

# Leukotrienes provide an NFAT-dependent signal that synergizes with IL-33 to activate ILC2s

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Group 2 innate lymphoid cells (ILC2s) and type 2 helper T cells (Th2 cells) are the primary source of interleukin 5 (IL-5) and IL-13 during type 2 (allergic) inflammation in the lung. In Th2 cells, T cell receptor (TCR) signaling activates the transcription factors nuclear factor of activated T cells (NFAT), nuclear factor  $\kappa$ B (NF- $\kappa$ B), and activator protein 1 (AP-1) to induce type 2 cytokines. ILC2s lack a TCR and respond instead to locally produced cytokines such as IL-33. Although IL-33 induces AP-1 and NF- $\kappa$ B, NFAT signaling has not been described in ILC2s. In this study, we report a nonredundant NFAT-dependent role for lipid-derived leukotrienes (LTs) in the activation of lung ILC2s. Using cytokine reporter and LT-deficient mice, we find that complete disruption of LT signaling markedly diminishes ILC2 activation and downstream responses during type 2 inflammation. Type 2 responses are equivalently attenuated in IL-33- and LT-deficient mice, and optimal ILC2 activation reflects potent synergy between these pathways. These findings expand our understanding of ILC2 regulation and may have important implications for the treatment of airways disease.

## INTRODUCTION

Type 2, or allergic, inflammation in the lung requires the cytokines IL-5, IL-13, and IL-9, which collectively elicit eosinophilia, alternative activation of macrophages, goblet cell hyperplasia, smooth muscle hypercontractility, and tissue remodeling, and may contribute to wound healing (Licona-Limón et al., 2013; Gour and Wills-Karp, 2015). As long-lived tissue-resident cells, group 2 innate lymphoid cells (ILC2s) are a critical early source of these type 2 cytokines before type 2 helper T cell (Th2 cell) recruitment. The role of ILC2s in promoting type 2 lung inflammation has been established in mice using models such as parasitic worm infection, chitin, papain, fungal, or house dust mite challenge, and allergic asthma (Neill et al., 2010; Price et al., 2010; Barlow et al., 2012; Bartemes et al., 2012; Halim et al., 2012; Klein Wolterink et al., 2012; Van Dyken et al., 2014). In humans, ILC2s accumulate in nasal polyps of patients with chronic sinusitis (Mjösberg et al., 2011; Ho et al., 2015), and genome-wide association studies have implicated the ILC2-activating cytokine IL-33 in airways disease (Ober and Yao, 2011).

The mechanisms of ILC2 activation upon encounter of a type 2 agonist remain incompletely understood. Because Th2 cells and ILC2s require the same transcription factors for their

differentiation and secrete many of the same cytokines, our extensive understanding of gene regulation in Th2 cells may be informative for ILC2 biology. In Th2 cells, signaling through the TCR activates a phospholipase C (PLC)-dependent signaling cascade that drives three primary responses: (1) cytosolic  $\text{Ca}^{2+}$  influx, calcineurin activation, and translocation of NFAT into the nucleus, (2) MAPK-dependent translocation of activator protein 1 (AP-1) to the nucleus, and (3) protein kinase C (PKC)-dependent activation of NF- $\kappa$ B. In the nucleus, NFAT, AP-1, and NF- $\kappa$ B cooperatively drive expression of type 2 cytokines (Hermann-Kleiter and Baier, 2010). In contrast, ILC2s lack antigen receptors and instead integrate numerous locally produced signals to drive cytokine production. We hypothesize that in doing so, ILC2s respond to perturbations in tissue homeostasis that are common to the diverse set of type 2 agonists (von Moltke and Locksley, 2014). To date, most studies have focused on the ILC2-activating cytokines thymic stromal lymphopoietin, IL-33, and IL-25, of which IL-33 is particularly important in the lung (Barlow et al., 2013). More recently, the TNF family cytokine TL1A was also implicated in ILC2 activation (Yu et al., 2014). Notably, although these signals can activate AP-1 and NF- $\kappa$ B (Parnet et al., 1996; Brint et al., 2002), none have been linked to rapid cytosolic  $\text{Ca}^{2+}$  flux. Therefore, whether calcineurin and NFAT contribute to ILC2 activation remains an open question.

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Abbreviations used: AP-1, activator protein 1; BAL, bronchoalveolar lavage; cysLT, cysteinyl LT; ILC, innate lymphoid cell; LT, leukotriene; MFI, median fluorescence intensity; PKC, protein kinase C; PLC, phospholipase C.

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Eicosanoids are a family of arachidonic acid metabolites that includes the prostaglandins and leukotrienes (LTs). Eicosanoids are rapidly synthesized and degraded and are potent drivers of inflammation that act on numerous target cells. For example, LT signaling induces contraction of smooth muscle, chemokine production in mast cells, and permeabilization of vasculature. Eicosanoid biosynthesis is initiated by phospholipase A2, which releases arachidonic acid from membrane phospholipids (Fig. S1 A). Arachidonic acid then serves as the substrate for the cyclooxygenase enzymes, leading to prostaglandin production, or for 5-lipoxygenase (ALOX5), which catalyzes the first step in all LT synthesis by generating LTA<sub>4</sub>. LTA<sub>4</sub> is rapidly converted to LTB<sub>4</sub> by LTA<sub>4</sub> hydrolase or to LTC<sub>4</sub> by LTC<sub>4</sub> synthase (LTC4S), which conjugates glutathione. Peptidase cleavages convert LTC<sub>4</sub> to LTD<sub>4</sub> and then LTE<sub>4</sub>, and these three LTs are collectively known as the cysteinyl LTs (cysLTs).

Both LTB<sub>4</sub> and the cysLTs bind G protein–coupled receptors. LTB<sub>4</sub> R1 is the primary LTB<sub>4</sub> receptor and in immune cells predominantly mediates chemotaxis. LTB<sub>4</sub> R2 binds LTB<sub>4</sub> with much lower affinity and can respond to other arachidonic acid metabolites as well (Yokomizo et al., 2001). CYSLTR1 is the best-characterized cysLT receptor, and in transfected cells, it exhibits the following ligand preference: LTD<sub>4</sub> >> LTC<sub>4</sub> > LTE<sub>4</sub>. Like the TCR, CYSLTR1 signals through PLC to induce Ca<sup>2+</sup> flux and activate PKC (Peres et al., 2007). CYSLTR2 expressed in transfected cells binds LTD<sub>4</sub> and LTC<sub>4</sub> with equal affinity, but like CYSLTR1, it does not bind strongly to LTE<sub>4</sub> (Heise et al., 2000; Laidlaw and Boyce, 2012). The high-affinity LTE<sub>4</sub> receptor was recently identified as OXGR1, also known as GPR99 (Kanaoka et al., 2013; Bankova et al., 2016).

The link between LTs and type 2 airway inflammation is well established. CYSLTR1 is up-regulated on activated Th2 cells (Spinozzi et al., 2004; Prinz et al., 2005; Parmentier et al., 2012), and both *Ltc4s*<sup>−/−</sup> and *Ltb4r1*<sup>−/−</sup> mice are protected in ovalbumin-induced models of allergic lung inflammation (Henderson et al., 1996, 2002; Miyahara et al., 2005; Terawaki et al., 2005; Kim et al., 2006). Clinically, LT levels are elevated in asthmatic patients (Wardlaw et al., 1989; Hanazawa et al., 2000), and CYSLTR1 inhibitors provide symptomatic relief, particularly for patients with aspirin-exacerbated respiratory disease or exercise-induced asthma (Leff et al., 1998; Reiss et al., 1998; Dahlén et al., 2002).

Although the roles of both ILC2s and eicosanoids are established in type 2 lung inflammation, studies that integrate the two remain incomplete. Prostaglandin D2 has been reported as a chemoattractant for mouse and human ILC2s, whereas prostacyclin inhibits cytokine production in ILC2s (Chang et al., 2014; Xue et al., 2014; Tait Wojno et al., 2015; Zhou et al., 2016). Among LTs, studies in human and mouse ILC2s demonstrated CYSLTR1 expression and LTD<sub>4</sub>–dependent cytokine induction (Doherty et al., 2013; Xue et al., 2014; Pelly et al., 2016). However, analysis of the entire set of LTs and their signaling mechanisms in ILC2 activation remains incomplete and is therefore the focus of this study.

The analysis of secreted cytokines at the single-cell level presents significant technical challenges. Ex vivo restimulation and secretion inhibitors are commonly used before intracellular staining, but these approaches rely on antibody sensitivity and specificity and often reveal the cytokine potential of cells rather than their in situ cytokine production. To address this problem, we have developed genetically encoded cytokine reporters. Smart13 (*Il13*<sup>Smart/Smart</sup>) mice express nonsignaling human CD4 (hCD4) from an internal ribosomal entry site inserted downstream of the *Il13* coding sequence. Surface staining for hCD4 therefore allows direct quantification of the frequency (percent Smart13<sup>+</sup>) of IL-13–producing cells and the level (Smart13 median fluorescence intensity [MFI]) of IL-13 production in each cell directly ex vivo (Liang et al., 2012). This approach also has the benefit of avoiding fluorescent reporter proteins with long half-lives that obscure subtleties in gene regulation.

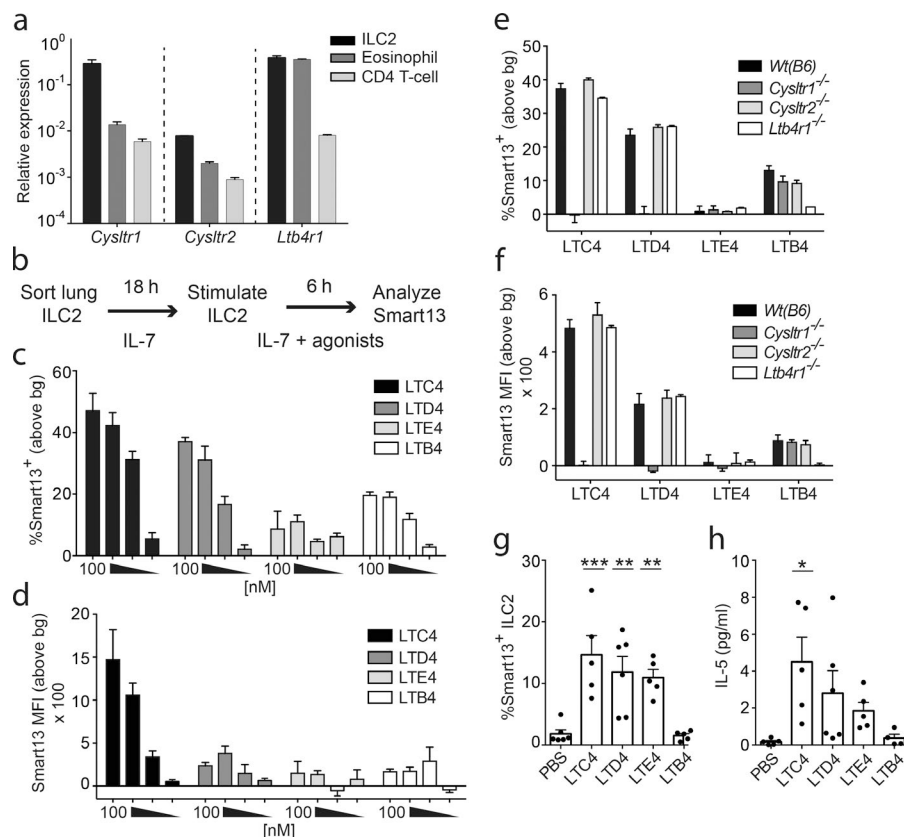
Here, we use two models to induce ILC2-driven type 2 immune responses in the mouse lung. In the first, intranasal delivery of chitin induces an acute, short-lived, type 2 response driven entirely by innate immune cells, particularly ILC2s (Van Dyken et al., 2014). In the second, the intestinal helminth *Nippostrongylus brasiliensis* briefly transits through the lung during the first 1–2 d after infection, inducing a sustained type 2 response involving both ILC2s and adaptive cells. By combining the Smart13 reporter allele with LT synthesis and/or receptor knockout alleles, we dissect the role of LT signaling in ILC2 activation in the lung.

## RESULTS AND DISCUSSION

### LTs are sufficient to activate ILC2s in vitro and in vivo

To assess the complete LT-sensing potential of lung ILC2s, we performed quantitative RT-PCR on sorted ILC2s (lineage<sup>−</sup>; THY1.2<sup>+</sup>; IL-33R<sup>+</sup>; Fig. S2, A and B). Indeed, *Cysltr1* and *Ltb4r1* were expressed much higher in ILC2s than in CD4<sup>+</sup> cells and at levels equal to or higher than in blood eosinophils (Fig. 1 a). Expression of *Cysltr2* was low in all three cell populations tested, whereas *Ltb4r2* and the LTE<sub>4</sub> receptor *Oxgr1* were undetectable.

To test the expressed receptors functionally, we sorted ILC2s from the lungs of Smart13 reporter mice crossed to mice deficient in specific LT receptors, rested them overnight in IL-7 to ensure viability, and stimulated them for 6 h, a time frame that limited variability from agonist stability, cell survival, and cell replication (Fig. 1 b, Fig. S2 C, and Fig. S3 A). In this assay, LTs rapidly induced IL-13 production in ILC2s with the following hierarchy: LTC<sub>4</sub> > LTD<sub>4</sub> >> LTB<sub>4</sub> > LTE<sub>4</sub> (Fig. 1, c and d). The greater potency of LTC<sub>4</sub> was particularly apparent in the Smart13 MFI, demonstrating that although LTD<sub>4</sub> and LTC<sub>4</sub> activate a similar number of cells, each LTC<sub>4</sub>–activated cell makes more IL-13. Given its prominent role in chemotaxis, the ability of LTB<sub>4</sub> to induce any cytokine production in ILC2s was notable but is perhaps explained by its ability to couple with the α-q G protein (Peres et al., 2007). As expected, the activity of LTC<sub>4</sub> and LTD<sub>4</sub> was completely



**Figure 1. LTs are sufficient to rapidly activate lung ILC2s.** (A) Gene expression measured by quantitative RT-PCR on cells sorted from lungs (CD4 and ILC2) or blood (eosinophils) of naive mice. (B) Experimental workflow for in vitro ILC2 stimulation assay. (C–F) Frequency (C and E) and MFI (D and F) of Smart13 reporter-positive lung ILC2s sorted from naive wild-type (C and D) or the indicated (E and F) mice and treated in vitro for 6 h with the indicated LTs. (C and D) Four 10-fold dilutions from 100 to 0.1 nM are shown. (E and F) 10 nM of the indicated LTs. (G and H) Frequency of Smart13 reporter-positive ILC2s in lungs (G) and amount of IL-5 protein in BAL (H) of mice treated for 6 h with 200 ng of the indicated LTs. Data are representative of two experiments (A, E, and F) or pooled from at least three experiments (C, D, G, and H).  $n = 3$  technical replicates (A, E, and F) or at least 3 biological replicates (C and D) or biological replicates as shown (G and H). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (compared with PBS by one-way ANOVA). Data are means  $\pm$  SEM. bg, background.

dependent on CYSLTR1, whereas LTB<sub>4</sub> required LTB4R1 (Fig. 1, e and f). Consistent with its low expression level, we could not detect any role for CYSLTR2 using this assay.

To confirm our findings in vivo, we treated Smart13 reporter mice intranasally with LTs. Here too, LTC<sub>4</sub> and LTD<sub>4</sub> activated ILC2s by 6 h after treatment, and this was accompanied by accumulation of IL-5 protein in the bronchoalveolar lavage (BAL; Fig. S3 B and Fig. 1, g and h). However, unlike in vitro, LTE<sub>4</sub> did activate ILC2s in vivo, whereas at this dose, activation of ILC2s by LTB<sub>4</sub> alone was negligible in vivo. Collectively, these findings demonstrate that LTs are individually sufficient to induce at least modest cytokine production in ILC2s, although LTE<sub>4</sub> can do so only in vivo and likely requires an intermediate cell, possibly an epithelial cell (Bankova et al., 2016). In contrast to a previous study using cells other than ILC2s (Lynch et al., 1999), we found that LTC<sub>4</sub> signaling through CYSLTR1 was more potent than LTD<sub>4</sub>. In vivo, LTC<sub>4</sub> is rapidly hydrolyzed to LTD<sub>4</sub> and then LTE<sub>4</sub>; perhaps this happened in our tissue culture media as well, providing ILC2s with successive stimulation by LTC<sub>4</sub>, LTD<sub>4</sub>, and/or LTE<sub>4</sub>. Alternatively, ILC2s may encode a novel LTC<sub>4</sub>- and CYSLTR1-dependent signaling pathway, perhaps requiring additional receptors.

#### LTs are largely dispensable for ILC2 homeostasis

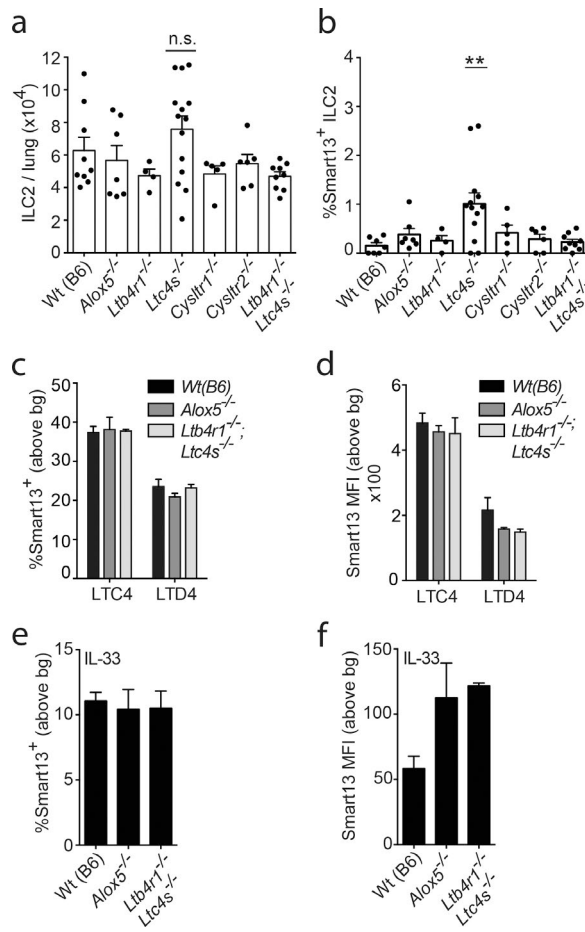
To test the necessity of LTs in ILC2 activation, we crossed receptor (*Cysltr1*, *Cysltr2*, *Ltb4r1*) and synthase (*Alox5*, *Ltc4s*)

knockout mice to Smart13 reporter mice. We also generated *Ltc4s*;*Ltb4r1* double knockout mice to control for shunting of excess arachidonic acid into the cyclooxygenase pathway in *Alox5*<sup>-/-</sup> animals. The development and seeding of ILC2s to the lung were normal, and IL-13 production was negligible in these mice under resting conditions, with the exception of *Ltc4s*<sup>-/-</sup> mice, discussed in the next paragraph (Fig. 2, a and b). Furthermore, ILC2s from *Alox5*<sup>-/-</sup> and *Ltb4r1*;*Ltc4s*<sup>-/-</sup> mice remained fully responsive to IL-33, LTC<sub>4</sub>, and LTD<sub>4</sub> (Fig. 2, c–f). As expected, tissue-resident eosinophils were absent in the resting mouse lungs.

Interestingly, in *Ltc4s*<sup>-/-</sup> mice, we noted a trend toward more ILC2s and a small but significant increase in basal IL-13 production (Fig. 2, a and b). These results suggest mild ILC2 activation in *Ltc4s*<sup>-/-</sup> mice, perhaps resulting from shunting of excess LTA<sub>4</sub> into LTB<sub>4</sub> production, as the activation phenotype is eliminated in *Ltb4r1*;*Ltc4s* doubly deficient mice.

#### LTB<sub>4</sub> and cysLTs cooperate to activate ILC2s during type 2 inflammation in the lung

To test the role of LTs during type 2 lung inflammation, we used chitin or *N. brasiliensis*. 8 h after chitin challenge, a fraction of ILC2s in wild-type mice became Smart13<sup>+</sup>; this response was intact in *Ltb4r1*<sup>-/-</sup> and *Cysltr2*<sup>-/-</sup> mice and only mildly reduced in *Cysltr1*<sup>-/-</sup> and *Ltc4s*<sup>-/-</sup> mice (Fig. 3 a), with the caveat that some ILC2s in *Ltc4s*<sup>-/-</sup> mice were activated before treatment (Fig. 2 b). However,



**Figure 2. Lung ILC2 homeostasis is largely intact in the absence of LTs.** (A and B) Number of ILC2s (A) and frequency of Smart13 reporter-positive ILC2s (B) in the lungs of naive mice of the indicated genotypes. (C–F) Frequency (C and E) and MFI (D and F) of Smart13 reporter-positive lung ILC2s sorted from naive mice of the indicated genotypes and treated in vitro for 6 h with 10 nM of the indicated LTs (C and D) or 10 ng/ml IL-33 (E and F). Data are pooled from at least three experiments (A and B) or representative of two experiments (C–F).  $n = 3$  technical replicates (C–F) or biological replicates as shown (A and B). \*\*,  $P < 0.01$  (compared with WT[B6] by one-way ANOVA). One high *Ltc4s*<sup>-/-</sup> outlier was excluded from B by Grubb's test ( $P < 0.01$ ). Data are means  $\pm$  SEM. bg, background.

when LT signaling was completely disrupted (*Alox5*<sup>-/-</sup> or *Ltc4s*;*Ltb4r1*<sup>-/-</sup>), ILC2 activation was nearly absent. To assess whether this early requirement for LTs was maintained during adaptive type 2 responses, we examined lungs 7 d after *N. brasiliensis* infection. We again observed some impact from the loss of cysLT generation or function alone, but the loss of both cysLT generation and LTB4R1 function had much more profound effects. There was a partial decrease in ILC2 accumulation in *Alox5*<sup>-/-</sup> and *Ltc4s*;*Ltb4r1*<sup>-/-</sup> mice (Fig. 3 b), and IL-13 production was nearly absent in those cells that were present, as both the frequency of Smart13<sup>+</sup> ILC2s and their MFI of Smart13 was near background levels (Fig. 3, c and d).

Infection by *N. brasiliensis* leads to eosinophil accumulation and hemorrhage during larval migration through the lung, the latter of which can be detected by the presence of blood in the BAL. In wild-type mice, the BAL is free of blood by 7 d after infection, but in the absence of signaling through IL-4RA (required for both IL-4 and IL-13), hemorrhage is more severe and prolonged, perhaps because of a loss of type 2-dependent tissue repair (Chen et al., 2012). Therefore, both the absence of eosinophilia in *Alox5*<sup>-/-</sup> and *Ltc4s*;*Ltb4r1*<sup>-/-</sup> mice and the presence of blood in the BAL of *Alox5*<sup>-/-</sup> mice 7 d after infection provide in vivo evidence of defective ILC2-mediated type 2 immune responses in the absence of LT signaling (Fig. 3, e–g).

Activation of ILC2s by LTC<sub>4</sub> and LTD<sub>4</sub> appears completely CYSLTR1 dependent, but the response to LTE<sub>4</sub> in vivo is likely CYSLTR1 independent, which may in part explain why ILC2 activation is only partially reduced in *Cysltr1*<sup>-/-</sup> mice. LTE<sub>4</sub> signaling is absent in *Ltc4s*<sup>-/-</sup> mice, but it is difficult to interpret results in this background given the homeostatic activation we observed. However, most notably, the profound defects revealed in *Alox5*<sup>-/-</sup> and *Ltc4s*;*Ltb4r1*<sup>-/-</sup> mice suggest that both cysLT and LTB<sub>4</sub> signaling contribute to ILC2 activation in vivo. This is perhaps surprising given the modest effects observed after 6-h stimulation with LTB<sub>4</sub> in vitro and in vivo. We speculate that LTB<sub>4</sub> provides a weak but more durable stimulus for ILC2s or that the well characterized chemotactic activity of LTB<sub>4</sub> helps to position ILC2s near sources of cysLT.

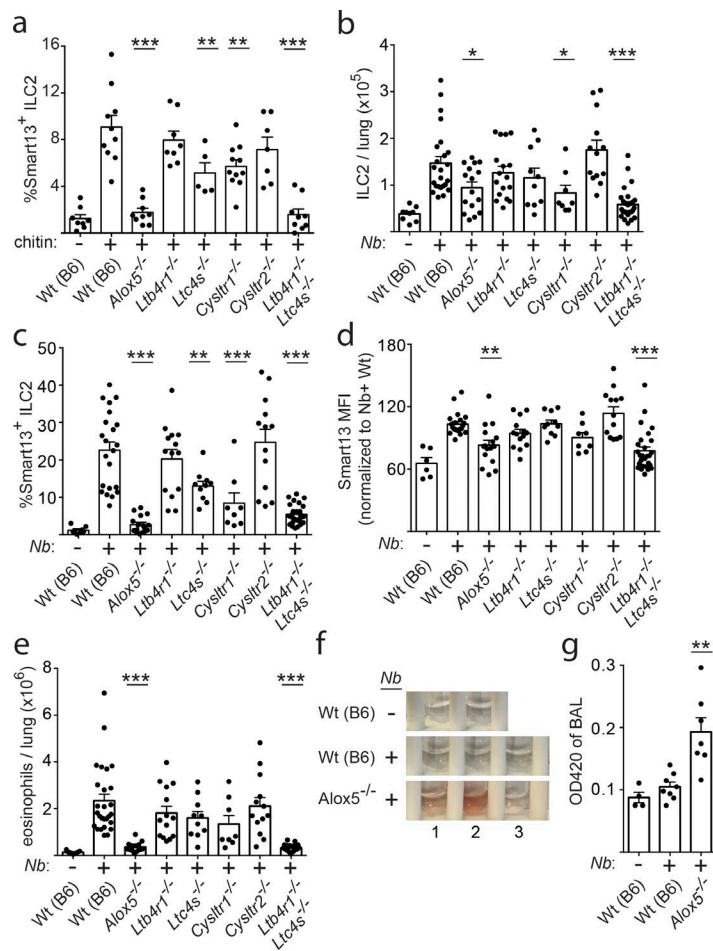
It should be noted that we cannot rule out the possibility that other LT-responsive cells contribute to ILC2 activation in vivo. If tools for selective targeting of LT receptors in ILC2s become available, it will be interesting to assess the extent to which the phenotypes we observed result from ILC2-intrinsic LT signaling.

### CysLT and IL-33 signaling are nonredundant in ILC2s

A previous study identified IL-33 as a critical signal for ILC2 activation in the lung and demonstrated substantial protection from type 2 inflammation in IL-33-deficient mice (Barlow et al., 2013). However, these findings do not preclude the existence of nonredundant signals that are also required for ILC2 activation. LTs represent such a nonredundant signal, as BAL levels of IL-13 and IL-5, tissue eosinophilia, and ILC2 expansion were all equivalently reduced in the lungs of *Il33*<sup>-/-</sup>, *Alox5*<sup>-/-</sup>, and *Ltb4r1*;*Ltc4s*<sup>-/-</sup> mice 7 d after *N. brasiliensis* infection (Fig. 4, a–d). These defects were not caused by changes in expression of *Cysltr1* or *Il33r*; a modest decrease in *Ltb4r1* expression is also unlikely to account for the attenuated inflammation (Fig. 4, e–g).

To better understand how IL-33 and LTC<sub>4</sub> interact to activate ILC2s in vivo, we first compared the two agonists in vitro. To avoid using anti-IL-33 receptor antibody, we generated *Arg1*<sup>eYFP/eYFP</sup>;*Il13*<sup>Smart/Smart</sup> mice and sorted ILC2s using the previously characterized *Arginase1* marker (Fig. S2, D and E; Bando et al., 2013). We found that although IL-33 activates ILC2s across a wider range of concentrations, LTC<sub>4</sub> achieves greater maximal ILC2 activation than IL-33 at this 6-h time point (Fig. 4, h





**Figure 3. LTs are required for lung ILC2 activation during type 2 inflammation.** (A) Frequency of Smart13 reporter-positive ILC2s in the lungs of mice of the indicated genotypes treated 8 h with intranasal chitin. (B–E) Mice of the indicated genotypes infected for 7 d with *N. brasiliensis* (Nb) and then analyzed for number of lung ILC2s (B), frequency of Smart13 reporter-positive ILC2s (C), MFI of Smart13 reporter on ILC2s (D), and number of lung eosinophils (E). (F) BAL from mice of the indicated genotypes infected 7 d with *N. brasiliensis*. (G) OD420 of BAL in F to quantify red blood cell infiltrate. Data are representative of two experiments (F) or pooled from two (G) or at least three experiments (A–E). *n* = biological replicates as shown. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001 (compared with treated WT[B6] by one-way ANOVA). Data are means ± SEM.

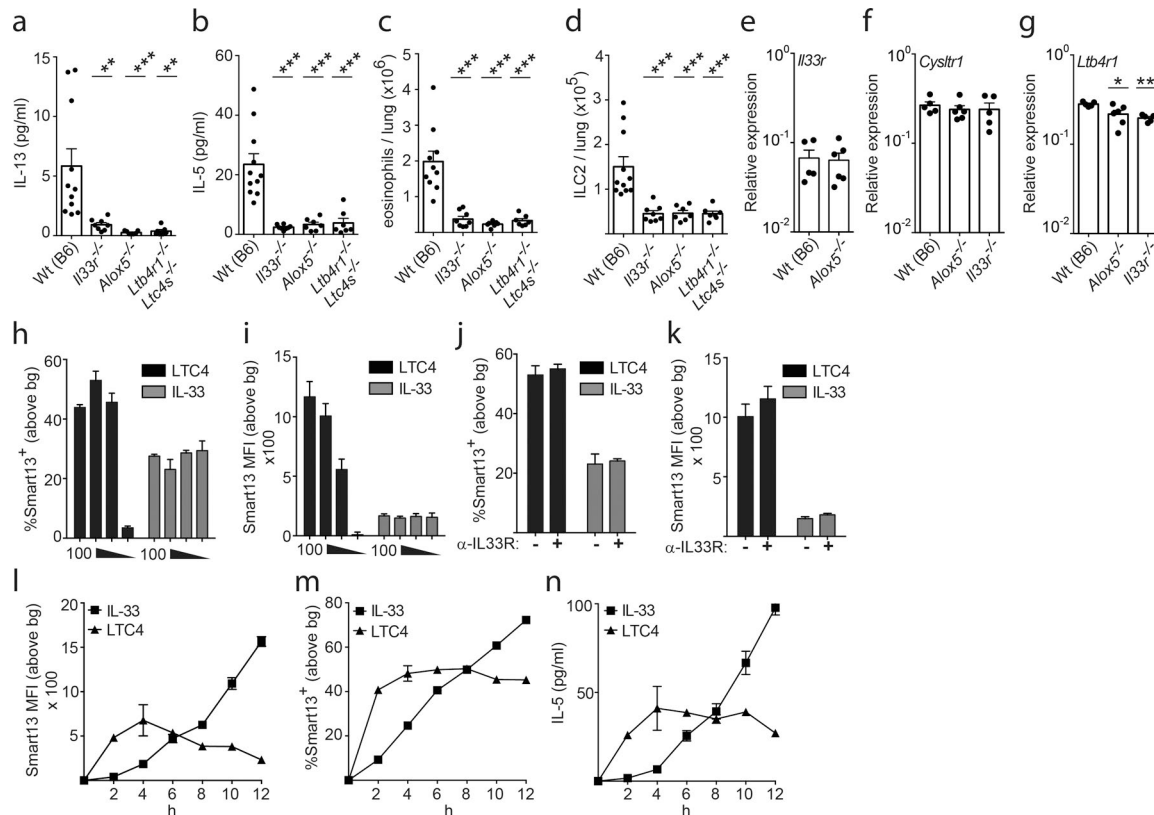
and i). Because the availability of *Arg1<sup>eYFP/cYFP</sup>;Il13<sup>Smart/Smart</sup>* mice was limited, we also confirmed that ILC2s sorted using anti-IL-33 receptor antibody respond normally to IL-33 (Fig. 4, j and k). Next, we compared ILC2 activation by LTC<sub>4</sub> and IL-33 across a 12-h time course and found strikingly different kinetics (Fig. 4, l–n). LTC<sub>4</sub> rapidly activated ILC2s, with cytokine production peaking by 4 h after stimulation. In contrast, IL-33 was a weaker agonist for the first 8 h but surpassed LTC<sub>4</sub> by 12 h.

#### CysLT signaling in ILC2s activates NFAT and synergizes with IL-33

Given the reported PKC activation and Ca<sup>2+</sup> flux downstream of CYSLTR1, we examined whether LTC<sub>4</sub> might provide ILC2s with a signal analogous to the TCR signal that drives cytokine expression in Th2 cells. PMA (a PKC agonist) and ionomycin (a Ca<sup>2+</sup> ionophore) are routinely used to mimic PLC signaling downstream of the TCR. Therefore, it was notable that whereas IL-33 enhanced IL-13 production in ILC2s treated with these agonists, the addition of LTC<sub>4</sub> was either neutral or even inhibitory, suggesting that LTC<sub>4</sub> and PMA + ionomycin share a common signaling pathway, whereas IL-33 and LTC<sub>4</sub> activate ILC2s through different mechanisms (Fig. 5 a).

Signaling through both IL-33R and CYSLTR1 can activate NF-κB and PKC (Parnet et al., 1996; Peres et al., 2007; Loegering and Lennartz, 2011). Consistently, the PKC inhibitor sotrastaurin and the NF-κB inhibitor caffeic acid phenethyl ester each individually blocked both LTC<sub>4</sub>- and IL-33-mediated ILC2 activation (Fig. 5, b and c). In contrast, cytosolic Ca<sup>2+</sup> influx occurs only downstream of CYSLTR1, and the calcineurin inhibitor cyclosporin A accordingly inhibited cytokine production in LTC<sub>4</sub>- but not IL-33-treated ILC2s (Fig. 5, b and c). NFAT, the primary substrate of calcineurin, has not been previously implicated in ILC2 gene regulation. However, we detected NFATC1 and NFATC2 in ILC2s by immunofluorescence and found that LTC<sub>4</sub> but not IL-33 induced their translocation to the nucleus, as predicted by our findings using cyclosporin A (Fig. 5, d–f). Collectively, our results demonstrate that the nonredundant role of LTs in ILC2 activation is explained at least in part by the selective capacity of LTC<sub>4</sub> signaling to translocate NFAT to the nucleus.

Given the nonredundant nature of IL-33 and LT signaling in ILC2s, we hypothesized that these two ligands might synergize to mediate maximal ILC2 activation. Therefore, we stimulated ILC2s with suboptimal levels of IL-33, LTC<sub>4</sub>, or LTD<sub>4</sub> alone and in combination. As expected, each signal



**Figure 4. IL-33 and LTs are nonredundant and signal with different kinetics in lung ILC2s.** (A–D) IL-13 (A) or IL-5 (B) protein in the BAL or number of eosinophils (C) or ILC2s (D) in the lungs of the indicated mice infected 7 d with *N. brasiliensis*. (E–G) Expression of *Il33r* (E), *Cysltr1* (F), or *Ltb4r1* (G) measured by quantitative RT-PCR in ILC2s sorted from naive lungs of the indicated mice. (H and I) Frequency (H) or MFI (I) of Smart13 reporter-positive ILC2s sorted from the lungs of *Arg1<sup>Yarg/Yarg</sup>;Il13<sup>Smart/Smart</sup>* mice and treated in vitro for 6 h with IL-33 or LTC<sub>4</sub>, as indicated. Four 10-fold dilutions from 100 nM (LTC<sub>4</sub>) and 100 ng/ml (IL-33) are shown. (J and K) Frequency (J) or MFI (K) of Smart13 reporter-positive ILC2s sorted from lungs of *Arg1<sup>Yarg/Yarg</sup>;Il13<sup>Smart/Smart</sup>* mice with or without anti-IL-33R antibody, as indicated. Cells were treated 6 h in vitro with 10 nM LTC<sub>4</sub> or 10 ng/ml IL-33. (L and M) MFI (L) or frequency (M) of the Smart13 reporter on lung ILC2s sorted from *Il13<sup>Smart/Smart</sup>* mice and treated in vitro for the indicated times with 10 nM LTC<sub>4</sub> or 10 ng/ml IL-33. (N) IL-5 protein in the supernatants of cells in L and M. The data shown are pooled from two experiments (A–G) or representative of two experiments (H–N). *n* = 3 technical replicates (H–N) or biological replicates as shown (A–G). \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001 (compared with WT[B6] by one-way ANOVA). One high WT(B6) outlier was excluded from C by Grubb's test (*P* < 0.01). Data are means ± SEM. bg, background.

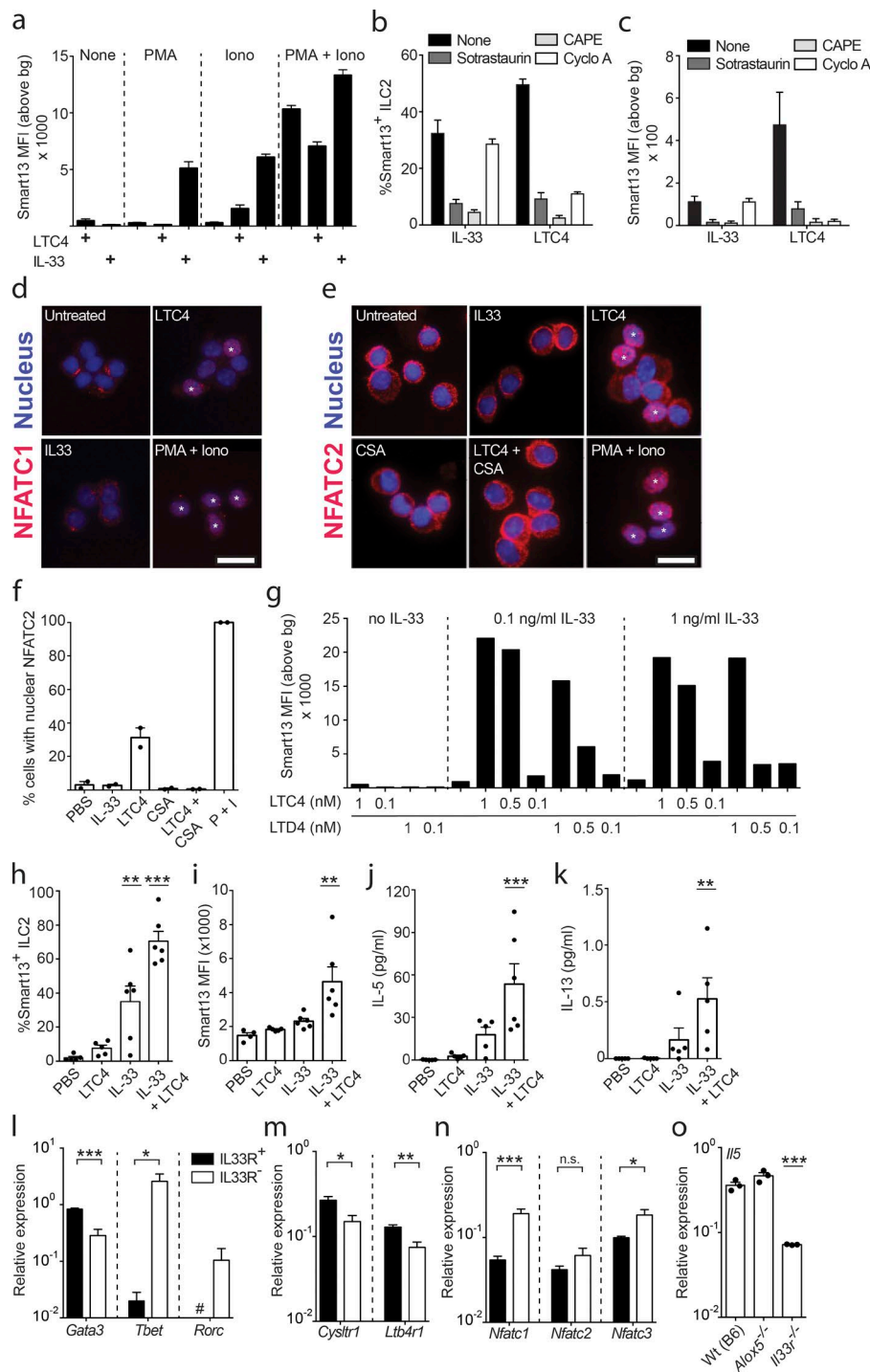
alone induced only minimal ILC2 activation (Fig. 5 g). However, when IL-33 was combined with LTC<sub>4</sub> or LTD<sub>4</sub>, ILC2 activation was not just additive but potentially synergistic. This synergy was also evident in vivo. The combination of low-dose IL-33 and LTC<sub>4</sub> given intranasally induced Smart13 in >60% of lung ILC2s in just 6 h, and each cell produced high levels of cytokines, as assessed by Smart13 MFI and accumulation of IL-5 and IL-13 in the BAL (Fig. 5, h–k).

### Concluding remarks

In summary, our findings demonstrate a critical, nonredundant role for LTs in ILC2 activation, provide the first report of NFAT signaling in these cells, and suggest that ILC2s integrate multiple inputs using pathways analogous to those downstream of the TCR. Optimal ILC2 activation reflects the synergy between LT-dependent NFAT activation, IL-33-dependent AP-1 and NF-κB activation, and perhaps also

STAT5 signaling downstream of thymic stromal lymphopoietin and/or IL-25. As the only NFAT-activating signal identified in ILC2s to date, LTs perform a nonredundant function in ILC2s and warrant closer attention in other settings where ILC2s are important.

Our findings may also have implications for other ILC subsets. ILC2s represent the dominant ILC population in the resting lung, whereas ILC1 and ILC3 populations have not been well characterized in this setting. We performed quantitative RT-PCR to compare sorted lung ILC2s (lineage<sup>+</sup>;Thy1.2<sup>+</sup>;IL-33R<sup>+</sup>) with other ILCs in the lung (lineage<sup>+</sup>;Thy1.2<sup>+</sup>;IL-33R<sup>+</sup>). As expected, we found that the ILC2-specific transcription factor *Gata3* is enriched in IL-33R<sup>+</sup> cells, whereas *Tbet* and *Rorc*, which are required for ILC1s and ILC3s, respectively, are detected almost exclusively in IL-33R<sup>+</sup> cells (Fig. 5 l). Compared with ILC2s, and consistent with intestinal ILC analysis by the Immunological



**Figure 5. NFAT-dependent LTC<sub>4</sub> signaling synergizes with IL-33 for optimal ILC2 activation.**

(A) MFI of Smart13 reporter on ILC2s sorted from lungs of *IL13<sup>Smart/Smart</sup>* mice and treated 6 h in vitro with 10 nM LTC<sub>4</sub>, 10 ng/ml IL-33, 30 ng/ml PMA, and/or 500 ng/ml ionomycin (Iono), as indicated. (B and C) Frequency (B) or MFI (C) of Smart13 reporter-positive ILC2s sorted from lungs of *IL13<sup>Smart/Smart</sup>* mice and treated in vitro for 30 min with 10  $\mu$ M sotrastaurin, 100  $\mu$ M caffeic acid phenethyl ester (CAPE), or 100 nM cyclosporine A (Cyclo A) as indicated, followed by 6-h treatment with 10 nM LTC<sub>4</sub> or 10 ng/ml IL-33 in the presence of the indicated inhibitors. (D and E) Fluorescence microscopy of ILC2s sorted from lungs of WT(B6) mice and treated 30 min with cyclosporine A (CSA) as indicated followed by 90-min treatment with 100 nM LTC<sub>4</sub>, 100 ng/ml IL-33, 30 ng/ml PMA, and/or 500 ng/ml ionomycin, as indicated. Cells were stained with anti-NFATC1 (D) or anti-NFATC2 (E) and DAPI (blue). White asterisks indicate nuclear localization of NFATC2. Bars, 10  $\mu$ m. (F) Quantification of cells in E with nuclear localization of NFATC2. At least 40 cells were counted for each condition. P + I, PMA + ionomycin. (G) MFI of the Smart13 reporter on ILC2s sorted from the lungs of *IL13<sup>Smart/Smart</sup>* mice and treated in vitro for 6 h with the indicated combinations of IL-33 with LTC<sub>4</sub> or LTD<sub>4</sub>. (H–K) *IL13<sup>Smart/Smart</sup>* mice treated 6 h with 50 ng IL-33 or LTC<sub>4</sub> given intranasally alone or in combination and analyzed for frequency (H) or MFI (I) of Smart13 reporter-positive ILC2s in lungs or IL-5 (J) or IL-13 (K) in the BAL. (L–N) Quantitative RT-PCR analysis of the indicated genes in ILC2s (lineage<sup>+</sup>;Thy1.2<sup>+</sup>;IL-33R<sup>+</sup>) or other ILCs (lineage<sup>+</sup>;Thy1.2<sup>+</sup>;IL-33R<sup>+</sup>) sorted from the lungs of naive WT(B6) mice. (O) Expression of *Il5* measured by quantitative RT-PCR in ILC2s sorted from naive lungs of the indicated mice. The data shown are pooled from three (A–C and H–K) or two (L–N) experiments or are representative of two (D–F and O) or three (G) experiments.  $n = 3$  (A–C), 2 (F) or 1 (G) technical replicates, or 5–6 (L–N) or biological replicates as shown (F, H–K, and O). #, not detected. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (compared with PBS by one-way ANOVA [H–K and O] or pairwise comparison by Student's  $t$  test [L–N]). Data are means  $\pm$  SEM. bg, background.

Genome Consortium, these IL-33R<sup>+</sup> lung ILCs expressed somewhat lower levels of *Cysltr1* and *Ltb4r1* and somewhat higher levels of *Nfat* genes than lung ILC2s (Fig. 5, m and n). Other ILC subsets may therefore also rely on NFAT signaling for cytokine induction, and LT signaling may contribute to its activation. It will be interesting to determine whether optimal

activation of ILC1s and ILC3s also results from an integration of multiple signals, analogous to our findings in ILC2s.

To our knowledge, this is the first study to demonstrate cooperative functions for LTB<sub>4</sub> and cysLTs in the induction of cytokine expression, which has important implications for clinical practice. Currently, most therapies targeting LTs rely

on the potent CYSLTR1 inhibitors montelukast, pranlukast, and zafirlukast, which bind their targets with nanomolar affinity (Lynch et al., 1999). Zileuton, the only approved 5-lipoxygenase inhibitor, requires 4× daily dosing and is less frequently prescribed. However, our results suggest that a renewed focus on 5-lipoxygenase inhibitors (or LTB<sub>4</sub> inhibitors in combination with CYSLTR1 inhibitors) may be warranted. Indeed, there is evidence that despite achieving only partial inhibition of systemic LT levels, zileuton may be particularly valuable in treating nasal dysfunction and bronchial hyperresponsiveness, the latter of which is often refractory to treatment with CYSLTR1 inhibitors alone (Dahlén et al., 1998).

The distinct kinetics and effective doses of IL-33 and LTC<sub>4</sub> lead us to propose a model in which these two agonists serve qualitatively different functions in ILC2 activation. IL-33, which remains active at 0.1 ng/ml (Fig. 4 h), may be important not just during inflammation, but also during homeostasis to provide a tonic signal that maintains ILC2s in a primed state. However, LTC<sub>4</sub> is rapidly synthesized and degraded and therefore likely represents a more labile signal for rapid cytokine induction during inflammation. Consistent with this model, the constitutive production of IL-5 by ILC2s in naive mice is strongly IL-33 dependent but LT independent (Fig. 5 o). Interestingly, as Th2 cells mature in the tissue, their cytokine production becomes TCR independent, whereas IL-33R and CYLSTR1 are both up-regulated (Prinz et al., 2005; Parmentier et al., 2012; Guo et al., 2015; Van Dyken et al., 2016). Perhaps IL-33–LTC<sub>4</sub> synergy is an innate substitute for TCR signaling in ILC2s and these TCR-independent effector Th2 cells.

The results presented here demonstrate a critical, nonredundant role for LTs in the activation of ILC2s and suggest several avenues for further research. In particular, it will be important to identify the cellular sources of LTs that act on ILC2s and to understand the upstream signals that drive LT synthesis. Given that IL-33 release has been linked to mechanical stretch or damage of tissue (Moussion et al., 2008; Kakkar et al., 2012), it will be interesting to determine whether LTs are similarly induced by tissue perturbation, which would be consistent with our model that ILC2s integrate multiple host-derived signals to assess the status of the surrounding tissue.

## MATERIALS AND METHODS

### Mice

Mice were maintained in the University of California, San Francisco specific pathogen-free animal facility in accordance with the guidelines established by the Institutional Animal Care and Use Committee and Laboratory Animal Resource Center. Mice aged 6–12 wk were used for all experiments. Mice were age- and sex-matched in figures displaying a single representative experiment. Pooled results include both male and female mice of varying ages. Unless otherwise noted, all mice used in these studies were homozygous for the Smart13 reporter allele (*Il13<sup>Smart/Smart</sup>*), which was generated as previ-

ously described (Liang et al., 2012). In brief, a cDNA fragment encoding truncated hCD4 (with a point mutation that results in the substitution of isoleucine for phenylalanine at position 43 of the protein, to abrogate binding of mouse major histocompatibility complex class II) preceded by an internal ribosomal entry sequence was introduced between the stop codon of *Il13* and the 3' untranslated region. Smart13 mice were generated on a BALB/c background and backcrossed for at least eight generations to C57BL/6J. BALB/c.*Arg1<sup>eYFP/cYFP</sup>* mice were generated as previously described (Reese et al., 2007) and backcrossed to C57BL/6J for at least eight generations. *Alox5<sup>-/-</sup>* (B6.129S2-Alox5<sup>tm1Fnn</sup>/J; 004155) and *Ltb4r1<sup>-/-</sup>* (B6.129S4-Ltb4r1<sup>tm1Adl</sup>/J; 008102) mice were purchased from The Jackson Laboratory. B6.*Cysltr1<sup>-/-</sup>*, B6.*Cysltr2<sup>-/-</sup>*, and B6.*Ltc4s<sup>-/-</sup>* mice were generated as previously described (Kanaoka et al., 2001; Maekawa et al., 2002; Beller et al., 2004). B6.*Ltb4r1<sup>-/-</sup>;Ltc4s<sup>-/-</sup>* mice were bred in house.

### In vivo treatments

Pure chitin beads (New England Biolabs, Inc.) ranging from 50 to 70 μm in diameter were prepared by size filtration through nylon mesh, washed, and reconstituted in sterile PBS (Ca<sup>2+</sup> and Mg<sup>2+</sup> free) at a final concentration of 10<sup>5</sup> chitin beads/ml. Mice were briefly anesthetized with isoflurane, and 50 μl of this suspension (5,000 beads) was aspirated by intranasal administration, followed by euthanasia and analysis at various time points after instillation. Separately, mice were infected subcutaneously with 500 third-stage *N. brasiliensis* larvae, which were raised and maintained as previously described (Liang et al., 2012). LTB<sub>4</sub>, LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub> (Cayman Chemicals), and IL-33 (R&D Systems) were diluted in sterile PBS and delivered intranasally to isoflurane-anesthetized mice in a final volume of 50 μl.

### Tissue preparation

For lung analysis, mice were euthanized, and BAL was collected in 1 ml PBS, if necessary. Then, the lungs were perfused through the heart with 10 ml PBS and harvested. Single-cell suspensions were prepared with an automated tissue dissociator (gentleMACS; Miltenyi Biotec), running program lung\_01 followed by incubation for 35 min at 37° C in HBSS (Ca<sup>2+</sup> and Mg<sup>2+</sup> free) containing 0.2 mg/ml Liberase (Roche) and 25 μg/ml DNase I (Roche), and then running gentleMACS program lung\_02. The tissue was further dispersed by passing through 70-μm nylon filters, washed, and subjected to red blood cell lysis (PharmLyse; BD) before final suspension in PBS with 3% fetal calf serum. For eosinophil sorting, 1–2 ml of blood was subjected to red blood cell lysis in PharmLyse for 5 min before washing and antibody staining.

### Flow cytometry and cell sorting

Single-cell suspensions prepared as described in the Tissue preparation section were incubated with anti-CD16 and CD32 monoclonal antibodies (University of California, San Francisco Antibody Core Facility) for 10 min at 4°C. The cells were



stained with antibodies to surface markers for 20 min at 4°C followed by DAPI (Roche) for dead cell exclusion. See Fig. S1 for a list of antibodies used in this study. CountBright Absolute Counting Beads (Thermo Fisher Scientific) were used according to the manufacturer's instructions to determine total cell numbers in each sample. Samples were analyzed on a Fortessa flow cytometer (BD) with five lasers (355 nm, 405 nm, 488 nm, 561 nm, and 640 nm) or sorted on a MoFlo XDP cell sorter (Beckman Coulter). Samples were gated by forward and side scatter to exclude debris, forward scatter width by forward scatter area-gated to select single cells, and gated to exclude DAPI<sup>+</sup> dead cells. Data were analyzed with FlowJo 10 (Tree Star).

### In vitro ILC2 culture and analysis

ILC2s were sorted from lungs of naive *Il13<sup>Smart/Smart</sup>* mice, as described in the Flow cytometry and cell sorting section. Basal media for ILC2 culture was high-glucose DMEM supplemented as follows: 4.5 g/L glucose, 0.584 g/L L-glutamine, 3.7 g/L NaHCO<sub>3</sub>, 1× nonessential amino acids, 1× minimal essential vitamins, 0.116 g/L L-arginine HCl, 0.036 g/L L-asparagine, 0.006 g/L folic acid, 10% FBS, 100 mg/ml streptomycin, 100 U/ml penicillin, 10 mM Hepes, 1 mM Na pyruvate, 100 μM 2-ME, and 2 mM L-glutamine. Cells were plated at 4,000–5,000 cells/well in 96-well plates and incubated overnight in 10 ng/ml IL-7 (R&D Systems). The next morning, media was replaced with fresh media containing 10 ng/ml IL-7 and the indicated LT (Cayman Chemical) or cytokine (R&D Systems) agonists. The following chemical agonists and inhibitors were used where indicated: 30 ng/ml PMA (Sigma-Aldrich), 500 ng/ml ionomycin (Sigma-Aldrich), 10 μM sotrastaurin (Cayman Chemical), 100 μM caffeic acid phenethyl ester (Cayman Chemical), and 100 nM cyclosporin A (Cayman Chemical). After 6-h incubation at 37°C, cells were stained with 1 μl/well of PE-conjugated anti-hCD4 for 30 min on ice. Cells were washed, resuspended in DAPI, and analyzed on a flow cytometer (Dual Fortessa; BD).

### Cytokine quantification

Levels of IL-5 and/or IL-13 in BAL or cell culture supernatant were measured using Cytokine Bead Array Enhanced Sensitivity Flex Sets (BD) according to the manufacturer's protocol.

### Quantitative RT-PCR

ILCs and CD4<sup>+</sup> cells were sorted from the lungs and eosinophils from the blood of naive mice, as described in the Flow cytometry and cell sorting section. RNA was isolated using the Micro Plus RNeasy kit (QIAGEN) and subjected to reverse transcription using SuperScript Vilo Master Mix (Thermo Fisher Scientific). The resulting cDNA was used as a template for quantitative PCR with the Power SYBR green reagent on a StepOnePlus cycler (Applied Biosystems). Transcripts were normalized to *Rps17* (40S ribosomal protein S17) expression. See Fig. S1 for a list of primers used in this study.

### NFAT imaging

ILC2s were sorted from the lungs of naive wild-type mice and cultured overnight in 10 ng/ml IL-7, as described in the In vitro ILC2 culture and analysis section. The next morning, cells were treated for 90 min as indicated and affixed to slides by Cytospin followed by 5 min in ice-cold methanol. Slides were blocked for 30 min with 10% goat serum and then incubated 60 min with 1:100 polyclonal rabbit anti-NFATC2 (NFAT1; clone D43B1; Cell Signaling Technology) or 1:50 with Alexa Fluor 488 anti-NFATC1 (NFAT2; clone 7A6; BioLegend). For NFATC2 staining, cells were next incubated 40 min with Alexa Fluor 555 goat anti-rabbit IgG Fc (Thermo Fisher Scientific). All cells were counterstained for 5 min with DAPI. Images were acquired with a camera (AxioCam HR) on an upright microscope (AxioImagerM2; ZEISS).

### Statistical analysis

All experiments were performed using randomly assigned mice without investigator blinding. Where noted in the figures, statistical significance was calculated using one-way ANOVA with Dunnett's correction or two-tailed Student's *t* test. All statistical analysis was performed using Prism 6 (GraphPad Software). Figures display means ± SEM.

### Online supplemental material

Fig. S1 shows the LT biosynthesis pathways and lists antibodies and primers used in this study. Figs. S2 and S3 show gating strategies.

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Author contributions: J. von Moltke conceived the study, designed and performed experiments, analyzed data, and wrote the manuscript with R.M. Locksley. C.E. O'Leary performed experiments and analyzed data. N.A. Barrett, Y. Kanaoka, and K.F. Austen provided mice and reviewed the manuscript. R.M. Locksley conceived and directed the study and wrote the manuscript with J. von Moltke.

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