IL-4, IL-5, or IL-13 stimulation.^{26,27,40-42} We have previously reported that mice exposed to the fungal allergen *Alternaria* species have rapid increases in

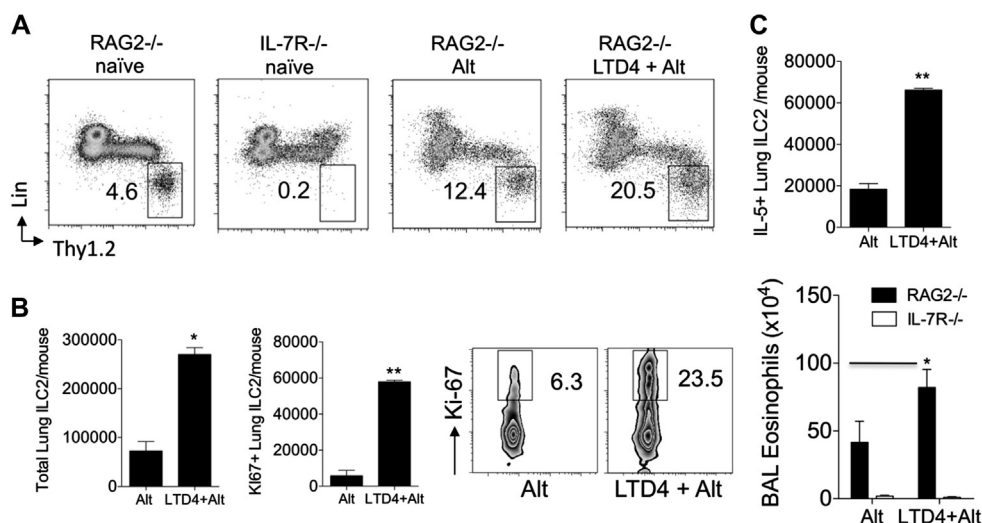


FIG 5. LTD₄ induces accumulation and proliferation of lung ILC2s and potentiates *Alternaria* species–induced eosinophilia. RAG2^{−/−} and IL-7R^{−/−} mice received 4 intranasal challenges with *Alternaria* species alone or with LTD₄. **A**, Representative FACS plots of ILC2 cell percentages from pooled lungs of naive RAG2^{−/−} (left) and IL-7R^{−/−} (middle left) and RAG2^{−/−} mice after 4 challenges with *Alternaria* species alone (middle right) or with LTD₄ (right). **B**, Total ILC2 numbers per mouse (left), Ki-67⁺ ILC2 numbers (middle), and percentage of Ki-67 ILC2s in mice receiving *Alternaria* species alone or with LTD₄ (right). **C**, Total IL-5⁺ lung ILC2 numbers (top) from RAG2^{−/−} mice and BAL fluid eosinophil numbers (bottom) from RAG2^{−/−} and IL-7R^{−/−} mice receiving 4 intranasal challenges with *Alternaria* species alone or with LTD₄. Total ILC2s and Ki-67⁺ and IL-5⁺ ILC2s per mouse are from 2 independent experiments with pooled lungs from 2 mice per group. **P* < .05 and ***P* < .005, *t* test. BAL fluid eosinophils from 8 mice per group. **P* < .05, Mann-Whitney test.

airway IL-5 and IL-13 levels,¹⁴ although we did not detect an increase in ILC2 CysLT1R expression levels at several time points after *Alternaria* species challenge compared with levels seen in ILC2s in naive mice, even in the absence of STAT6. In contrast, we found that STAT6 regulated the induction of CysLTs in airways after *Alternaria* species challenge, despite not affecting ILC2 CysLT1R expression. Thus ILC2 CysLT1R expression is constitutive and stable, suggesting that ILC2s are primed to respond to CysLTs that are found at increased levels in the asthmatic airway.

We found that LTD₄ strongly induces purified lung ILC2s to produce IL-4, IL-5, and IL-13. Previously, ILC2s have been shown to produce large amounts of IL-5 and IL-13 but very little, if any, IL-4.^{1-3,31} In these reports IL-33 was used to stimulate ILC2s, and we obtained similar results after purified lung ILC2s were cultured with IL-33. The finding that IL-33 induces IL-5 and IL-13, but not IL-4, might not be specific to ILC2s because a previous report demonstrated that conventional CD4⁺ T cells stimulated with IL-33 differentiate into IL-5– and IL-13–producing, but not IL-4–producing, cells.⁴³ The IL-33–induced T_H2 differentiation was dependent on the known IL-33 receptor signaling molecules p38 and MyD88.⁴⁴ Furthermore, mouse ILC2s have been shown to produce IL-4 after stimulation with phorbol 12-myristate 13-acetate and ionomycin¹ and when human ILC2s were stimulated with TSLP.³² Taken together, this suggests that ILC2s have the capacity to produce a significant amount of IL-4 and that the lack of IL-4 production from ILC2s in previous studies might be more related to the downstream effects of IL-33 on lymphocytes. Consistent with our findings that LTD₄ stimulates ILC2 T_H2 cytokine production, CysLTs have been previously shown to induce IL-4 production from eosinophils⁴⁵ and T_H2 cells,²⁹ as well as IL-5 from mast cells.^{46,47}

Our results demonstrate that administration of LTC₄, LTD₄, or LTE₄ *in vivo* induces ILC2 IL-5 production and that montelukast pretreatment inhibited the IL-5 production induced by LTC₄ and LTD₄ but not LTE₄. A few possibilities might explain these findings. LTC₄ could directly bind to ILC2 CysLT1R or undergo conversion *in vivo* to LTD₄, resulting in higher affinity binding to CysLT1R. In contrast, LTE₄ might bind to a receptor other than CysLT1R on ILC2s to induce IL-5 production. Consistent with this, one report demonstrated that LTE₄–potentiated airway inflammation was dependent on the purinergic receptor P2Y₁₂, but not CysLT1R or CysLT2R,⁴⁸ in an adaptive immune model using ovalbumin as the antigen. Thus it is interesting to speculate that there might be several receptors on ILC2s that bind leukotrienes and potentially regulate ILC2 cytokine production and proliferation.

We detected increased accumulation and proliferation of ILC2s in the lungs when LTD₄ was repetitively administered along with *Alternaria* species to RAG2^{−/−} mice. CysLTs have been shown *in vitro* to induce proliferation of mast cells,^{34,49} and LTE₄ has been shown to be more potent than LTD₄ in promoting mast cell proliferative responses.^{49,50} As discussed, LTD₄ can be rapidly converted to LTE₄ *in vivo*, and it is possible that LTD₄, LTE₄, or both can act directly or indirectly to promote ILC2 proliferation and accumulation.⁴⁸ Additional receptors for CysLTs independent of CysLT1R and CysLT2R likely exist⁵¹ and might help to explain some of our findings once characterized and applied to future ILC2 studies. Interestingly, we administered LTD₄ repetitively with and without *Alternaria* species to WT mice (not shown) and found very little effect on LTD₄–induced ILC2 proliferation. WT mice administered repetitive *Alternaria* species challenges alone have a very strong adaptive T_H2 responses, as well as robust ILC2 proliferation, that might not

be further enhanced by exogenous LTD₄. Furthermore, ILC2 proliferation might not be sufficiently induced by LTD₄ alone *in vivo* and likely requires other signals to be present during allergen challenge. Finally, although our results suggest that ILC2s accumulate in the lung through local proliferation (Ki-67⁺ cells), we cannot exclude that LTD₄ might promote chemotaxis of ILC2s, as previously reported with CD34⁺ progenitor cells.⁵²

In summary, we present novel findings that ILC2s, a recently discovered population of cells that includes natural helper cells and nuocytes,^{1,2} can be activated by LTD₄ to rapidly produce large amounts of T_H2 cytokines in a CysLT1R-dependent manner. If such findings can be translated to human subjects, a leukotriene-ILC2 pathway might promote tissue eosinophilia and hyperresponsiveness in patients with diseases such as asthma and aspirin-exacerbated respiratory disease in which CysLT levels are increased.

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Key messages

- Lung ILC2s express CysLT1R.
- LTD₄ rapidly and robustly induces lung ILC2 calcium influx and IL-4, IL-5, and IL-13 production in a CysLT1R-dependent manner.
- LTD₄ potentiates *Alternaria* species-induced ILC2 accumulation, proliferation, and airway eosinophilia.

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