

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Type 2 Innate Lymphoid Cell Activation by Cysteinyl Leukotrienes: a Novel
Mechanism of Lipid-Mediated Allergic Inflammation

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by

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University of California, San Diego

2014

DEDICATION

I would like to dedicate this thesis to my Mom and Dad for their unwavering and total support. I could not have accomplished what I have been able to without them.

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LIST OF ABBREVIATIONS

2-MeSADP – 2-Methylthioadenosine Diphosphate

5-LO – 5-Lipoxygenase

ADP – Adenosine Diphosphate

APC – Allophycocyanin

BAL – Bronchoalveolar Lavage

CD – Cluster of Differentiation

CysLTs – Cysteinyl Leukotrienes

CysLT1R – Cysteinyl Leukotriene Receptor 1

CysLT2R – Cysteinyl Leukotriene Receptor 2

DMSO – Dimethyl Sulfoxide

FACS – Fluorescence-Activated Cell Sorting

FitC – Fluorescein Isothiocyanate

GPR99 – G protein-coupled Receptor 99

IL-4 – Interleukin 4

IL-5 – Interleukin 5

IL-13 – Interleukin 13

IL-25 – Interleukin 25

IL-33 – Interleukin 33

ILC2 – Type 2 Innate Lymphoid Cell

LTA4 – Leukotriene A4

LTB4 – Leukotriene B4

LTC4 – Leukotriene C4

LTD4 – Leukotriene D4

LTE4 – Leukotriene E4

Mk – Montelukast

PCR – Polymerase Chain Reaction

PerCP – Peridinin Chlorophyll

PE – Phycoerythrin

qPCR – Quantitative Polymerase Chain Reaction

RAG2 – Recombination Activating Gene 2

STAT6 – Signal Transducer and Activator of Transcription 6

T_H2 – Type 2 T helper cell

WT – Wild Type

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The Abstract, Materials and Methods, Results, and Discussion sections contain contributions to the manuscript “Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates T_H2 cytokine production” published in *The Journal of Allergy and Clinical Immunology*. The thesis author made significant contributions to the paper as a researcher, including generating most of the data in this thesis.

ABSTRACT OF THE THESIS

Type 2 Innate Lymphoid Cell Activation by Cysteinyl Leukotrienes: a Novel Mechanism of Lipid-Mediated Allergic Inflammation

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Type 2 innate lymphoid cells (ILC2) are a novel population of innate lymphocytes lacking expression of lineage markers. ILC2 lack antigen specificity and are instead activated by epithelial cytokines IL-33 and IL-25. Similar to type 2 T helper cells, ILC2 produce IL-5 and IL-13, two cytokines shown to be important in the development and pathology of asthma. Cysteinyl leukotrienes are important inflammatory mediators and potent bronchoconstrictors derived from arachidonic acid. 5-lipoxygenase converts arachidonic acid into leukotriene A4 (LTA4) and LTC4

synthase subsequently generates the cysteinyl leukotrienes, LTC₄, LTD₄, and LTE₄. Expression of the cysteinyl leukotriene receptor 1 (CysLT₁R) on ILC2 was shown both by flow cytometric analysis and PCR of sorted ILC2. Leukotriene C₄, D₄, and E₄ administered intranasally induced ILC2 activation *in vivo*, and leukotriene D₄ induced ILC2 IL-4, IL-5, and IL-13 production *in vitro*. Here, we show that the cysteinyl leukotrienes play a role as regulators and activators of type 2 innate lymphoid cells apart from known roles in bronchoconstriction and activation of other inflammatory cells.

INTRODUCTION

Asthma is an allergic disease of the small and medium sized airways in the lungs, with symptoms including coughing, wheezing, and shortness of breath¹. Certain triggers including allergens can provoke an acute asthma attack through the activation of different subsets of leukocytes and the release of cytokines. Mast cells, T cells, and a new type of leukocyte termed type 2 innate lymphoid cells (ILC2), in the lung secrete cytokines and leukotrienes^{2, 3}. Interleukins are predominantly messenger molecules that act on and are released by leukocytes⁴. The list of interleukins is extensive⁴, and we will focus on IL-5, IL-13, and IL-33, as these three interleukins have been shown to be important in the pathogenesis and exacerbation of asthma as well as either being produced by or activating type 2 innate lymphoid cells.

Discovery and function of type 2 innate lymphoid cells

In 2010, Moro et al. discovered and characterized a novel population of cells in murine adipose tissue; they termed these cells ‘natural helper cells’ due to their lack of antigen specificity which indicates an effector cell of the innate immune system⁵. The authors reported this population to be morphologically similar to lymphocytes (small cells with relatively little cytoplasm) and functionally similar to T helper cells, especially T_H2 polarized T helper cells. In addition, a microarray performed on these natural helper cells revealed that the T_H2 cytokines, IL-4, IL-5, and IL-13, were more heavily expressed than other cytokines, resembling T_H2 type T helper cells⁵. After this initial discovery, other studies have found similar populations of cells with many of the same characteristics and functions as natural helper cells and include nuocytes⁶ and innate helper type 2 cells⁷. These subpopulations are now referred to as type 2

innate lymphoid cells (ILC2) by consensus⁸. Since the discovery of type 2 innate lymphoid cells, ILC2 have been detected in several different anatomical locations including peripheral blood, lungs, mediastinal lymph nodes, intestinal tract, and mesenteric fat^{5, 6, 7, 9, 10, 11}.

Inflammatory Mediators

Since the discovery of ILC2, many studies have looked at the role of ILC2 in asthma, and in particular have focused on several inflammatory mediators that either stimulate and activate ILC2 or are produced by ILC2. The most important mediators for ILC2 are summarized below.

Interleukin-33 (IL-33) has been widely implicated in allergic disease^{12, 13}. IL-33 is a somewhat novel class of interleukin, in that it is considered an alarmin¹⁴ being mostly produced in airway epithelial cells¹⁵. Upon tissue damage, IL-33 is released by the epithelium where it binds to T1/ST2, a heterodimeric IL-33 receptor found on the extracellular surface of type 2 innate lymphoid cells¹⁵. Studies have shown that mice challenged with allergens such as the spores of the fungal allergen *Alternaria alternata*, show increased levels of IL-33 in the airway and increased proliferation of ILC2 and production of the T_H2 cytokines, interleukins 5 and 13¹⁶. Other studies have shown that IL-33 is the more potent than IL-25, another activating factor of type 2 innate lymphoid cells¹⁷. IL-33 is a major cytokine involved in the pathogenesis of asthma, and studies have shown the proportionality of serum IL-33 levels and severity of asthma¹⁸.

After activation by IL-33 via the ST2 receptor, ILC2 produce large amounts of IL-5 and IL-13⁵. Interleukin-5 (IL-5) is a classical allergic cytokine important in

asthma¹⁹, primarily responsible for the recruitment, proliferation, growth, and survival of eosinophils²⁰. Eosinophils are cells containing granules filled with toxic proteins, the most important of which is major basic protein, that are primarily responsible for clearing helminthic worm infections, but are also heavily implicated in allergic disease and asthma in particular²¹. One study has detected major basic protein in necrotic sections of bronchial walls, suggesting that proliferation of eosinophils and release of toxic products contributes to damaged epithelium and the pathogenesis of asthma²².

Interleukin-13 is another important cytokine released by ILC2 following activation by IL-33. IL-13 has been found to play a diverse role in asthma pathology, with studies reporting its importance in airway hyperresponsiveness²³, or ease of contractility of smooth muscle found around the airway, and mucus production²⁴. These changes are important in the pathogenesis and are present during asthma attacks.

Apart from the interleukins, the cysteinyl leukotrienes represent another important class of inflammatory mediators. The cysteinyl leukotrienes are lipid inflammatory mediators derived from arachidonic acid and include LTC₄, LTD₄, and LTE₄. The pathway involves conversion to leukotriene A₄ by 5-lipoxygenase and then subsequently into leukotriene C₄ by LTC₄ synthase²⁵. LTD₄ and LTE₄ are breakdown products of LTC₄, with LTE₄ being the most stable of the leukotrienes²⁵, and is detectable in the urine of asthmatic patients^{26, 27}. The cysteinyl leukotrienes are known to be bound by two G protein-coupled receptors, CysLT₁R and CysLT₂R. CysLT₁R exhibits the highest affinity for LTD₄, moderate affinity for LTC₄, and the lowest affinity for LTE₄²⁸. CysLT₂R binds LTC₄ and LTD₄ with equal affinity, and

CysLT2R has low affinity for LTE4²⁹. Interestingly, recent studies have shown that CysLT2R can have an inhibitory effect upon interaction with CysLT1R in mast cells and dendritic cells³⁰. Despite an overlap in ligands, the two cysteinyl leukotriene receptors have an interesting relationship that perhaps could be exploited for therapeutic gain. Also of great interest is characterization of an unknown receptor for LTE4. LTE4 has been reported to signal through several different receptors, including CysLT1R, a receptor named CysLT_ER, the newly identified cysteinyl leukotriene receptor 3 (CysLT3R) also known as GPR99, and the purinergic receptor P2Y12^{31, 32},³³. The cysteinyl leukotrienes are important mediators of asthma pathology. Several studies have reported the cysteinyl leukotrienes as being potent bronchoconstrictors in humans³⁴ and have found that mice deficient in either LTC4 synthase or CysLT1R show decreased airway hyperresponsiveness and diminished bronchoconstriction^{35, 36}, indicating the importance of the cysteinyl leukotrienes in bronchoconstriction in murine models of asthma. Leukotrienes are a major contributor to the pathology of asthma, thus a major therapeutic target in human asthma is the blockade of CysLT1R by the pharmacologic agent montelukast³⁷.

The aims of these studies are the elucidation of the role of cysteinyl leukotrienes in type 2 innate lymphoid cell activation, the role of the CysLT1R and CysLT2R receptors in ILC2 regulation, as well as potential identification of other receptors for LTE4 present on type 2 innate lymphoid cells. Studies have shown that two possible LTE₄ receptors exist including purinergic receptor P2Y12³¹ and the G-protein coupled receptor 99 (GPR99)³². LTE4 is the most stable and longest lasting of the leukotrienes³⁸, with some recommendations that it be used to monitor asthma³⁹.

Therefore it is of great clinical importance that other pathways through which LTE4 can act, including via ILC2 activation, be identified so that new treatment options for asthma can be researched and implemented.

MATERIALS AND METHODS

Mice

6 week to 8 week old, male and female wild-type C57BL/6, RAG2 knockout, STAT6 knockout, and IL-7R knockout mice were obtained from Jackson Laboratories (Bar Harbor, ME) and bred in house. All knockout mouse strains were bred on a C57BL/6 background.

Intranasal Administration and Pharmacological Treatment

In some experiments, 25µg of the fungal allergen *Alternaria alternata* (Greer, NC) was suspended in 40µL PBS and administered via the intranasal route, either once, on days 0, 3, and 6 of an 8 day protocol, or on days 0, 3, 6, and 9 of a 10 day protocol. Mice were euthanized either 12 hours, 24 hours, or 3 days after receiving a single administration of *Alternaria alternata*, on day 8 of the 8 day protocol, or on day 10 of the 10 day protocol.

In some experiments, wild-type mice received 100ng of the leukotrienes C4, D4, and E4 (Cayman Chemical, Ann Arbor, MI) in 2.5% ethanol and were euthanized 3 hours later. In some experiments, mice received either 0.2mg montelukast (Cayman Chemical, Ann Arbor, MI) in 1% DMSO or PBS in 1% DMSO intragastrically for two days prior to challenge and once two hours before challenge for a total of three times. All animal studies were approved by the University of California, San Diego Institutional Animal Care and Use Committee.

BAL and Lung Processing

Airways were flushed six times with 0.5 mL of BSA (Sigma Aldrich) and collected. The resulting BAL was then washed and centrifuged for 4 minutes at 4°C

and 1400 RPM (Beckman Coulter). Following centrifugation, the resulting cell pellet was resuspended in 500 μ L RPMI (Gibco, Life Technologies).

Lungs were collected and then processed using the gentleMACS lung digestion system according to the manufacturer's protocol (Miltenyi Biotec).

Flow Cytometry

After obtaining single-cell lung suspension, cell density was analyzed using an Accuri C6 Flow Cytometer (BD Biosciences) and used to calculate total numbers of cells. Following cell density analysis, Fc receptors were first blocked with an anti-mouse CD16/32 antibody (BioLegend, San Diego, CA) for 10 minutes at 4°C. After blocking, lung cells were stained with anti-mouse CD45.2 conjugated to PerCP (eBioscience, San Diego, CA), anti-mouse Thy1.2 conjugated to APC (eBioscience, San Diego, CA), and Lineage cocktail (BioLegend, San Diego, CA), anti-mouse TCR β , anti-mouse TCR $\gamma\delta$, anti-mouse CD11c, anti-mouse Fc ϵ R1, anti-mouse Nk1.1, and anti-mouse CD5 all conjugated to FitC to identify type 2 innate lymphoid cells as lineage-negative CD45⁺ Thy1.2⁺ cells. Expression of CysLT1R on ILC2 was analyzed using a anti-mouse CysLT1R antibody kindly provided by Dr. Joshua Boyce (Harvard), followed by a PE-conjugated rabbit F'ab antibody. Expression of P2Y12 on ILC2 was assessed using an anti-mouse P2Y12 antibody (Alomone Labs, Jerusalem). Expression of GPR99 on ILC2 was assessed using an anti-mouse GPR99 antibody (Bioss, Boston, MA). To assess ILC2 proliferation, following surface staining, cells were permeabilized and fixed using the FoxP3 Staining Buffer Set according to the manufacturer's protocol (eBioscience, San Diego, CA). After permeabilization and fixation, cells were stained intracellularly with anti-mouse/rat KI-67 conjugated to

eFluor 660. To assess cytokine production in ILC2, following surface staining, cells were permeabilized and fixed using the BD intracellular staining kit according to the manufacturer's protocol (BD Biosciences). Cells were stained intracellularly with either a PE-conjugated Rat IgG1 isotype control antibody (eBioscience, San Diego, CA), a PE-conjugated anti-mouse IL-5 antibody, or a PE-conjugated anti-mouse IL-13 antibody. All stains were done at 4°C for 30 minutes and analyzed using an Accuri C6 Flow Cytometer (BD Biosciences).

Cell Sorting

Prior to sorting, ILC2 were expanded *in vivo* after mice were given 25µg intranasal *Alternaria alternata* 3 to 4 times over 10 days. CD45 positive, lineage negative, Thy1.2 positive ILC2 were sorted using an Aria cell sorter (BD Biosciences).

Cell Culture

After sorting ILC2, 50,000 cells were cultured per well in a 96-well, flat-bottom plate. Prior to use, sorted ILC2 were cultured with 10ng/mL IL-2 and IL-7 (R&D Systems). After 48 hours, media was changed and ILC2 were cultured with either IL-33 (30ng/mL, R&D Systems) or LTC₄, LTD₄, LTE₄ (all at either 10⁻⁶ or 10⁻⁸ mol/L, Cayman Chemical, Ann Arbor), or media. In some experiments, sorted ILC2 were cultured with either ADP (MP Biomedicals) or 2-MeSADP (Tocris Bioscience, UK) In some wells, montelukast was added 2 hours prior to culture with cysteinyl leukotrienes. Six hours post culture, plates were centrifuged for 5 minutes at 1500rpm, and supernatants were collected for ELISA analysis.

Calcium Signaling

Analysis of calcium signaling in ILC2 was conducted with sorted ILC2 using the Fluo-4 Direct Calcium Assay Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol in conjunction with an Accuri C6 Flow Cytometer (BD Biosciences). In some cases, purified ILC2 were incubated with montelukast for 2 hours prior to analysis at 37°C.

ELISA

ELISA analysis was performed on BAL samples and cell culture supernatants using IL-4, IL-5, and IL-13 DuoSets (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. A model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA) was used to read the plates and Microsoft Excel was used to analyze data.

RT-PCR and qPCR

RNA was isolated from sorted ILC2 using TRIzol (Life Technologies). cDNA was reverse transcribed from the isolated RNA using the Transcriptor First Strand cDNA synthesis kit (Roche, Mannheim, Germany). After obtaining cDNA, diluted cDNA with SYBR Green I Master (Roche) and the primers: **CysLT1R forward**, 5'-CAA CGA ACT ATC CAC CTT CACC-3'; **CysLT1R reverse**, 5'-AGC CTT CTC CTA AAG TTT CCAC-3'; **L32 forward**, 5'-GAA ACT GGC GGA AAC CCA-3'; and **L32 reverse**, 5'-GGA TCT GGC CCT TGA ACC TT-3'. Data is shown as compared to the housekeeping gene L32.

Statistical Analysis

Statistical analysis was performed on all experiments using GraphPad Prism Software (La Jolla, CA). In most cases the Mann-Whitney test was used, in some

experiments the unpaired t-test was used, p values of <0.05 were considered statistically significant.

RESULTS

***Alternaria alternata* induces IL-33 release and is a potent activator of the innate immune system**

Alternaria alternata is a fungal allergen known to play a role in the sensitization and exacerbation of human asthma⁴⁰, even being linked to the development of more severe asthma⁴¹. In mouse models of asthma, *Alternaria* is frequently used along with several other fungal allergens and non-fungal allergens such as house dust mite. We have shown previously that *Alternaria* induces a rapid and robust innate response characterized by airway eosinophilia and type 2 cytokines, significantly more than *Aspergillus*, *Candida*, and house dust mite^{16, 42}. In addition, this innate response is driven by IL-33¹⁶, a known activator of ILC2^{17, 43}. In contrast to *Alternaria*, the allergens *Aspergillus*, *Candida*, and house dust mite did not induce IL-33. Previously, our group has shown that intranasal administration of *Alternaria* leads to rapid ILC2 cytokine production in as little as 3 hours¹⁶. With such a robust change in ILC2 brought about by *Alternaria* challenge, we were interested in identifying changes in gene expression in ILC2. We performed an mRNA expression array on sorted ILC2 from PBS and *Alternaria*-challenged ILC2. Differences in gene expression are calculated relative to PBS challenged ILC2 (Fig 1B). Strikingly, the cysteinyl leukotriene receptors were among the most upregulated mRNA transcripts and higher than IL-5. Thus, we set out to confirm expression of CysLT receptors on ILC2 and whether there was functional significance.

ILC2 express the cysteinyl leukotriene receptor 1 on the plasma membrane

Following the detection of high levels of CysLT1R, cysteinyl leukotriene receptor 1, transcripts on our ILC2 mRNA array, we sought to elucidate a potential role for the cysteinyl leukotrienes in ILC2 biology. Our first aim was to determine if the receptor was expressed on ILC2. To accomplish this, flow cytometric analysis was performed on whole lung from WT, RAG2 knockout, and STAT6 knockout mice. ILC2 CysLT1R expression was analyzed on naïve mice as well as after 24 hour, 3 day, and 10 day allergen challenge protocols (Fig 2A, 2B, 24 hour and 3 day protocol data not shown). The expression of CysLT1R is not upregulated or downregulated following allergen challenge, nor is it dependent on RAG2 indicating that adaptive immunity was not required for ILC2 expression. An earlier report showed that CysLT1R was upregulated following type 2 cytokine signaling by IL-4 and IL-13 via phosphorylation of STAT6 in human monocytes and macrophages⁴⁴. We did not detect a reduction in ILC2 CysLT1R expression in naïve STAT6 knockout mice or after a 10 day allergen challenge protocol (Fig. 2A, 2B). Further, ILC2 had higher levels of CysLT1R mRNA compared with conventional T_H2 cells (CD4⁺ T1/ST2⁺) (Fig 2C). Quantitative PCR was also done to detect whether or not CysLT2R, the second of the cysteinyl leukotriene receptors, is present on ILC2. The results of the PCR are less clear and seem to indicate that CysLT2R is not as highly expressed as CysLT1R (Fig 2D). Taken together, these findings suggest that cysteinyl leukotrienes could play a major role in ILC2 activation.

The cysteinyl leukotrienes induce ILC2 cytokine production *in vitro*

To assess a role for leukotrienes in ILC2 activation, mice were challenged 3 times over 7 days with *Alternaria alternata* to induce ILC2 expansion *in vivo* prior to

fluorescence-activated cell sorting. After sorting, a purity of at least 97% was obtained in all instances (Supplementary Fig 1). ILC2 were cultured with IL-33, LTD4, or LTD4 and Montelukast, and repeated with LTE4. The cysteinyl leukotrienes, LTD4 and LTE4 induced IL-5 and IL-13 production to the same degree as IL-33, a known activator of ILC2 (Fig 3A, 3C). Further, a calcium signaling assay showed that addition of LTD4 or LTE4 results in calcium influx. Blockade of CysLT1R with montelukast prevents signaling and calcium influx (Fig 3B, 3D). Remarkably, LTD4 also induced IL-4 production (Fig 3A). IL-33 is not known to induce IL-4¹⁶; therefore the capability of LTD4 to induce production of IL-4 could lead to a broader role of ILC2 in allergic responses.

The cysteinyl leukotrienes induce ILC2 cytokine production *in vivo*

Following *in vitro* cell culture, we sought to identify whether leukotrienes could induce ILC2 activation *in vivo*. Wild-type mice were challenged one time with 100ng of leukotriene, LTC4, LTD4, or LTE4 and sacrificed 3 hours later. In all experiments, a subset of mice received intragastric montelukast twice before initial challenge. Mice receiving a leukotriene without montelukast showed increased IL-5 production, while the increased IL-5 production is prevented upon addition of montelukast, except in the case of LTE4 (Fig 4A). CysLT1R exhibits a higher affinity for LTD4 and LTC4 than it does for LTE4; another receptor with which LTE4 has a higher affinity could explain why LTE4 still seems to activate ILC2 even in the presence of a CysLT1R antagonist. Recently, studies have shown that LTE4 can bind two additional receptors, P2Y12 and GPR99. GPR99 has been referred to as the third cysteinyl leukotriene receptor, or CysLT3R³². The presence of P2Y12, GPR99, or both

on the surface of ILC2 could explain why LTE4 was able to activate ILC2 in the presence of a CysLT1R antagonist. To test this possibility, we analyzed the expression of both P2Y12 and GPR99 on ILC2. We found that ILC2 strongly express P2Y12, and to a lesser extent express GPR99 (Supplementary Fig 3). The P2Y receptors are a family of purinergic receptors that exhibit binding affinity for extracellular nucleotides; in particular these receptors have been shown to be important in platelet activation, platelet clumping, and immune cell signaling^{45, 46, 47, 48}. With recent reports demonstrating the importance of P2Y receptors in immune cells, we were interested in seeing what effects stimulation of P2Y12 would have on ILC2. Interestingly, after culture with ADP and 2-MeSADP, two potent agonists of P2Y12, ILC2 had increased IL-5 production (Supplementary Fig 3).

The administration of the fungal allergen *Alternaria alternata* along with exogenous cysteinyl leukotrienes potentiates ILC2 activation and proliferation

We wanted to assess the role or contribution of leukotrienes in an *Alternaria alternata* model of asthma. Challenge with 100µg *Alternaria alternata* leads to production of cysteinyl leukotrienes that is dependent on the transcription factor STAT6 (Supplementary Fig. 2) supporting previously known induction of CysLTs by allergens⁴⁹. We next used RAG2 knockout mice to isolate the effects of cysteinyl leukotrienes on innate lymphocytes independent of adaptive immunity. IL-7R knockout mice that lack innate lymphocytes were used as controls for levels of eosinophil infiltration after leukotriene and allergen challenges. After challenge with *Alternaria alternata* alone, ILC2 proliferated and expanded in RAG2 knockout mice (Fig 5A, 5B). After allergen challenge and exogenous cysteinyl leukotriene

administration, ILC2 activation and proliferation (Fig 5B, 5C), as well as the resultant eosinophilia (Fig 5D) were further increased above *Alternaria* alone, indicating that cysteinyl leukotrienes can amplify the ILC2 response to allergen. Furthermore, response to both allergen and cysteinyl leukotriene occurred in RAG2 knockout mice ruling out any possibility of T cell interaction or T cell generated responses.

DISCUSSION

These studies have shown that following allergen challenge, the cysteinyl leukotriene receptor 1 mRNA is upregulated, more so than IL-5, a known product of ILC2. Type 2 innate lymphoid cells express the cysteinyl leukotriene receptor 1, CysLT1R, constitutively at protein level, and are activated by the cysteinyl leukotrienes *in vivo* and *in vitro*. Activation of ILC2 by leukotriene D4 induces IL-4 production in ILC2, in addition to IL-5 and IL-13. Additionally, allergen challenge with *Alternaria alternata* as well as administration of exogenous leukotrienes led to an amplification in ILC2 activation and proliferation. Further, the resultant eosinophilia was shown in RAG2 knockout mice, indicating the potential of ILC2 to amplify immune responses independent of adaptive immunity. The activation of type 2 innate lymphoid cells by cysteinyl leukotrienes is novel and presents a new mechanism of both ILC2 activation and of lipid-mediated allergic inflammation.

The activation of ILC2 by cysteinyl leukotrienes presents a novel mechanism of allergic disease. Previously, ILC2 were known to be activated by IL-25 and IL-33^{17, 50, 51}, two interleukins derived from the airway epithelium that are important in allergic inflammatory diseases. Our novel findings that ILC2 express the cysteinyl leukotriene receptor 1 (and possibly CysLT2R) present another mechanism by which ILC2 can be activated. Cysteinyl leukotrienes are derived from arachidonic acid and are primarily produced by macrophages, mast cells, eosinophils, and basophils³⁸. The fact that ILC2 respond so readily to early mediators of inflammation, the alarmin cytokine IL-33 as well as cysteinyl leukotrienes, would suggest that ILC2 are early amplifiers of inflammation.

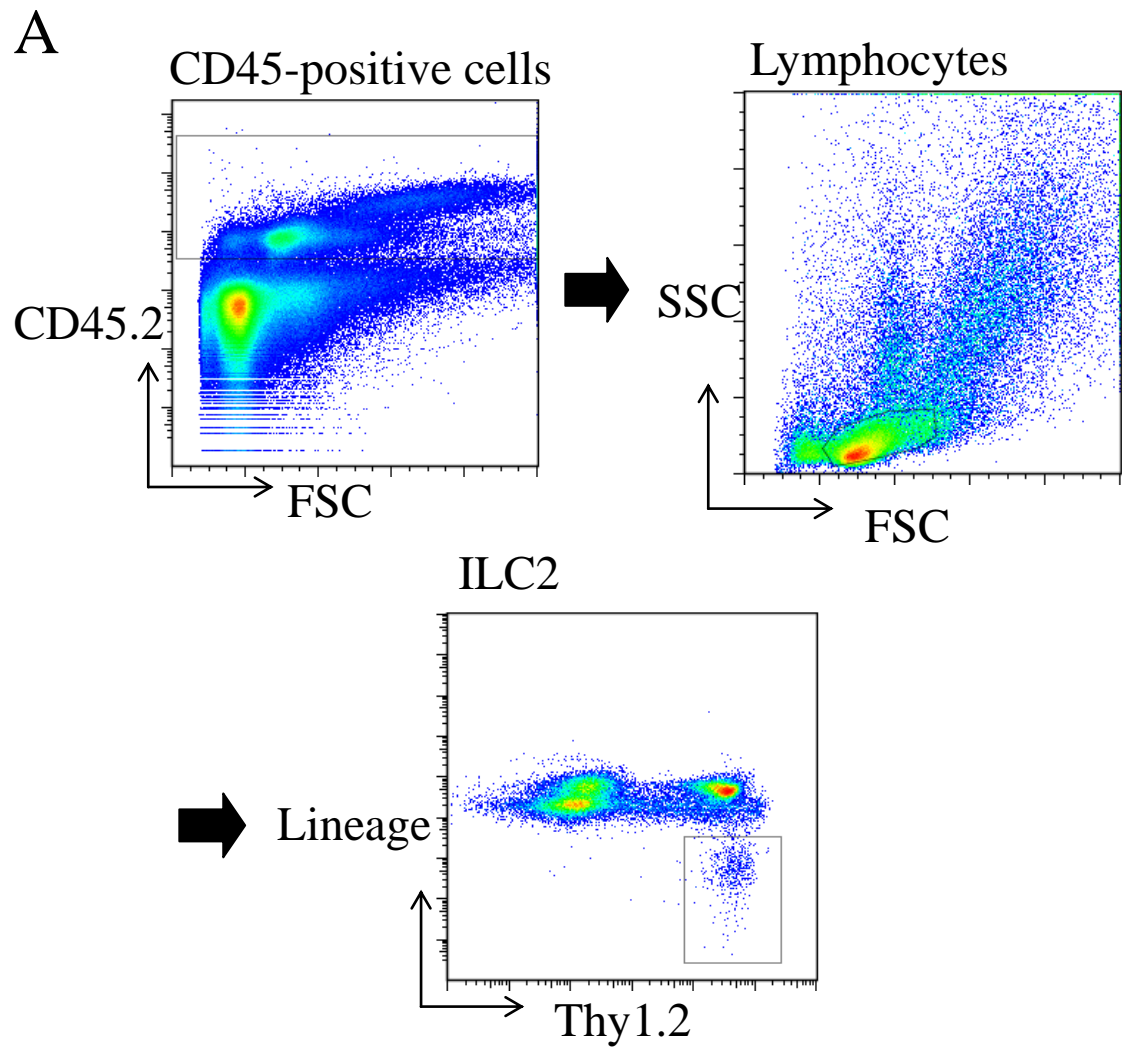
One of the major targets of asthma therapeutics is the cysteinyl leukotriene pathway and its receptors^{52,53}. Thus, the finding that cysteinyl leukotrienes prolong inflammatory responses by the activation of ILC2 provides additional insight into asthma pathogenesis in addition to the known role of CysLTs in bronchoconstriction³⁴. Additionally, further elucidation of the novel LTE4 receptor that may be present on ILC2 may provide an additional pharmacologic target than the current CysLT1R antagonist Montelukast³¹. However, recently a controversy has emerged regarding the putative LTE4 receptor. In 2009, Parachuri et al. reported that the purinergic receptor P2Y12 is necessary for pulmonary inflammation mediated by LTE4³¹. Further, Parachuri et al. showed that although P2Y12 is necessary for development of inflammation, P2Y12 does not directly bind to LTE4 and may form a complex with another receptor that allows it to recognize LTE4³¹. Using gene deficient mice, the authors showed that *in vivo* responses to exogenous LTE4 including eosinophilia, goblet cell metaplasia, and IL-13 production were dependent on P2Y12, but not CysLT1R and CysLT2R. This suggests that P2Y12 is necessary for LTE4 mediated inflammation³¹. In 2013, Foster et al. found that P2Y12 was not involved in LTE4 signaling. The author utilized a human embryonic kidney cell line, human platelets, and Chinese hamster ovary cells to assess the relationship between LTE4 and the P2Y12 receptor. The study evaluated a number of different markers of G protein-coupled receptor activation including calcium influx, β -arrestin signaling, and cAMP signaling. LTE4 did not induce any change consistent with G protein-coupled receptor activation. While the receptor for LTE4 may remain unknown, the importance of LTE4 is beginning to be understood. LTE4 is the most available cysteinyl leukotriene

in tissues and the elucidation of the receptor for LTE4 will likely provide invaluable insight into mechanisms of asthma and allergic inflammation.

Our findings have prompted additional questions that remain to be addressed. As mentioned, LTE4 is perhaps the most important leukotriene clinically as it is the most stable leukotriene³⁸. Further investigation is needed to determine whether LTE4 induces a similar response when bound to P2Y12, GPR99, or CysLT1R on ILC2, and the relative potency of LTE4 when bound by each receptor. Additionally, the presence of P2Y12 on the surface of ILC2 would seem to indicate that extracellular ADP, the natural ligand of the purinergic receptor P2Y12, could also play a role in ILC2 regulation. We have shown that culturing both sorted ILC2 and whole lung cells with ADP led to IL-5 production in ILC2. Recent studies have focused on the role of danger-associated molecular patterns (DAMPs) in innate immune cell activation. ADP is one such DAMP that could further characterize ILC2 as early regulators of an immune response based on activation by a variety of cytokines, inflammatory molecules, and seemingly innocuous cellular materials.

APPENDIX: FIGURES

Figure 1. *Alternaria alternata* induces changes in gene expression in ILC2. (A) Parent gating of ILC2, gating shown on *Alternaria* challenged murine lung cells. Gating is representative of all experiments. (B) mRNA array performed on naïve and *Alternaria* challenged ILC2, the red region indicates upregulation, the green region indicates downregulation, and the blue region is no change in regulation.



B

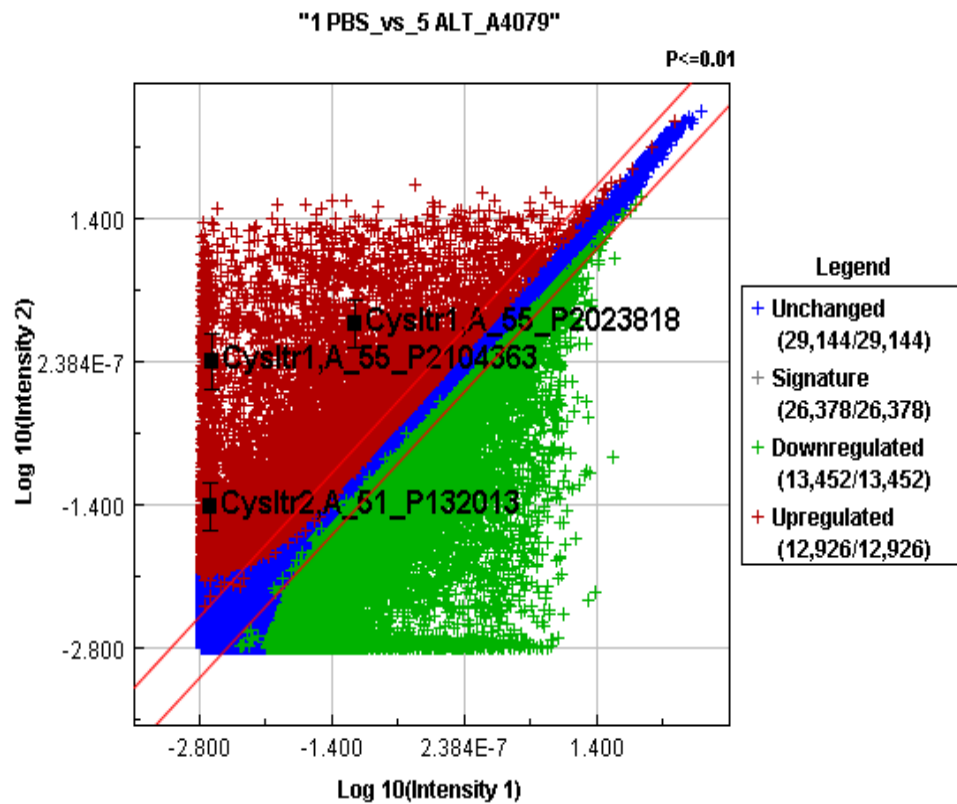


Figure 1. continued

Figure 2. CysLT1R is expressed on the surface of type 2 innate lymphoid cells. (A) Expression of CysLT1R on naïve wild-type, RAG2 knockout, and STAT6 knockout mice analyzed by flow cytometry. Gated on Lineage negative, thy1.2 positive ILC2. Gray shaded curve is the isotype control. (B) Expression of CysLT1R on wild-type, RAG2 knockout, and STAT6 knockout mice after a 10 day intranasal *Alternaria alternata* protocol, analyzed by flow cytometry. Gated on CD45 positive, Lineage negative, thy1.2 positive ILC2. Gray shaded curve is the isotype control. (C) Levels of CysLT1R mRNA as compared to the L32 housekeeping gene in ILC2, CD4+/T1ST2+ cells, and CD4+/T1ST2- cells. (D) Levels of CysLT2R mRNA as compared to the L32 housekeeping gene in ILC2, CD4+/T1ST2+ cells, and CD4+/T1ST2- cells. qPCR was performed on sorted populations. * indicates a p-value of less than 0.05, Mann-Whitney U Test.

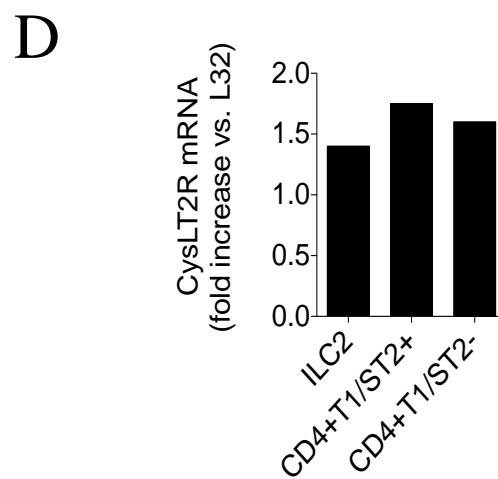
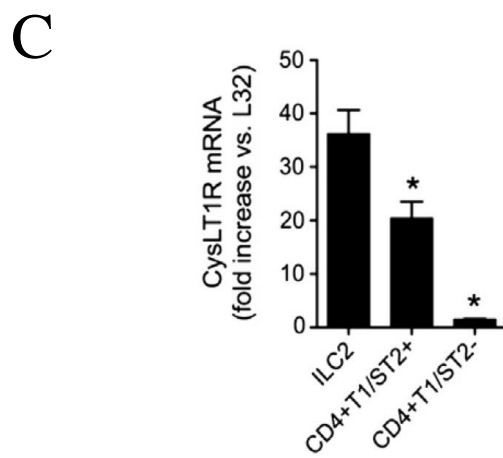
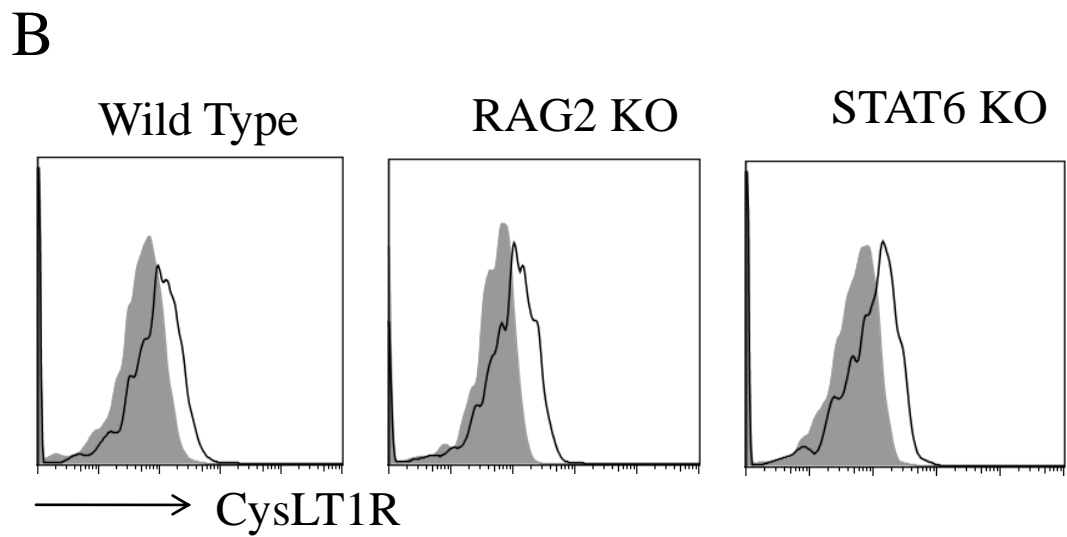
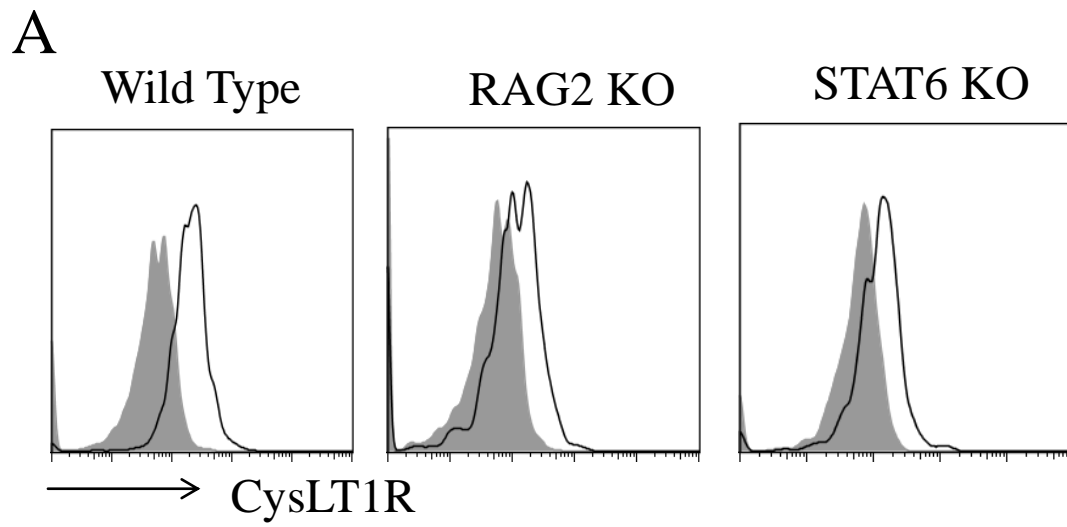
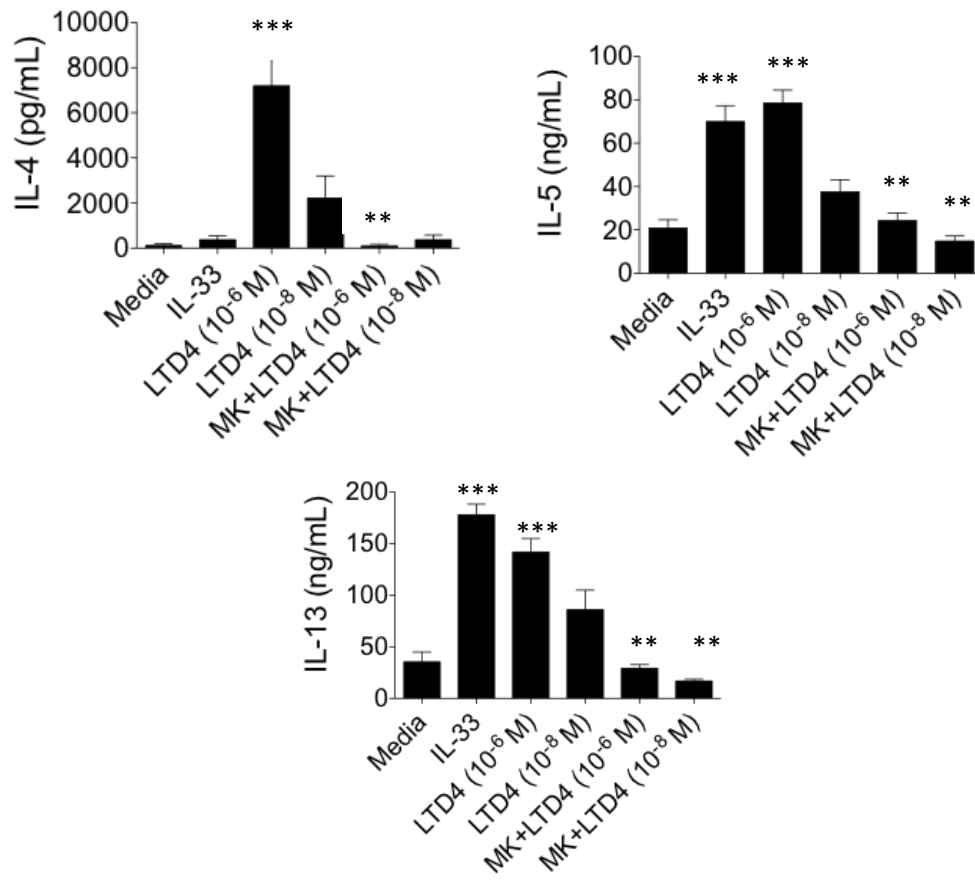
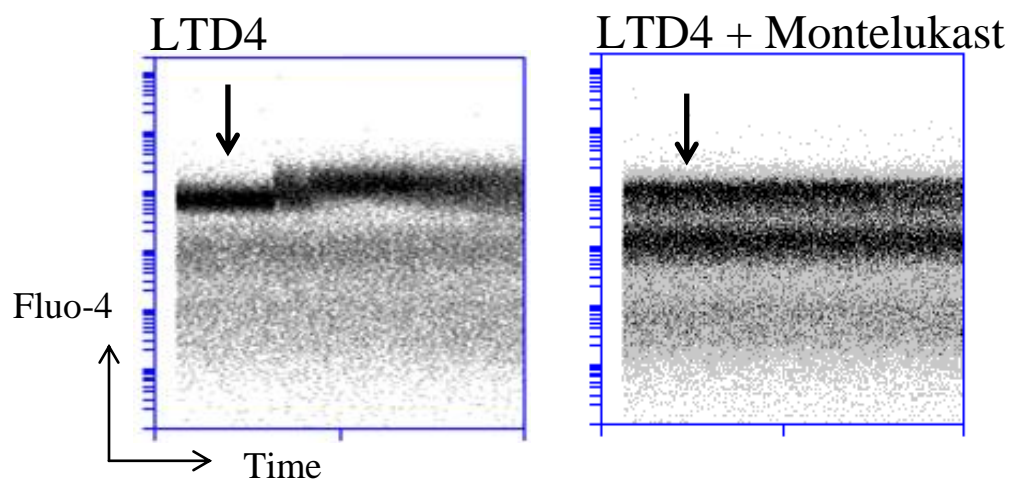


Figure 3. Purified ILC2 cultured with the cysteinyl leukotrienes results in production of type 2 cytokines. (A) Concentration of IL-4, IL-5, or IL-13 after culture with IL-33, LTD4, or LTD4 with montelukast. (B) Calcium signaling assay performed on sorted ILC2, stimulated by LTD4 with and without blockade of CysLT1R. Addition of LTD4 marked by arrow. (C) Concentration of IL-4, IL-5, or IL-13 after culture with IL-33, LTE4, or LTE4 with montelukast. Unpaired t test used to assess statistical significance in IL-4 and IL-13 ELISA (D) Calcium signaling assay performed on sorted ILC2, stimulated by LTE4 with and without blockade of CysLT1R. Addition of LTE4 marked by arrow. * indicates a p-value of less than 0.05, Mann-Whitney t Test. * indicates a p-value of less than 0.05, ** indicates a p-value of less than 0.01, *** indicates a p-value of less than 0.005, Mann-Whitney U Test was used unless otherwise noted above.

A



B



C

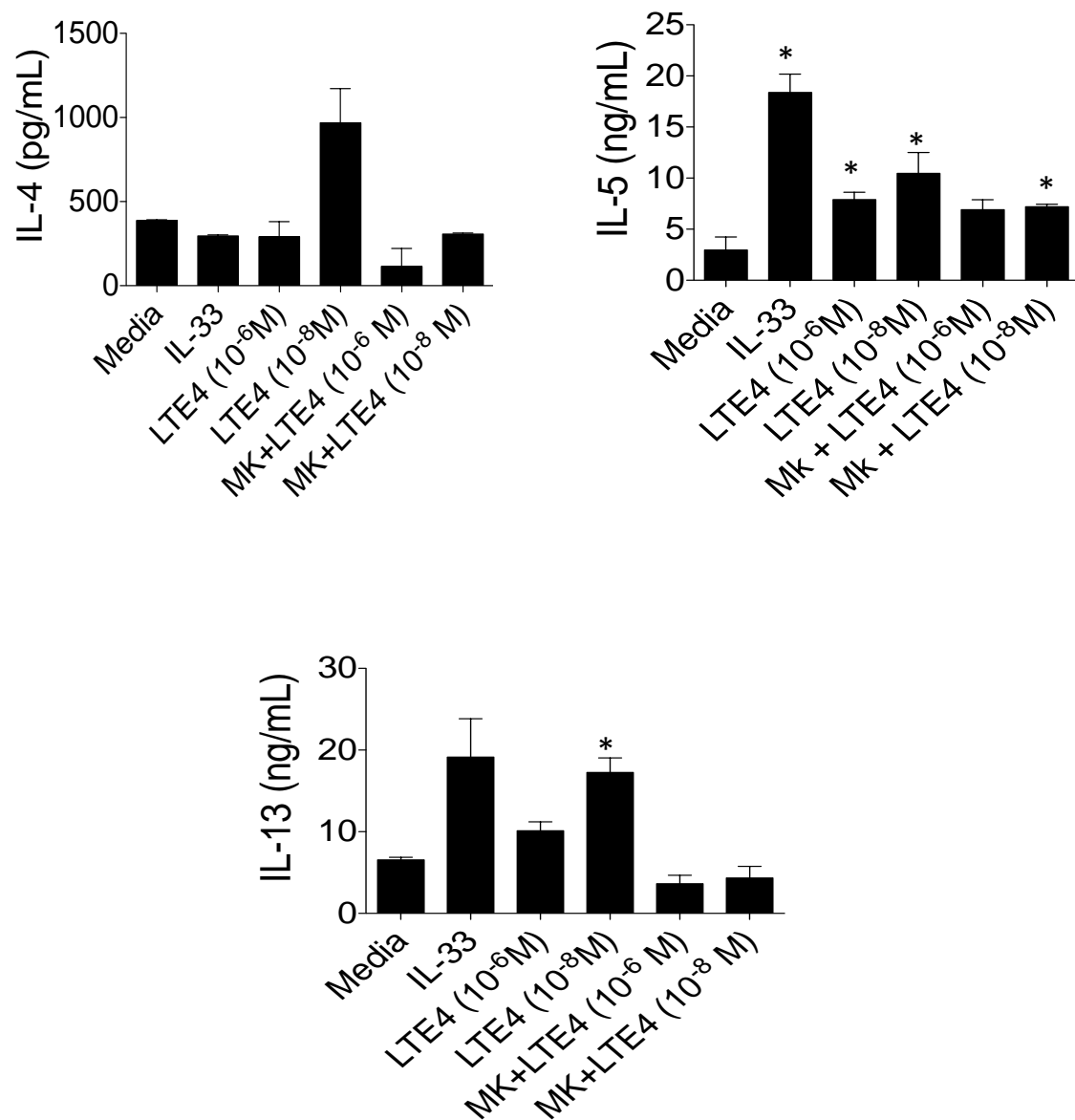


Figure 3. continued

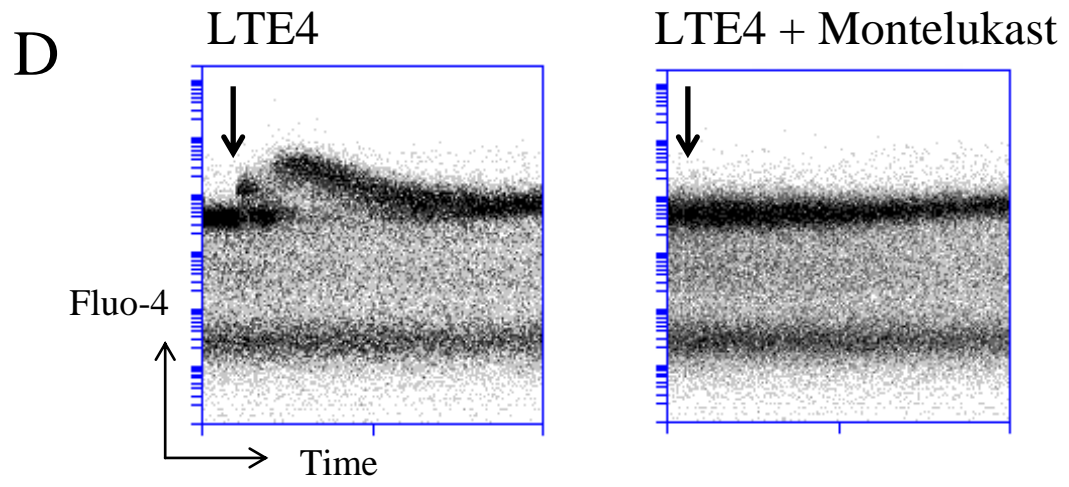


Figure 3. continued

Figure 4. Exogenous leukotrienes induce ILC2 IL-5 production *in vivo* (A)

Intracellular staining for IL-5 production in ILC2. Wild type mice were given 0.2mg Montelukast or DMSO intragastrically on days -2, -1, and 0, then subsequently challenged on day 0 one time with 100ng of LTC₄, LTD₄, or LTE₄ and were sacrificed three hours later.

A

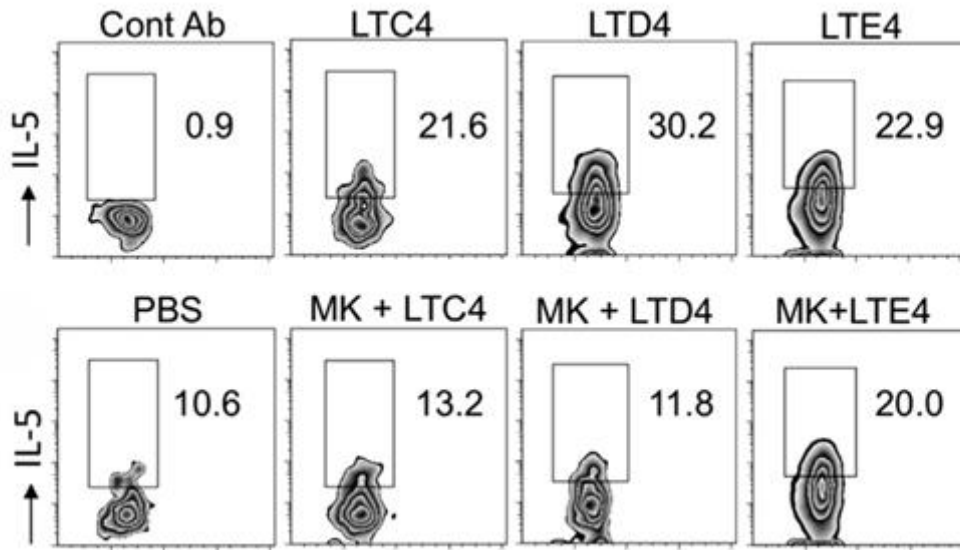
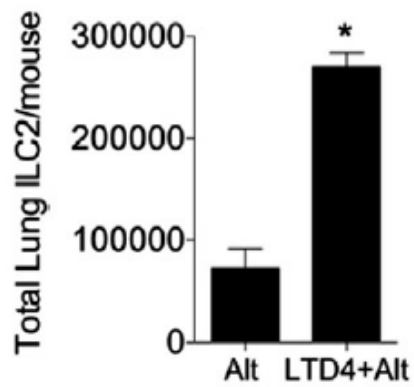
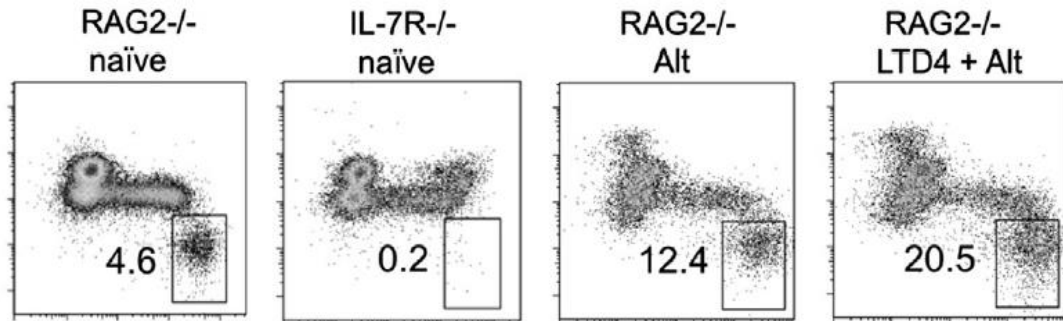
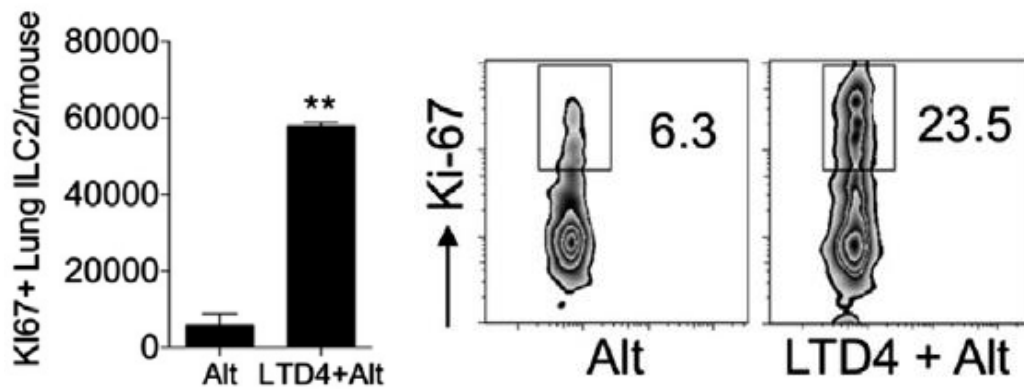


Figure 5. Leukotriene D4 potentiates *Alternaria*-induced ILC2 proliferation *in vivo* independent of adaptive immunity. (A) Expansion of ILC2 following *Alternaria alternata* challenge alone or *Alternaria alternata* challenge with leukotriene D4 in RAG2 knockout and IL-7R knockout mice. (B) ILC2 proliferation analyzed by intracellular staining for Ki67. (C) Total numbers of IL-5+ ILC2 and, (D) eosinophils following *Alternaria alternata* challenge or *Alternaria alternata* challenge with exogenous leukotriene D4 in RAG2 knockout and IL-7R knockout mice. * indicates a p-value of less than 0.05, ** indicates a p-value of less than 0.01, Mann-Whitney U Test.

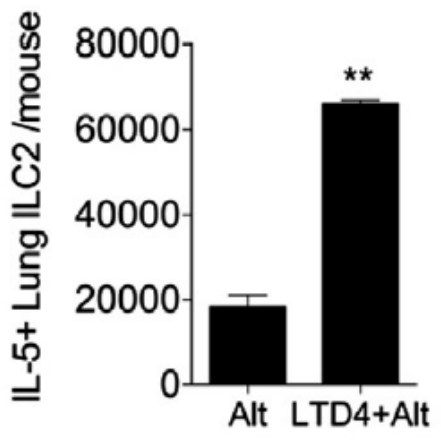
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D

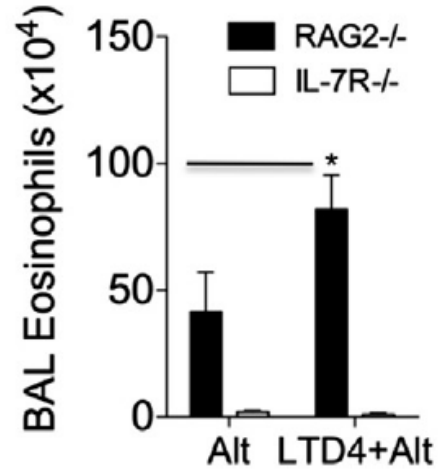


Figure 5. continued

Figure S1. Representative pre-sort and post-sort plots of ILC2. (A) ILC2 staining pre and post sorting. Sorted on CD45+ Lineage negative Thy1.2+ lymphocytes.

A

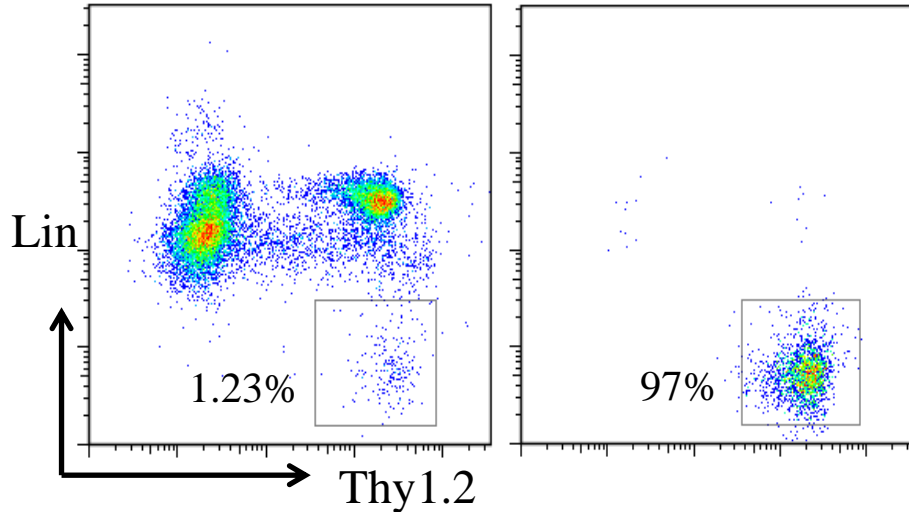


Figure S2. *Alternaria* induces cysteinyl leukotrienes. (A) WT C57BL/6 mice were challenged one time with 100 μ g of *Alternaria alternata* then sacrificed 12 hours later. A cysteinyl leukotriene ELISA was performed on collected BAL. * indicates a p-value of less than 0.05, ** indicates a p-value of less than 0.01, Mann-Whitney U Test.

A

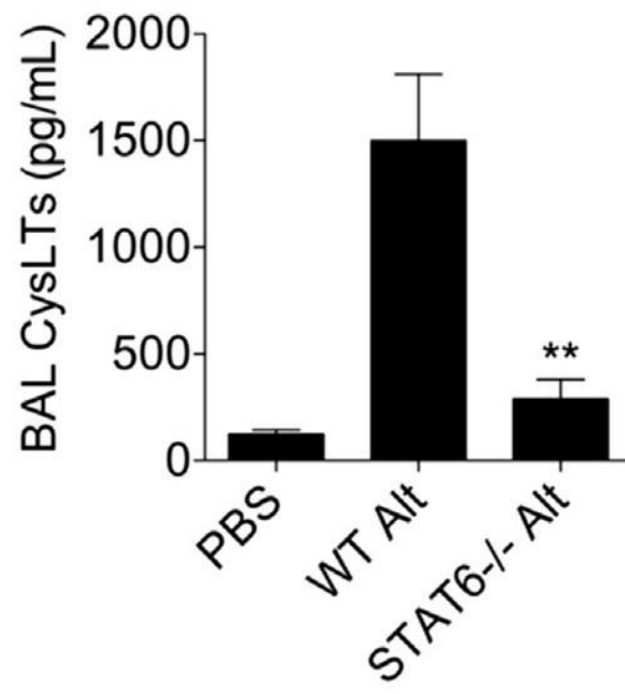
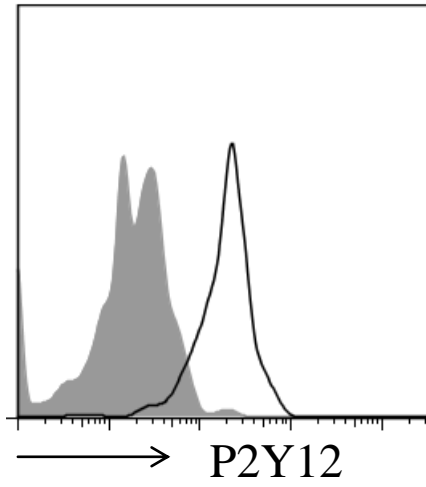
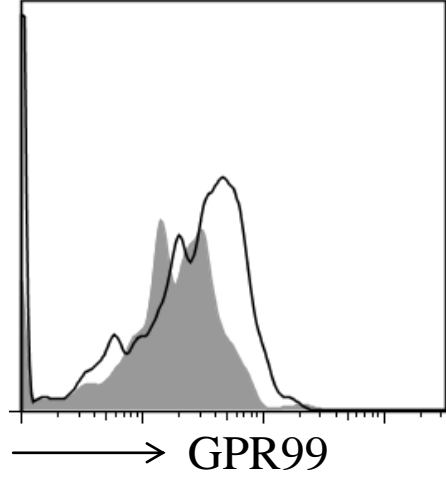


Figure S3. ILC2 express P2Y12 and GPR99, and produce IL-5 in response to ADP stimulation. (A) Expression of P2Y12 on naïve ILC2. (B) Expression of GPR99 on naïve ILC2. Gated on CD45.2 positive, Lineage negative, Thy1.2 positive lymphocytes in panels A and B. (C) Intracellular flow cytometry staining for IL-5 production in ILC2, gated on Lineage negative, thy1.2 positive ILC2. (D) Sorted ILC2 were cultured with either media alone, IL-33, or two concentrations of ADP. Concentrations of IL-5 and IL-13 by ELISA.

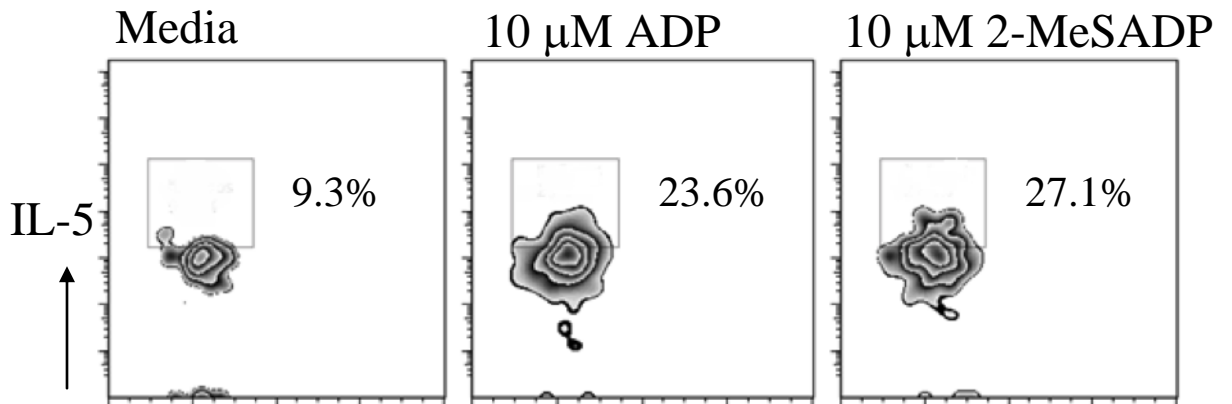
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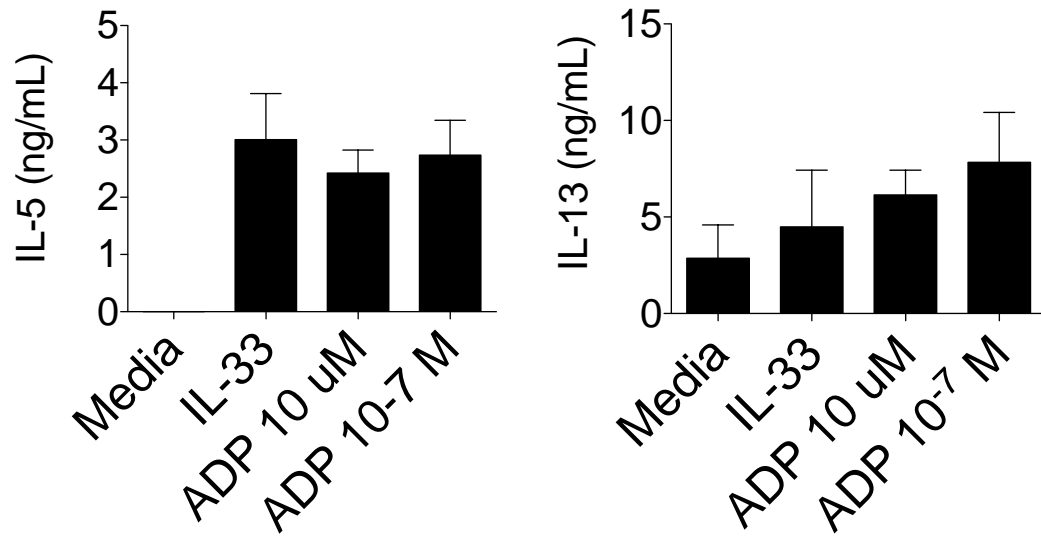


Figure S3. continued

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