Macrophages regulate lung ILC2 activation via Pla2g5-dependent mechanisms

M Yamaguchi1,3, SK Samuchiwal1,3, O Quehenberger2, JA Boyce1 and B Balestrieri1

INTRODUCTION

Alternaria Alternata is a common fungus that is a source of allergens associated with the development of asthma and asthma exacerbations. In mice, Alternaria allergens trigger the accumulation of eosinophils and the development of airway hyperreactivity,1,2 each of which prominently involves effectors of the innate immune system.1,3,4 Acute exposure of the airways of naive mice to Alternaria causes the rapid release of IL-33 by epithelial cells, followed by the activation of group 2 innate lymphoid cells (ILC2s).3,5 Long-term repetitive administration of Alternaria upregulates whole lung expression of IL-33, and promotes incremental ILC2-dependent lung eosinophilic inflammation.1 ILC2s lack cell surface markers associated with major hematopoietic lineages (Lin−).6–8 In the lung they express Thy1.2 (CD45+ Lin− Thy1.2+),9 and inducible molecules including ST2 (IL1R1), Sca-1, CD278 (ICOS), CD25 (IL-2Rα), CD127 (IL-7Rα), CD117 (c-Kit), and IL-17RB (IL-25R).1,10–12 Following activation, ILC2s produce IL-5 and IL-13 (as well as other cytokines), which mediate pulmonary eosinophilia, airway hyperreactivity1,12,13 and macrophage activation.14 Although IL-33 in naive mouse lung is principally derived from structural cells,15 hematopoietic cells (including macrophages) can express IL-33 inducibly.16,17 Macrophages can activate ILC2s through an IL-33-dependent mechanism in a model of influenza-induced airway hyperreactivity.10 Whether macrophages contribute to activating ILC2s in allergic inflammation in general, or in Alternaria-induced pulmonary inflammation in particular is not known.

Phospholipases A2 (PLA2) are a family of enzymes that release lysophospholipids and free fatty acids (FFAs) from membrane glycerophospholipids.18,19 While FFAs such as arachidonic acid (AA) can be converted to receptor-active eicosanoids (including prostaglandins and leukotrienes), other FFAs can act directly at cognate receptors to regulate metabolic...
processes and inflammatory responses. PLA₈s may have substrate preferences and specific cell and tissue expression, therefore serving context-specific functions. Group V PLA₃ (Pla2g5) preferentially releases lysophosphatidylcholine and the FFAs linoleic acid (LA) and oleic acid (OA), and is prevalently expressed by innate immune cells, including dendritic cells and macrophages. Using a mouse model of allergic lung inflammation induced by the allergens of house dust mite *Dermatophagoides farinae*, we found that Pla2g5 was necessary for the effector functions of both dendritic cells and macrophages. Adoptive transfer studies showed that Pla2g5 expression by macrophages was required for their generation of CCL22 and recruitment of T cells into the lungs. While the defects in cellular functions resulting from cell intrinsic absence of Pla2g5 suggest critical roles for endogenous lipids, neither the identity of the lipids nor their potential paracrine cellular targets are known.

Because eicosanoids may contribute to ILC2-mediated pulmonary inflammation, we hypothesized that Pla2g5-derived lipids generated from macrophages may contribute to ILC2 activation and subsequent pulmonary inflammation. Here we demonstrate that ILC2 activation is impaired in Pla2g5-null mice exposed to *Alternaria*. Moreover, adoptive transfers of macrophages restored ILC2 activation by a mechanism that is at least in part dependent on Pla2g5-dependent production of IL-33 and releases of LA, OA and AA by macrophages, which sustain ILC2 activation, and on Pla2g5-dependent expression of the LA-prefering FFA-receptor-1 (FFAR1) by ILC2s.

**RESULTS**

**Pulmonary inflammatory response to *Alternaria* requires Pla2g5**

To investigate the role of Pla2g5 in activation of ILC2s, we used a model of allergic pulmonary inflammation induced by *Alternaria*, which relies on ILC2 activation to cause eosinophilic inflammation. We administered *Alternaria* (25 μg per dose) every 2 days for four doses and lungs were collected 18 h after the last dose. Wt mice treated with *Alternaria* had significantly increased total lung cell numbers compared to *Alternaria*-treated Pla2g5-null mice ([Figure 1a](#)). The number of eosinophils (identified as CD45⁺/CD11c⁻/SiglecF⁺ cells) in *Alternaria*-treated Pla2g5-null mice ([Figure 1a](#)) significantly increased total lung cell numbers compared to *Alternaria*-treated Pla2g5-null lungs ([Figure 1b](#)). *Alternaria*-treated Wt mice had a significantly higher number of ILC2s, identified as CD45⁺/Lin⁻, Thy1.2⁺ cells ([see Supplementary Figure 1a](#) for staining controls), than equivalently treated Pla2g5-null mice ([Figure 1c](#)), although the percentages were similar ([Figure 1c](#) and data not shown). The expression of the inducible markers Sca-1, ST2, CD25, ICOS by lung ILC2s was drastically reduced in *Alternaria*-treated Pla2g5-null mice compared to ILC2s isolated from equivalently treated Wt mice ([Figure 1d](#) and [Supplementary Figure 1B](#)). The numbers ([Figure 1e](#)) and percentages ([Supplementary Figure 1B](#) and data not shown) of ILC2s expressing IL-5 or IL-13 were also significantly reduced in Pla2g5-null mice treated with *Alternaria* compared to Wt mice. Similar results were obtained by gating ILC2s as CD45⁺/Lin⁻/Thy1.2⁺ cells to exclude at least contaminating ILC3 ([Supplementary Figure 1C](#) and data not shown). These data suggest that the absence of Pla2g5 affects both numbers and activation of lung ILC2s.

**Induced IL-33 expression requires Pla2g5**

Whereas IL-33 is constitutively expressed by lung barrier cells, its expression can also be upregulated during sustained inflammatory responses, in part reflecting the contributions from hematopoietic cells. To investigate whether the reduced ILC2 activation in Pla2g5-null mice was associated with a lack of either constitutive or inducible pools of IL-33, we measured IL-33 release into the bronchoalveolar lavage fluids of naive mice after administration of a single *Alternaria* dose. We also monitored the content of IL-33 in the lung at baseline and after four doses of *Alternaria* using western blotting. We found that naive Wt and Pla2g5-null mice released similar amounts of IL-33 into bronchoalveolar lavage at 1 and 3 h after *Alternaria* challenge ([Figure 2a](#)), and showed equivalent amounts of immunoreactive IL-33 in lung lysates ([Figure 2b](#)). Only the full-length IL-33 was detected in naive mice. After 10 days and four doses of *Alternaria*, Wt lungs had increased amounts of IL-33 protein compared to naïve mice, and both the preformed full-length form (34 kDa) and the proteolytically processed short length form (18 kDa) were present. Compared with the *Alternaria*-treated Wt controls, the lungs of Pla2g5-null mice showed sharply diminished induction of both the 18 and 34 kDa forms of IL-33 ([Figure 2b](#)). To identify the cellular source(s) responsible for the constitutive and inducible pools of IL-33, we stained frozen sections of Wt and Pla2g5-null lungs with anti-IL-33. Since alveolar type 2 pneumocytes (AT2) are one of the major sources of IL-33 in *Alternaria* challenged mice, we counterstained the lung sections with Abs against the AT2 cell marker surfactant protein C (SPC). Lungs of both Wt and Pla2g5-null mice showed IL-33 in the nuclei of SPC⁺ AT2 cells at baseline, with no differences between Wt and Pla2g5-null *Alternaria* challenged mice ([Figure 2c](#)). Since lung macrophages can also express IL-33 in a model of prolonged exposure to viral allergens and in the recovery phase of IAV infection, we used intracellular staining and flow cytometry to determine whether macrophages contributed to the *Alternaria*-inducible pool of IL-33. Intracellular staining showed that *Alternaria* increased the number of CD68⁺/IL-33⁺ macrophages in Wt mice ([Figure 2d](#)). The number of CD68⁺/IL-33⁺ cells was significantly reduced in *Alternaria*-treated Pla2g5-null mice. To determine whether cell-intrinsic Pla2g5 was involved in inducing IL-33 expression by macrophages, we examined IL-33 expression by Wt and Pla2g5-null bone marrow (BM)-macrophages stimulated with granulocyte-macrophage-colony-stimulating factor (GM-CSF), IL-4, and IL-33, and also by lung macrophages enriched from *Alternaria*-treated Wt and Pla2g5-null mice using...
consecutive Percoll gradients. Wt BM-macrophages activated to full potential by GM-CSF/IL-4/IL-33 displayed robust induced expression of IL-33 mRNA compared to macrophages unstimulated or activated more weakly with GM-CSF/IL-4. In contrast, Pla2g5-null GM-CSF/IL-4/IL-33 BM-macrophages showed significantly reduced induction compared to equally treated Wt controls (Figure 2e, left panel). Wt lung macrophages enriched from Alternaria-treated mice expressed significantly higher IL-33 mRNA compared to equally treated Pla2g5-null lung macrophages (Figure 2e, right panel).

Figure 1. Alternaria-induced pulmonary inflammation requires Pla2g5. (a) Total cell counts from homogenate lungs of naïve and Alternaria-treated Wt and Pla2g5-null mice. Analysis by flow cytometry of lung cell from naïve and Alternaria-treated Wt and Pla2g5-null lung homogenates of (b) eosinophils gated as CD45+ CD11c–SiglecF–; (c) ILC2s gated as CD45+ Lin– Thy1.2+; (d) histograms of Sca-1, ST2, ICOS, CD25 gated on Thy1.2+ cells (isotype in gray, Wt in blue, Pla2g5-null in red), and (e) expression of intracellular IL-5 and IL-13 by Thy1.2+ ILC2s. Values are mean ± s.e.m. of at least three independent experiments with 5–9 ( naïve) or 10–21 ( Alternaria-treated) mice per group. Images are from one representative mouse per group. *** P < 0.0005, **P < 0.005, *P < 0.05. ILC, innate lymphoid cell.

Pla2g5-sufficient macrophages, but not IL-33 alone, can restore ILC2 activation and inflammation to Pla2g5-null mice

Next, we wanted to ascertain whether exogenous recombinant (r)-IL-33 would restore eosinophilia and ILC2 activation in Pla2g5-null mice. Administration of IL-33 over 10 days (100 ng per dose, Figure 3, inset) robustly increased the numbers of eosinophils, ILC2s and Sca-1+ ILC2s in Wt mice. Surprisingly, Pla2g5-null mice showed markedly diminished numbers of eosinophils, total ILC2s, and Sca-1+ ILC2s after treatment with...
IL-33 compared with Wt controls (Figure 3a). Exogenous IL-33 also induced substantial macrophage activation in Wt mice, as determined by the detection of resistin-like molecule alpha (RELMα) in macrophages. In contrast, macrophage activation was markedly impaired in IL-33-treated Pla2g5-null animals (Figure 3b). To determine whether the defect in ILC2 function reflected the effects of ILC2-intrinsic Pla2g5, we sorted ILC2s from the lungs of Wt mice and performed qPCR. Pla2g5 transcripts were not detected in ILC2s (data not shown).

Because macrophages require endogenous Pla2g5 for their functions in pulmonary inflammation, we wanted to investigate whether ILC2 activation and downstream lung inflammation could be restored to Pla2g5-null mice by reconstituting Pla2g5 function in macrophages. We adoptively transferred unstimulated Wt BM-macrophages into Wt and Pla2g5-null recipient mice 24 h before the second dose of Alternaria, then administered three more doses and analyzed eosinophil numbers and ILC2 activation (Figure 4, inset).
Compared to *Pla2g5*-null mice receiving *Alternaria* without macrophage transfer, *Pla2g5*-null mice receiving Wt BM-macrophages plus *Alternaria* had significantly higher numbers of eosinophils and significantly higher numbers of ILC2s expressing Sca-1, CD25 or intracellular IL-5 (Figure 4a). In contrast, the transfer of Wt BM-macrophages into *Alternaria*-treated Wt mice did not significantly increase the recruitment of eosinophils or activation of ILC2s compared to *Alternaria*-treated Wt mice. Accordingly, transfers of *Pla2g5*-null macrophages into *Pla2g5*-null mice were ineffective (Figure 4a). However, transfers of *Pla2g5*-null macrophages into Wt mice (Figure 4a) significantly reduced the numbers of activated ILC2s expressing Sca-1, CD25, and IL-5 and eosinophil numbers, suggesting that *Pla2g5*-null macrophages could downregulate ILC2 activation when exposed to Th2 inflammatory environment. To further prove this point, we fully activated *Pla2g5*-null macrophages, and Wt macrophages as controls, with GM-CSF/IL-4/IL-33 and transferred them into *Alternaria*-treated Wt mice. As shown in Supplementary Figure 2, activated *Pla2g5*-null macrophages significantly reduced the numbers of IL-5⁺ ILC2s and eosinophils in the lungs of *Alternaria*-challenged Wt mice. Transfers of Wt macrophages activated with GM-CSF/IL-4/IL-33 were ineffective.

**Pla2g5-dependent generation of linoleic acid and oleic acid contribute to ILC2 activation and pulmonary inflammation**

To identify candidate *Pla2g5*-derived mediators generated by macrophages that could contribute to ILC2 activation, we performed an unbiased assessment of lipids constitutively released by Wt and *Pla2g5*-null BM-macrophages, using mass spectrometry. Compared to Wt BM-macrophages, *Pla2g5*-null BM-macrophages produced significantly lower quantities of medium- and long- chain FFAs, mostly represented by oleic acid (OA, 18:1), LA (18:2), and AA (20:4) (Figure 4b). Short chain FFAs were not different (data not shown). We also examined FFAs produced by lung macrophages enriched from *Alternaria* exposed mice by Percoll gradients, a technique previously shown to enrich lung macrophages 80%. We did not sort CD68⁺ cells because staining for CD68 requires fixation with paraformaldehyde and permeabilization with saponin (as in Figure 2d) which could alter the lipid composition of the cells. Compared to Wt lung macrophages, *Pla2g5*-null macrophages had reduced quantities of OA and LA (Figure 5a). AA was similar in both genotypes.

To determine whether LA and/or OA could restore the IL-33-mediated induction of eosinophilic inflammation and ILC2 expansion, we administered intranasal LA and/or OA, alone and in combination with IL-33 (4 doses in 10 days), to Wt and
Pla2g5-null mice. Neither LA nor OA alone caused pulmonary inflammation in either genotype (Figure 5b). The combination of LA + IL-33 increased the numbers of eosinophils in the lungs of Wt mice by ~3-fold when compared to IL-33 alone, and the combination of LA and OA + IL-33 further increased the numbers of eosinophils in this genotype (Figure 5b). In contrast, LA failed to potentiate IL-33-induced eosinophilia in Pla2g5-null mice, although OA + IL-33 was markedly active and the combination of LA and OA + IL-33 induced a modest further increase over IL-33 + OA. The effects of FFAs on the numbers of lung ILC2s expressing IL-5 paralleled their effects on eosinophil numbers (Figure 5c and Supplementary Figure 3 for representative fluorescence-activated cell sorting (FACS) plots), although the numbers of IL-5⁺ ILC2s in IL-33 + LA + OA treated Pla2g5-null mice were slightly lower than in equally treated Wt mice.

Because AA-derived eicosanoids were previously shown to contribute to ILC2 activation,9,28 to understand whether AA could increase the numbers of IL5⁺ ILC2s in FFAs treated Pla2g5-null mice to the same levels as equally treated Wt mice, in another set of experiments, we administered AA alone or in combination with IL-33 + LA + OA to Wt and Pla2g5-null mice. AA alone did not induce pulmonary inflammation in either genotype (Figure 5d and e). In combination with IL-33, AA induced in Wt mice a twofold increase in eosinophil numbers (Figure 5d) and IL5⁺ ILC2s (Figure 5e), but was ineffective in Pla2g5-null mice. The combination of IL-33 + LA + OA + AA induced in Wt mice a significant increase in numbers of eosinophils and IL-5⁺ ILC2s compared to IL-33-exposed mice (Figure 5d and e) and a nearly significant increase in Pla2g5-null mice. However, in both genotypes the effects of IL-33 + LA + OA + AA were similar to those of IL-33 + LA + OA (Figure 5b–e).

To determine whether LA and/or OA directly activated ILC2s, we sorted ILC2s from lungs of Alternaria-treated Wt and Pla2g5-null mice, rested them for 40 h, and stimulated with...
LA, OA, IL-33 or a combination for 8 h. Then we assayed ILC2s for their expression of intracellular IL-5. Staining controls and representative FACS plots are shown in Supplementary Figure 4. IL-33 significantly increased the percentage of IL-5-expressing ILC2s isolated from both Pla2g5-null and Wt mouse lungs. Neither LA nor OA induced significant IL-5 expression by ILC2s of either genotype. LA, but not OA, significantly potentiated IL-33-induced expression of IL-5 by Wt ILC2s, and the combination of LA + OA did not differ from the effects of LA (Figure 6a). In contrast LA suppressed the IL-33-induced increase in percentages of IL-5+ Pla2g5-null ILC2s (Figure 6b). OA was inactive. To determine whether LA and OA amplified the release of IL-5 by Wt ILC2s, we measured the quantity of secreted IL-5 from sorted lung ILC2s activated ex vivo (Figure 6c). IL-33 induced the release of large quantities of IL-5 in supernatant of sorted Wt ILC2s and the combination of IL-33 + LA + OA significantly potentiated this release. In contrast, Pla2g5-null ILC2s released significantly less amount of IL-5 and failed to exhibit potentiation in response to LA + OA (Figure 6c).
Medium and long chain FFAs signal through two G protein-coupled receptors, FFA receptor-1 (FFAR1) and FFA receptor-4 (FFAR4). To determine whether ILC2s expressed these receptors, and to determine the potential basis for the different responses of Wt and Pla2g5-null ILC2s to LA, we analyzed ILC2 expression of FFAR1 and FFAR4 in ILC2s sorted from Wt and Pla2g5-null Alternaria-treated mice. Wt ILC2s expressed FFAR1 mRNA and its expression was significantly higher compared to Pla2g5-null ILC2s (Figure 6d). Wt and Pla2g5-null ILC2s also expressed FFAR4 mRNA to similar extents (Figure 6d).

DISCUSSION
It is now well established that ILC2s are key effectors of pulmonary inflammation. Their contribution is particularly evident in models triggered by the release of alarmins (IL-33, IL-25, TSLP) from epithelial cells in response to environmental proteases, many of which are relevant to asthma in humans. While macrophages can also express IL-33, and other innate cell types have been proposed to interact with ILC2s, no previous studies had established whether macrophages can activate ILC2s in Alternaria-induced pulmonary inflammation and which mediators might be involved.
Pla2g5-null mice show markedly impaired type 2 pulmonary inflammation that reflects, at least in part, a requirement for cell-intrinsic Pla2g5 for macrophage effector functions.\textsuperscript{25,26} We therefore investigated the role of Pla2g5 in general and macrophage-associated Pla2g5 in particular, in lipid-generating function and its potential downstream effects on ILC2 activation in a model of pulmonary inflammation induced by Alternaria.

We subjected Wt and Pla2g5-null mice to a protocol involving the administration of Alternaria four times over a 10-day period, which elicits prominent contributions from IL-33 and ILC2s. The marked pulmonary eosinophilia and increases in the numbers of total and activated ILC2s observed in Wt mice (Figure 1) were all sharply reduced in Pla2g5-null mice. The reduced levels of both eosinophils and ILC2s were paralleled by reduced levels of IL-33 induction (Figure 2b), but not constitutively levels of IL-33 (Figures 2a, 2b), or by release of IL-33 in response to a single Alternaria dose (Figure 2a). AT2 cells are the dominant source of pre-formed IL-33 in the mouse lung, as well as of the pre-formed IL-33 in response to a single dose of Alternaria.\textsuperscript{15} In our study, AT2 cells showed equivalent staining for IL-33 in Wt and Pla2g5-null mice (Figure 2c), suggesting that Pla2g5 functions are not required by AT2 to store or release IL-33. In marked contrast, IL-33 expression by lung macrophages was substantially induced in Alternaria-treated Wt mice but not in Pla2g5-null mice (Figure 2d), suggesting that macrophages may be one of the cell types accounting for the impaired induction of IL-33 in Pla2g5-null lungs. Our previous studies demonstrated that macrophage-intrinsic Pla2g5 was necessary for inducible expression of Th2 cell-active chemokines\textsuperscript{25}. Our current results, supported by our ex vivo data (Figure 2e), suggest that this may also be the case for IL-33 induction.

When administered exogenously to naive Wt mice, r-IL-33 is sufficient alone to drive a robust type 2 inflammatory response that depends on ILC2s.\textsuperscript{3,40} Despite the evident role of Pla2g5 in IL-33 induction by macrophages, the direct administration of IL-33 to naive Pla2g5-null mice was insufficient to induce inflammation, ILC2 expansion, and macrophage activation (Figure 3). Combined with the fact that transfer of Wt macrophages almost fully restored these parameters in Pla2g5-null mice in response to Alternaria challenges (Figure 4a), we suspected the involvement of additional Pla2g5-dependent factors that could enable macrophages to activate ILC2s, alone or in concert with IL-33. We identified at least two candidate FFAs (LA and OA) as Pla2g5-dependent factors derived from BM and lung macrophages (Figures 4b and 5a). Both of these FFAs can signal to immune and non-immune cells through the GPCRs FFAR1 and FFAR4, although their potential roles as mediators of allergic inflammation in general and stimulants of ILC2 activation in particular had not been explored. The sharp potentiation of IL-33-driven eosinophilic inflammation and ILC2 expansion in Wt mice by LA, alone and in combination with OA (Figure 5b and c), was parallel by its effects on IL-33-induced IL-5 generation (Figure 6a) and secretion (Figure 6c) by ILC2s ex vivo. Interestingly in Pla2g5-null mice this LA-induced potentiation of inflammation in vivo (Figure 5b and c) and ex vivo (Figure 6b and c) was absent. The lack of LA responsiveness by Pla2g5-null ILC2s is consistent with the lack of ILC2-intrinsic expression of FFAR1 (Figure 6d), which exhibits a preference for LA over OA.\textsuperscript{44} Notably, although unable to directly activate Wt or Pla2g5-null ILC2s, OA did substantially enhance IL-33-induced eosinophilic inflammation and expand lung ILC2s in Pla2g5-null mice, reflecting a potential compensatory mechanism involving ILC2 activation by a yet-to-be-determined OA-responsive cell. Since FFAR1 and FFAR4 are broadly expressed by immune and non-immune cell types,\textsuperscript{45} it is likely that Pla2g5-derived FFAs potentiate innate type 2 immune responses and ILC2 activation by both direct and indirect pathways. We speculate that ILC2s require conditioning in vivo by one or more inductive factors that are deficient in Pla2g5-null mice in order to express FFAR1 and respond to LA. Moreover, the fact that FFAs including AA could not restore ILC2 activation in Pla2g5-null mice to the levels of equally treated Wt mice (Figure 5c and e), suggests that the presence of Pla2g5-null macrophages (Figure 4a and Supplementary Figure 2) through a yet unknown mechanism might limit ILC2 activation in Pla2g5-null mice.

Our data clearly identify a role for macrophages, and Pla2g5-derived FFAs, as activators of ILC2s, acting in concert with IL-33. It is likely that the coordinate action of ILC2s, macrophages and epithelial cells induces pulmonary inflammation, highlighting a complex interplay of innate cells in the lung.\textsuperscript{4,12,46} These data also suggest that FFAs directly activate ILC2s through FFAR1, which is expressed on ILC2s in a yet to be identified Pla2g5-dependent fashion. However, it is likely that additional Pla2g5-generated factors might regulate type 2 immunity. Thus, our observations suggest that macrophage-derived FFAs amplify innate, IL-33-triggered type 2 immunopathology in diseases such as asthma. We speculate that LA, derived at least in part from Pla2g5-expressing macrophages, may contribute to the function of ILC2s in other circumstances, such as homeostasis of adipose tissue and glucose metabolism where macrophages, Pla2g5, IL-33, and ILC2s have all been implicated\textsuperscript{22,47}.  

**METHODS**

**Lung inflammation.** C57BL/6 Wt and Pla2g5-null mice\textsuperscript{48,49} (9–12-week-old males) received 25 μg of Alternaria alternata extract (Greer Laboratories, Lenoir, NC) in 20 μl of PBS or PBS alone intranasally (i.n.) on days 0, 3, 6 and 9 and killed 18 h later\textsuperscript{4} or a single dose of 100 μg and were killed after 1 or 3 h.\textsuperscript{2} Alternatively, Wt and Pla2g5-null naive mice were given mouse rIL-33 (R&D Systems, Minneapolis, MN) i.n. 100 ng per dose on days 0, 3, 6 and 9 with or without LA (132 nmol\textsuperscript{20} OA (106 nmol) or AA (99 nmol), and mice were killed 18 h after the last dose. All animal experiments were approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute and Brigham and Women’s Hospital (Boston, MA).

**Flow cytometry.** Lungs were manually chopped to approximately 10 mm pieces, then digested in RPMI containing 428 U ml\textsuperscript{−1} Collagenase IV (Worthington, Lakewood, NJ) and 20 mg ml\textsuperscript{−1} DNase I (Roche, Mannheim, Germany) (30 min, 37 °C). After red cell lysis, the obtained cell suspension from single mouse was washed and counted. Cells were blocked (1 h, 4 °C) with 1% of rat anti mouse CD16/CD32 (BD Biosciences, San Jose, CA) and 10% donkey serum and then
stained (1 h, 4 °C) with appropriate Abs: CD45 PerCP/Cy5.5 (clone 30-F11, BioLegend, San Diego, CA), CD19 FITC (6D5, BioLegend), CD3 FITC (145-2C11, BioLegend), CD11b FITC (M1/70, BioLegend), CD11c PE-Cy7 FITC (N418, BioLegend), Ly6G/C FITC FITLISA) (B8-R5, eBiosciences, San Diego, CA), Nk1.1 FITC (PK136, BioLegend), FcεR1 FITC (MAR-1, BioLegend), Siglec-F PE (E50-2440, BD Biosciences), Thy 1.2 APC (53-2.1, eBiosciences), ICOS (C398.4A, eBiosciences), Sca-1 (D7, eBiosciences), CD25 (PC61, eBiosciences), ST2 (clone D18, MD Biosciences, Oxford, UK) followed by PE streptavidin (eBiosciences). In selected experiments cells were fixed with 4% paraformaldehyde (7 min, 21 °C), washed, permeabilized with 0.1% saponin (Sigma-Aldrich, St Louis, CA) (7 min, 21 °C) and stained with CD68 APC (FA-11, AbD Serotec, Raleigh, NC), IL-5 PE (TRFK5, Biolegend), IL-13 (eBio13A, Biolegend) antibodies or appropriate isotypes controls at 4 °C. Alternatively, cells were permeabilized with BD Cytofix/CytoPerm kit (BD Biosciences). The acquisition was performed on a FACSAria flow cytometer with FACSDiva software (BD Biosciences), and data were analyzed with FlowJo (Tree Star, Ashland, OR).

Airways analysis and lung cell processing. Bronchoalveolar lavage was performed with 0.7 ml PBS (Sigma-Aldrich) containing 0.5 mM ethylenediaminetetraacetic acid (EDTA) (three times). The bronchoalveolar lavage fluid was collected, and cell-free supernatant was aliquoted and frozen. Enzyme-linked immunosorbent assay (ELISA) was used to measure IL-33 (R&D Systems) and IL-5 (R&D Systems) in selected experiments. Lung macrophages were enriched by Percoll gradients of lung homogenates pulled from 3 to 4 Alternaria-treated WT or Pla2g5-null mice. Cells were then counted and assayed by qPCR or Mass Spectrometry.

Western blot. Right lungs were collected at the time of killing and snap frozen. Proteins were isolated from tissue homogenates in RIPA buffer (Boston Bioproducts, Ashland, MA) with protease inhibitors. The protein concentration in cell lysates was measured using the BCA Assay (Thermo Scientific, Rockford, IL). Twenty micrograms of protein concentration in cell lysates was measured using the BCA assay (Thermo Scientific, Rockford, IL). Twenty micrograms of protein concentration in cell lysates was measured using the BCA assay (Thermo Scientific, Rockford, IL). Twenty micrograms of protein concentration in cell lysates was measured using the BCA assay (Thermo Scientific, Rockford, IL). Twenty micrograms of protein concentration in cell lysates was measured using the BCA assay (Thermo Scientific, Rockford, IL). Twenty micrograms of protein concentration in cell lysates was measured using the BCA assay (Thermo Scientific, Rockford, IL). Twenty micrograms of protein concentration in cell lysates was measured using the BCA assay (Thermo Scientific, Rockford, IL). Twenty micrograms of protein concentration in cell lysates was measured using the BCA assay (Thermo Scientific, Rockford, IL). Twenty micrograms of protein concentration in cell lysates was measured using the BCA assay (Thermo Scientific, Rockford, IL). Twenty micrograms of protein concentration in cell lysates was measured using the BCA assay (Thermo Scientific, Rockford, IL). Twenty micrograms of protein concentration in cell lysates was measured using the BCA assay (Thermo Scientific, Rockford, IL). Twenty micrograms of protein concentration in cell lysates was measured using the BCA assay (Thermo Scientific, Rockford, IL).

ILC2 cells sorting and culture. WT and Pla2g5-null mice received four doses of 25 μg of Alternaria in 20 μl of PBS i.n. on days 0, 3, 6 and 9 and killed 18 h later in order to expand ILC2s prior to FACs sorting. Sorting of ILC2s (CD45+ Lin- (CD3, CD19, Ly6g, CD11c, CD11b, Nk1.1, FcεR1+), Thy1.2+ (Thy1.2+) ) was performed using a FACSDiva 8.0.1 cell sorter (BD Biosciences). Purified CD45+ Lin- Thy1.2+ cells (>98%) were rested for 40 h with 10 ng ml−1 IL-2 and IL-7 (R&D Systems) in 96 well round bottom plates (20,000 cells per well). Prior to stimulation, the medium was changed to fresh medium. ILC2s were cultured with 30 ng ml−1 IL-33 (R&D Systems), 200 μM Linoleic Acid (Cayman Chemical) or 200 μM Oleic Acid (Cayman Chemical, Ann Arbor, MI)22 or all together for 8 h. For intracellular cytokine staining, 1 μl ml−1 of Golgi Plug (BD Biosciences) was added to ILC2s 6 h before collection for FACS analysis.

ELISA. WT and Pla2g5-null mice sorted ILC2s were obtained as described above and rested for 40 h with 10 ng ml−1 IL-2 and IL-7 in a 96-well round bottom plates (40,000 cells per well). After changing to fresh media, ILC2s were stimulated with 30 ng ml−1 IL-33 or 30 ng ml−1 IL-33, 200 μM Linoleic Acid, 200 μM Oleic Acid22 for 8 h. Supernatants were then collected. IL-5 ELISA (R&D Systems, M5000) was performed as per the manufacturer’s protocol. For these experiments, during lung homogenization, Dispase (Gibco, Life Technologies, NY) was added (2 U ml−1) to increase the yield for ILC2s.

Real-time PCR. Total RNA was isolated from lysate with the RNeasy Micro Kit (Qiagen, Louisville, KY), reverse transcribed into cDNA (High-Capacity cDNA Reverse Transcription Kit; Thermo
science-Applied Biosystems, Foster City, CA) and measured by real-time PCR with the use of SYBR Green/ROX master mix (SA Biosciences, Frederick, MD) on an Mx3005P thermal cycler (Stratagene, Santa Clara, CA). The ratio of each mRNA to the Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) mRNA was calculated with the 2^(-ΔΔCT) threshold cycle method. The mouse primers used were GAPDH F: 5'-TCAACAGCAAATCCACCTTTCCA-3'; R: 5'-ACCTGGTGTCATGGACCTTATCCA-3'. Pla2g5 F: 5'-TGTGTCCTGGCCTGAGTGTG-3'; R: 5'-TTGCAGATGACTAGCGGCATTT-3'. IL-33 F: 5'-TCCACACAGGAAGACCAAGAAG-3'; R: 5'-GATAG TGCCAAAGCAAGGAT-3'. 

Real-time PCR products were run on a 1.5% agarose gel and visualized using chemilumager 4400 fluorescence system (Alpha Innotech, Missouri, TX).

**Statistical analysis.** Comparisons between two groups were made by using unpaired Student's t-test. To compare three or more groups, we performed one-way ANOVA or two-Way ANOVA with Sidak’s correction for multiple comparisons. Comparisons were performed with Prism software (GraphPad, La Jolla, CA). Data are expressed as mean ± s.e.m., and P < 0.05 was considered significant.

**SUPPLEMENTARY MATERIAL** is linked to the online version of the paper at [http://www.nature.com/mi](http://www.nature.com/mi)

**ACKNOWLEDGMENTS**

We thank Jennifer Zacharia and Jannatul Firdous for technical assistance.

**AUTHOR CONTRIBUTIONS**

Acquisition of data: M.Y., S.K.S., O.Q., J.A.B., B.B. Analysis and interpretation: M.Y., S.K.S., O.Q., J.A.B. Writing the paper: M.Y., S.K.S., O.Q., J.A.B., B.B.

**DISCLOSURE**

Dr Balestrieri received a patent relating to the subject matter discussed in this article. The other authors declared no conflict of interest.

© 2018 Society for Mucosal Immunology

**REFERENCES**

1. Doherty, T. A. et al. STAT6 regulates natural helper cell proliferation during lung inflammation initiated by Alternaria. *Am. j. physiol. Lung cell. mol. physiol.* 303, L577–L588 (2012).

2. Kouzaki, H., Iijima, K., Kobayashi, T., O'Grady, S. M. & Kita, H. The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses. *J. immunol.* 186, 4376–4387 (2011).

3. Bartemes, K. R., Iijima, K., Kobayashi, T., Kephart, G. M., McKenzie, A. N. & Kita, H. IL-33-responsive lineage-CD25+CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. *J. immunol.* 188, 1503–1513 (2012).

4. Halim, T. Y. et al. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 40, 425–435 (2014).

5. Doherty, T. A. et al. Alternaria induces STAT6-dependent acute airway eosinophilia and epithelial FIZZ1 expression that promotes airway fibrosis and epithelial thickness. *J. immunol.* 188, 2622–2629 (2012).

6. Neill, D. R. et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464, 1367–1370 (2010).

7. Moro, K. et al. Innate production of TH2 cytokines by adipose tissue-associated c-Kit+Scal-1+ lymphoid cells. *Nature* 463, 540–544 (2010).

8. Price, A. E. et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc. Natl. Acad. Sci. USA* 107, 11489–11494 (2010).

9. Doherty, T. A., Khorrham, N., Lund, S., Mehta, A. K., Crotf, M. & Broide, D. H. Lung type 2 innate lymphoid cells express cysteinyI leukotriene receptor 1, which regulates TH2 cytokine production. *J. allergy clin. immunol.* 132, 205–213 (2013).

10. Chang, Y. J. et al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat. immunol.* 12, 631–638 (2011).

11. Halim, T. Y., Krauss, R. H., Sun, A. C. & Takei, F. Lung natural helper cells are a critical source of TH2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* 36, 451–463 (2012).

12. Barlow, J. L. et al. In innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. *J. allergy clin. immunol.* 129, 191–198. e191–194 (2012).

13. Licona-Limon, P., Kim, L. K., Palm, N. W. & Flavell, R. A. TH2 allergy and group 2 innate lymphoid cells. *Nat. immunol.* 14, 536–542 (2013).

14. Molofsky, A. B. et al. Innate lymphoid type 2 cells sustain visceral adipose tissue eosinophils and alternatively activated macrophages. *J. exp. med.* 210, 535–549 (2013).

15. Hardman, C. S., Panova, V. & McKenzie, A. N. IL-33 citrine reporter mice reveal the temporal and spatial expression of IL-33 during allergic lung inflammation. *Eur. j. immunol.* 43, 488–498 (2013).

16. Kim, H. Y., Chang, Y. J., Subramanian, S., Lee, H. H., Alabacker, L. A. & Matangkasombut, P. et al. Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity. *J. allergy clin. immunol.* 129, 216–227. e211–216 (2012).

17. Polumuri, S. K. et al. Transcriptional regulation of murine IL-33 by TLR and non-TLR agonists. *J. immunol.* 189, 50–60 (2012).

18. Dennis, E. A., Cao, J., Hsu, Y. H., Magrilli, V. & Kokotos, G. Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chem. rev.* 111, 6130–6185 (2011).

19. Murakami, M., Taketomi, Y., Miki, Y., Sato, H., Yamamoto, K. & Lambeau, G. Emerging roles of secreted phospholipase A2 enzymes; the 3rd edition. *Biochimie* 107PA, 105–113 (2014).

20. Alvarez-Curto, E. & Milligan, G. Metabolism meets immunity: the role of free fatty acid receptors in the immune system. *Biochem. pharmacol.* 114, 3–13 (2016).

21. Murakami, M. et al. Distinct arachidonate-releasing functions of mammalian secreted phospholipase A2s in human embryonic kidney 293 and rat mastocytoma RBL-2H3 cells through hepatic sulfation and external plasma membrane mechanisms. *J. biol. chem.* 276, 10083–10096 (2001).

22. Sato, H. et al. The adipocyte-inducible secreted phospholipases PLA2G5 and PLA2G2E play distinct roles in obesity. *Cell metab.* 20, 119–132 (2014).

23. Singer, A. G. et al. Interfacial kinetic and binding properties of the complete set of human and mouse TLR1, TLR2, TLR4, and TLR8 secreted phospholipases A2. *J. biol. chem.* 277, 48535–48549 (2002).

24. Giannattasio, G., Fujikoa, D., Xing, W., Katz, H. R., Boyce, J. A. & Balestrieri, B. GroupV secretory phospholipase A2 reveals its role in house dust mite-induced allergic pulmonary inflammation by regulation of dendritic cell function. *J. immunol.* 185, 4430–4438 (2010).

25. Ohta, S., Imamura, M., Xing, W., Boyce, J. A. & Balestrieri, B. Group V secretory phospholipase A2 is involved in macrophage activation and is sufficient for macrophage effector functions in allergic pulmonary inflammation. *J. immunol.* 190, 5927–5938 (2013).

26. Yamaguchi, M., Zacharia, J., Laidlaw, T. M. & Balestrieri, B. PLA2G5 regulates transglutaminase activity of human IL-4–activated M2 macrophages through PGE2 generation. *J. leukoc. Biol.* 100, 131–141 (2016).

27. Munoz, N. M., Melton, A. Y., Arm, J. P., Bonventre, J. V., Cho, W. & Leff, A. R. Deletion of secretory group V phospholipase A2 attenuates cell migration and airway hyperresponsiveness in immunosensitized mice. *J. immunol.* 179, 4800–4807 (2007).

28. Wojno, E. D. et al. The prostaglandin D(2) receptor CRTH2 regulates accumulation of group 2 innate lymphoid cells in the inflamed lung. *Mucosal Immunol.* 8, 1313–1323 (2015).

29. Stevens, W. W., Kim, T. S., Pujanuskit, L. M., Hsieh, X. & Braciale, T. J. Detection and quantitation of eosinophils in the murine respiratory tract by flow cytometry. *J. immunol. methods* 327, 63–74 (2007).

30. Spits, H. et al. Innate lymphoid cells—a proposal for uniform nomenclature. *Nat. rev. Immunol.* 13, 145–149 (2013).
31. Walker, J. A., Barlow, J. L. & McKenzie, A. N. Innate lymphoid cells—how did we miss them? Nat. rev. Immunol. 13, 75–87 (2013).
32. Makrinioti, H., Toussaint, M., Jackson, D. J., Walton, R. P. & Johnston, S. L. Role of interleukin 33 in respiratory allergy and asthma. Lancet Respir. med. 2, 226–237 (2014).
33. Gorski, S. A., Hahn, Y. S. & Braciale, T. J. Group 2 innate lymphoid cell production of IL-5 is regulated by NKT cells during influenza virus infection. PLoS pathog. 9, e1003615 (2013).
34. Quehenberger, O., Armando, A. M. & Dennis, E. A. High sensitivity quantitative lipidomics analysis of fatty acids in biological samples by gas chromatography-mass spectrometry. Biochim. biophys. acts 1811, 648–656 (2011).
35. Briscoe, C. P. et al. The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. J. biol. chem. 278, 11303–11311 (2003).
36. Itoh, Y. et al. Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. Nature 422, 173–176 (2003).
37. Kotansky, K., Nilsson, N. E., Fodgren, E., Owman, C. & Olde, B. A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs. Biochem. biophys. res. commun. 301, 406–410 (2003).
38. Hirasawa, A. et al. Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. Nat. med. 11, 90–94 (2005).
39. Oh, & da, et al. A Gpr120-selective agonist improves insulin resistance and chronic inflammation in obese mice. Nat. med. 20, 942–947 (2014).
40. Barlow, J. L., Peel, S., Fox, J., Panova, V., Hardman, C. S. & Carmelo, A. et al. IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. J. allergy clin. immunol. 132, 933–941 (2013).
41. Halim, T. Y., Mano,M., Romanish, M. T., Gold, M. J., McNagny, K. M. & Takei, F. Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell development and allergic inflammation. Immunity 37, 463–474 (2012).
42. Wills-Karp, M. et al. Trefoil factor 2 rapidly induces interleukin 33 to promote type 2 immunity during allergic asthma and hookworm infection. J. exp. med. 209, 607–622 (2012).
43. Yu, S., Kim, H. Y., Chang, Y. J., DeKruyff, R. H. & Umetsu, D. T. Innate lymphoid cells and asthma. J. allergy clin. immunol. 133, 943–950 (2014). quiz 951.
44. Christiansen, E. et al. Activity of dietary fatty acids on FFA1 and FFA4 and characterisation of pinolenic acid as a dual FFA1/FFA4 agonist with potential effect against metabolic diseases. Br. j. nutr. 113, 1677–1688 (2015).
45. Heng, T. S. & Painter, M. W. Immunological Genome Project C. The Immunological Genome Project: networks of gene expression in immune cells. Nat. immunol. 9, 1091–1094 (2008).
46. Van Dyken, S. J. et al. Chitin activates parallel immune modules that direct distinct inflammatory responses via innate lymphoid type 2 and gamma-delta T cells. Immunity 40, 414–424 (2014).
47. Lee, M. W. et al. Activated type 2 innate lymphoid cells regulate beige fat biogenesis. Cell 160, 74–87 (2015).
48. Satake, Y. et al. Role of group V phospholipase A2 in zymosan-induced eicosanoid generation and vascular permeability revealed by targeted gene disruption. J. biol. chem. 279, 16488–16494 (2004).
49. Balestrieri, B. et al. Group V secretory phospholipase A2 translocates to the phagosome after zymosan stimulation of mouse peritoneal macrophages and regulates phagocytosis. J. biol. chem. 281, 6691–6698 (2006).
50. Quehenberger, O. et al. Lipidomics reveals a remarkable diversity of lipids in human plasma. J. lipid res. 51, 3299–3305 (2010).