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Macrophages regulate lung ILC2 activation via Pla2g5-dependent mechanisms

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Abstract

Group V phospholipase A_2 (*Pla2g5*) is a lipid-generating enzyme necessary for macrophage effector functions in pulmonary inflammation. However, the lipid mediators involved and their cellular targets have not been identified. Mice lacking Pla2g5 showed markedly reduced lung ILC2 activation and eosinophilia following repetitive *Alternaria Alternata* inhalation. While *Pla2g5*-null mice had Wt levels of immediate IL-33 release after one *Alternaria* dose, they failed to upregulate IL-33 in macrophages following repeated *Alternaria* administration. Unexpectedly, while adoptive transfer of bone marrow-derived (BM)-macrophages restored ILC2 activation and eosinophilia in *Alternaria*-exposed *Pla2g5*-null mice, exogenous IL-33 did not. Conversely, transfers of *Pla2g5*-null BM-macrophages reduced inflammation in *Alternaria*-exposed Wt mice. Mass spectrometry analysis of free fatty acids (FFAs) demonstrated significantly reduced FFAs (including linoleic acid (LA) and oleic acid (OA)) in lung and BM-macrophages lacking Pla2g5. Exogenous administration of LA or LA+OA to Wt mice sharply potentiated IL-33-induced lung eosinophilia and ILC2 expansion in-vitro and in-vivo. In contrast, OA potentiated IL-33-induced inflammation and ILC2 expansion in *Pla2g5*-null mice, but LA was inactive both in-vivo and in-vitro. Notably, *Pla2g5*-null ILC2s showed significantly reduced expression of the FFA-receptor-1

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Author contributions

Acquisition of data: MY, SS, OQ, BB. Analysis and interpretation: MY, SS, OQ, JB, BB. Drafting or revising manuscript: MY, SS, OQ, JB, BB. Conception and design: BB.

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compared to Wt ILC2s. Thus, macrophage-associated Pla2g5 contributes significantly to type-2 immunity through regulation of IL-33 induction and FFA-driven ILC2 activation.

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 hyperreactivity[1, 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 A_2 (PLA_2) 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[20]. PLA_2s may have substrate preferences and specific cell and tissue expression, therefore serving context-specific functions. Group V PLA_2 (Pla2g5) preferentially releases lysophosphatidylcholine (LPC) and the FFAs linoleic acid (LA) and oleic acid (OA)[21–23], and is prevalently expressed by innate immune cells, including dendritic cells and macrophages[24–26], as well as epithelial cells[25–27]. 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[24, 25]. Adoptive transfer studies showed that Pla2g5 expression by macrophages was required for their generation of CCL22 and recruitment of T cells into the lungs[25]. 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[9, 28], 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-preferring 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* (25μg/dose) every two days for four doses and lungs were collected 18h after the last dose[1]. 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)[29] in *Alternaria*-treated Wt lungs was significantly higher than in *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[9] (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 equally 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^- $Thy1.2^+$ $Sca\text{-}1^+$ cells to exclude at least contaminating ILC3[30, 31] (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[32]. 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 BAL 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 4 doses of *Alternaria* using western blotting. We found that naive Wt and *Pla2g5*-null mice released similar amounts of IL-33 into BAL at 1 and 3h 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 the naive mice. After ten days and four doses of *Alternaria*, Wt lungs had increased amounts of IL-33 protein compared to naive mice, and both the preformed full-length form (34 kDa) and the proteolytically processed short length form (18kDa) 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[15], 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[10] and in the recovery phase of IAV infection[33], 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 $CD68^+$/$IL\text{-}33^+$ 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 BM-macrophages stimulated with GM-CSF, IL-4, and IL-33[25], and also by lung macrophages enriched from *Alternaria*-treated Wt and *Pla2g5*-null mice using consecutive Percoll gradients[25]. 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).

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 (100ng/dose, Figure 3, inset) robustly increased the numbers of eosinophils, ILC2s and $Sca\text{-}1^+$ ILC2s in Wt mice[3]. Surprisingly, *Pla2g5*-null mice showed markedly diminished numbers of eosinophils, total ILC2s, and $Sca\text{-}1^+$ 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[25], 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 24h before the second dose of *Alternaria*, then administered 3 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 in 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[34]. 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%[25]. 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 FACS plots), although the numbers of IL5$^+$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 2-fold 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-esposed 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 40h, and stimulated with LA, OA, IL-33 or a combination for 8h. 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)[35–39]. 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[2, 4, 9, 11, 40, 41] in response to environmental proteases[11], many of which are relevant to asthma in humans[13]. IL-33, alone and in combination with IL-25, TSLP, and other cytokines can directly induce IL-5, IL-13, and IL-9 generation from ILC2s, promoting eosinophilic inflammation and goblet cell metaplasia that can occur independently of or in concert with adaptive immunity. The *Alternaria* model of pulmonary inflammation has been particularly useful to establish the contribution of innate, epithelial-derived alarmins and their downstream effects on ILC2 activation and subsequent development of airway inflammation[1, 3]. While macrophages can also express IL-33[42], and other innate cell types have been proposed to interact with ILC2s[43], 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[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 (Figure 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*[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[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[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 (Figure 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 5A and B) 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 to OA[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[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[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[22, 47].

Methods

Lung inflammation

C57/BL6 Wt and *Pla2g5*-null mice[48, 49] (9–12 wk-old males) received 25 μg of *Alternaria alternata* extract (Greer Laboratories, Lenoir, NC) in 20 uL of PBS or PBS alone intranasally (i.n.) on days 0,3,6 and 9 and euthanized 18h later[1] or a single dose of 100 μg and were euthanized after 1h or 3h[2]. Alternatively, Wt and *Pla2g5*-null naïve mice were given mouse rIL-33 (R&D Systems, Minneapolis, MN) i.n. 100 ng/dose on days 0,3,6 and 9 with or without LA (132 nM)[28] OA (106 nM) or AA (99 nM), and mice were euthanized 18h after the last dose. All animal experiments were approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute (Boston, MA).

Flow cytometry

Lungs were manually chopped to approximately 10mm pieces, then digested in RPMI containing 428 U/ml Collagenase IV (Worthington, Lakewood, NJ) and 20 mg/ml DNAse I (Roche, Mannheim, Germany) (30min, 37°C). After red cell lysis, the obtained cell suspension from single mouse was washed and counted. Cells were blocked (1h, 4 °C) with 1% of rat anti mouse CD16/CD32 (BD Biosciences, San Jose, CA) and 10% donkey serum and then stained (1h, 4 °C) with appropriate Abs: CD45 PercPCy5 (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 (RB6-8C5, eBiosciences), Nk1.1 FITC (PK136, Biolegend), FcεR1 FITC (MAR-1, Biolegend), Siglec-F PE (E50-2440, BD Bioscience), Thy 1.2 APC (53–2.1, eBioscience, San Diego, Ca), ICOS (C398.4A, eBiosciences), Sca-1 (D7, eBioscieces), CD25 (PC61, eBiosciences), ST2 biotin (clone DJ8, MD Bioscience) followed by PE streptavidin (eBiosciences). In selected experiments cells were fixed with 4% paraformaldehyde (7 min, 21°C), washed, permeabilized with 0.1% saponin (SigmaAldrich, 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 eBiosciences), IL-33 PE (396118, R&D Systems, Minneapolis, MN), rabbit polyclonal anti murine RELM-α (Peprotech, Rocky Hill NJ)) and corresponding isotypes as controls. Alternatively, cells were permeabilized with BD Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA). The acquisition was performed on a FACSCanto 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 (BAL) was performed with 0.7 mL PBS (Sigma-Aldrich) containing 0.5 mM EDTA (three times). The BAL fluid was collected, and cell-free supernatant was aliquoted and frozen. ELISA was used to measure IL-33 (R&D Systems). In selected experiments lung macrophages were enriched by Percoll gradients[25] of lung homogenates pulled from 3–4 *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 euthanasia and snap frozen. Proteins were isolated from tissue homogenates in RIPA buffer (Boston Bioproducts, Ashland, MA, USA) with protease inhibitors[26]. The protein concentration in cell lysates was measured using the BCA Assay (Pierce, Thermo Scientific). 20 μg of proteins were separated on a 10–20% Tris-Glycine gel (Novex, Life Technologies) and transferred to a PVDF membrane. After blocking overnight at 4°C in 5% milk, blots were incubated with a goat polyclonal IL-33 (1:500, R&D Systems) or mouse monoclonal β-actin (1:1000, Cell Signaling, Danvers, MA) antibodies diluted in TBST at RT for 2h, followed by a rabbit anti-goat or goat anti-mouse secondary antibody (1:3000, BioRad) diluted in TBST for 1h at RT. The blots were visualized using the Supersignal West Femto Chemiluminescent substrate (Thermo Scientific) and imaged by a KODAK M35A X-OMAT processor.

Frozen sections

Lungs of Wt and *Pla2g5*-null mice were excised and immersed in RPMI. Within 1h of surgery, the tissue was removed from RPMI and fixed in 4% paraformaldehyde, then embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Finetek), and kept at −80°C until sectioning. Sections of 5-μm thickness were freshly cut, thaw-mounted onto slides, and stained for confocal microscopy. Frozen sections were rehydrated for 1h at RT then blocked with 10% donkey serum, followed by incubation with goat polyclonal IL-33 (AF3626, R&D Systems) and rabbit polyclonal proSPC (AB3786, Millipore, Temecula, CA) antibodies or appropriate isotypes controls at 4°C, overnight. Samples were washed, incubated at RT for 1h with appropriate secondary antibodies, washed and covered with Fluoroshield mounting media (Electron Microscopy Sciences, Hatfield, PA). Sections were imaged using a Nikon C1 plus laser scanner confocal system with a 40× oil Plan-Fluor NA1.3 objective lens. 8–10 Z-stack images of 0.5 μm were acquired through a small pinhole using Nikon EZ-C1 software. Images were analyzed using Image J (U.S. National Institute of Health, Bethesda, MD).

BM macrophage transfer

Wt or *Pla2g5*-null bone marrow (BM) cells were collected from femurs and tibiae of mice. The disaggregated cells were counted and suspended in complete medium (DMEM F12, 5%FBS, 100U/ml penicillin, 100ug/ml streptomycin, 0.1mM nonessential amino acids, 2mM L-glutamine and 0.05 μM 2-ME) containing 50ng/ml murine r-MCSF (PeproTech) at a concentration of 4.0×10^6 cells/ml in a 10ml/Petri dish. On day 3, 10 ml of medium containing r-MCSF were added to each dish. On day 7, cells were harvested with PBS containing Lidocaine (4mg/ml, 15min, 37C) and resuspended at concentration of 5×10^6 cells/ml in PBS. For adoptive transfer, 1×10^5 Wt or *Pla2g5*-null BM-macrophages were transferred i.t. into Wt and *Pla2g5*-null mice two days after the first dose of *Alternaria* followed by 3 more doses of *Alternaria* (25 μg in 20 μl PBS) i.n. on day 3, 6 and 9. Mice were euthanized 18h after the last dose.

Mass Spectrometry of lipids

Wt and *Pla2g5*-null BM-macrophages were cultured for 7 days in r-MCSF. Adherent cells were collected, frozen and shipped for analysis by mass spectrometry. In another set of experiments lung macrophages were enriched by multiple Percoll gradients[25]. Free fatty acid analysis was performed according to a previously published method[34, 50]. Briefly, the cell pellet was homogenized in 500 ul of PBS/10% methanol. An aliquot of 200 μl corresponding to about 0.5×10^6 cells was withdrawn and a cocktail of internal standards consisting of 15 deuterated fatty acids was added. The extraction was initiated with 500 μl of methanol and 25 μl of 1N HCl and a bi-phasic solution is formed by addition of 1.5 ml of isooctane. The phases are separated by centrifugation and the isooctane phase containing the free fatty acids FFA fraction was removed. The extraction is repeated once and the combined extracts are evaporated to dryness. The free fatty acids were derivatized with pentafluorobenzyl (PFB) bromide and the resulting fatty acid PFB esters were analyzed by gas chromatography/mass spectrometry using a negative chemical ionization mode (Agilent 6890N gas chromatograph equipped with an Agilent 5973 mass selective detector; Agilent,

Santa Clara, CA). Standard curves for each of the fatty acids were acquired in parallel using identical conditions. The quantitative assessment of fatty acids in a sample was achieved by comparison of the mass spectrometric ion signal of the target molecule normalized to the internal standard with the matching standard curve according to the isotope dilution method and by protein content[34].

ILC2 cells sorting and culture

Wt and *Pla2g5*-null mice received four doses of 25ug of *Alternaria* in 20ul of PBS i.n. on day 0, 3, 6 and 9 and euthanized 18h later in order to expand ILC2s prior to FACs sorting. Sorting of ILC2s ($CD45^+$ Lin− (CD3, CD19, Ly6g, CD11c, CD11b, Nk1.1, $Fc\varepsilon R1^-$), $Thy1.2^+$) was performed using a FACSDiva 8.0.1 cell sorter (BD Bioscience). Purified $CD45^+$ lin-$Thy1.2^+$ cells (>98%) were rested for 40h with 10ng/mL rIL-2 and rIL-7 (R&D Systems, Minneapolis, MN) in 96 well around bottom plates (20000 cells per well). Prior to stimulation, the medium was changed to fresh medium. ILC2s were cultured with 30ng/mL rIL-33 (R&D Systems), 200 μM Linoleic Acid (Cayman Chemical) or 200 μM Oleic Acid (Cayman Chemical)[22] or all together for 8h. For intracellular cytokine staining, 1 μl/mL of Golgi Plug (BD Bioscience) was added to ILC2s 6h before collection for FACs analysis.

ELISA

Wt and *Pla2g5*-null mice sorted ILC2s were obtained as described above and rested for 40h with 10ng/mL rIL-2 and rIL-7 in a 96 well round bottom plates (40000 cells per well). After changing to fresh media, ILC2s were stimulated with 30ng/mL rIL-33 or 30ng/mL rIL-33, 200 μM Linoleic Acid, 200 μM Oleic Acid for 8h. Supernatants were then collected. IL-5 ELISA (R&D Systems, M5000) was performed as per manufacturer protocol. For these experiments, during lung homogenization, Dispase (Gibco, Life Technologies, NY) was added (2U/ml) to increase the yield for ILC2s.

Real-time PCR

Total RNA was isolated from lysate with the RNeasy Micro Kit (Qiagen, Louisville, KY, USA), reverse transcribed into cDNA (High-Capacity cDNA Reverse Transcription Kit; Thermo science-Applied Biosystems, Foster City, CA, USA) and measured by real-time PCR with the use of SYBR Green/ROX master mix (SABiosciences, Frederick, MD, USA) on an Mx3005P thermal cycler (Stratagene, Santa Clara, CA, USA). The ratio of each mRNA relative to the GAPDH mRNA was calculated with the Ct threshold cycle method. The mouse primers used were GAPDH F: TCAACAGCAACTCCCACTCTTCCA; R: ACCCTGTTGCTGTAGCCGTATTCA. Pla2g5 F: TGGTTCCTGGCTTGCAGTGTG; R: TTCGCAGATGACTAGGCCATT. IL-33 F: TCCCAACAGAAGACCAAAG; R: 5′-GATACTGCCAAGCAAGGAT. FFAR1/GPR40 and FFAR4/GPR120 were from Qiagen. Real-time PCR products were run on a 1.5% agarose gel and visualized using chemilmager 4400 fluorscience system (Alpha Innotech, Missouri, TEX, USA)

Statistical analysis

Comparisons between 2 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 ± SEM, and $P<0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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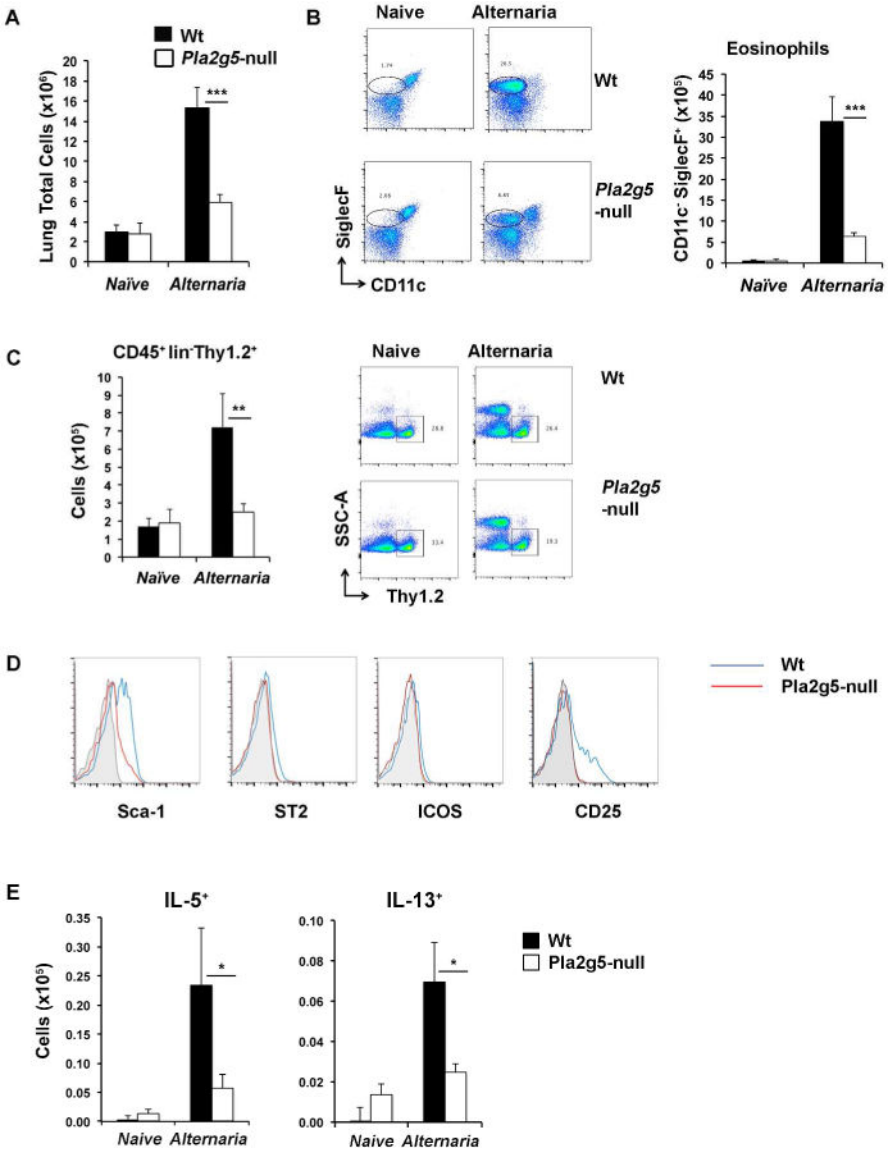

Figure 1. *Alternaria*-induced pulmonary inflammation requires *Pla2g5*

(A) Total cells counts from homogenate lungs of naive and *Alternaria*-treated Wt and *Pla2g5*-null mice. Analysis by flow cytometry of lung cell from naive 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 ± SEM 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.

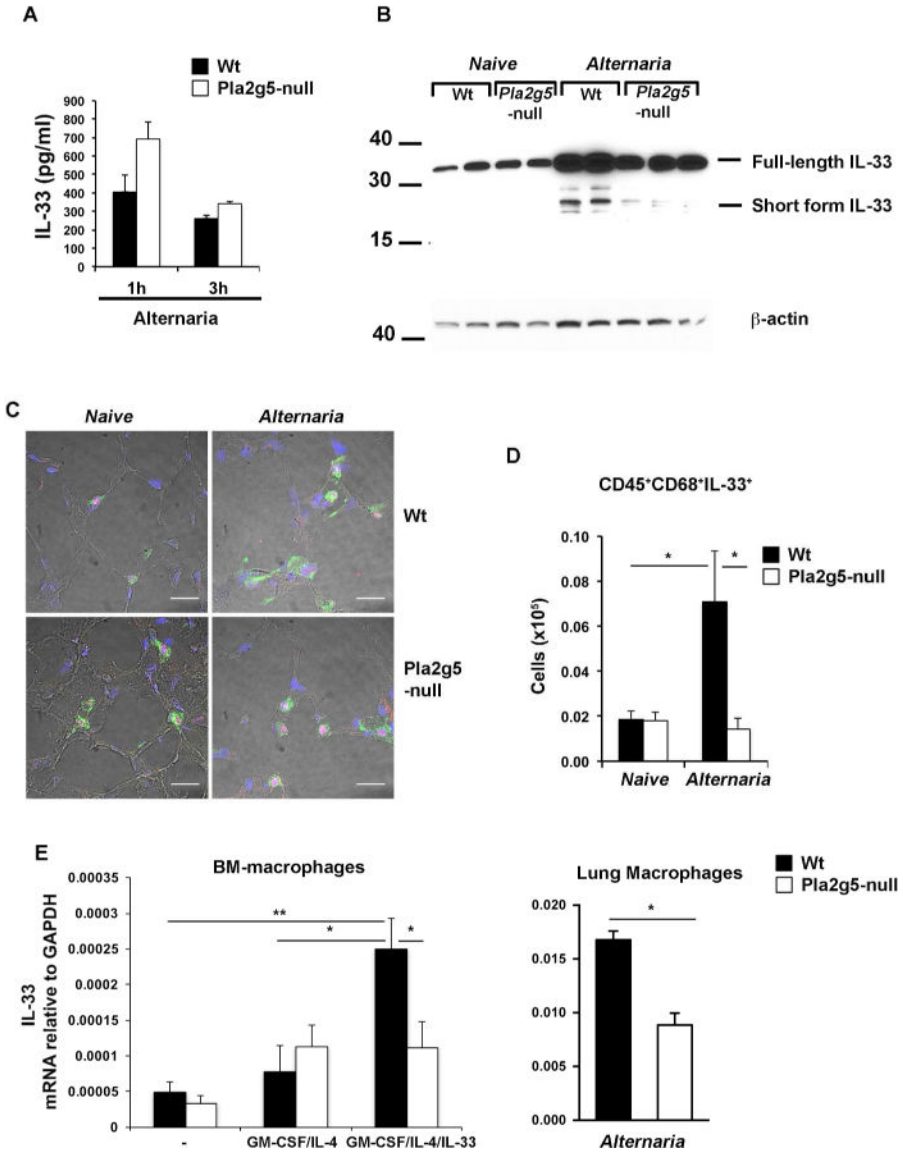

Figure 2. Induced IL-33 generation in the lung requires Pla2g5

(A) IL-33 levels determined by ELISA of BAL 1 or 3 h after *Alternaria* administration in Wt and *Pla2g5*-null mice. (B) Expression of IL-33 protein by Western blot in homogenized lungs of naive and *Alternaria*-treated Wt and *Pla2g5*-null mice treated with *Alternaria* for 10 days (4 doses). Equivalent loading was confirmed by immunoblot analysis for β-actin. (C) Frozen sections from the lungs of Wt and *Pla2g5*-null mice naive or treated with *Alternaria* for 10 days, were stained for IL-33 (red), SPC (green) and nuclei (blue). Original magnification ×40. Size bar 50 μm. (D) Expression of IL-33 on gated $CD68^+$ lung cells of naive and *Alternaria*-treated Wt and *Pla2g5*-null mice evaluated by flow cytometry. (E) Expression of IL-33 mRNA relative to GAPDH measured by qPCR in BM-macrophages unstimulated or stimulated with GM-CSF/IL-33/IL-4 or lung macrophages enriched by Percoll density gradients from 5–8 pulled lung homogenates of *Alternaria*-treated mice. Values are mean ± SEM from two or three independent experiments assayed in duplicate (E)

or with 5–8 mice per group (A and D). (B and C) Images and panels are from one experiment representative of two with similar results. **P< 0.005, *P< 0.05.

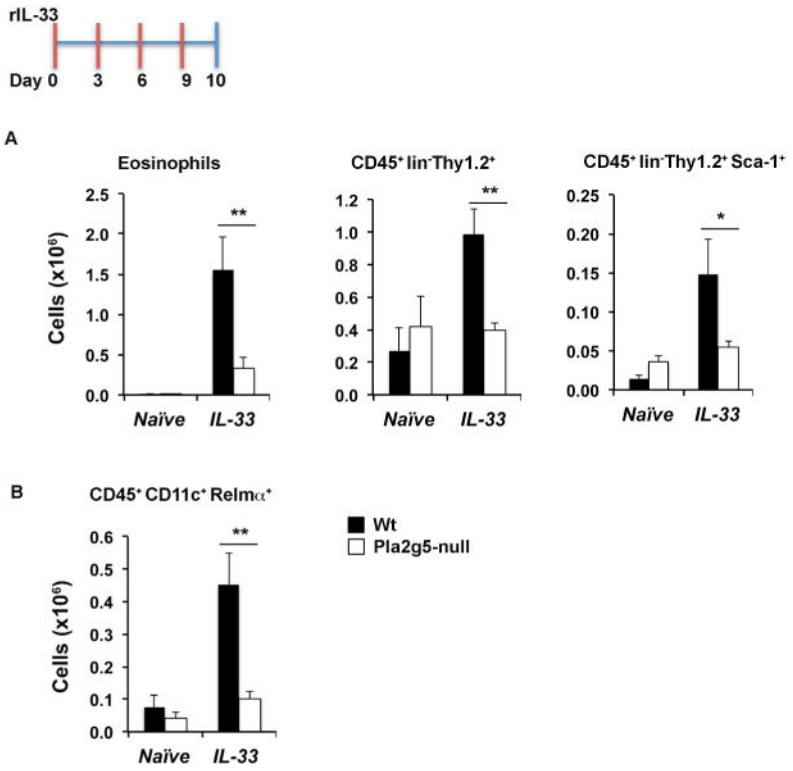

Figure 3. r-IL-33 induces pulmonary inflammation in Wt but not in *Pla2g5*-null mice

Flow cytometry analysis of (A) eosinophils, Thy1.2$^+$ ILC2s and Thy1.2$^+$ ILC2s expressing Sca-1, (B) Relm-α expression on gated CD45$^+$CD11c$^+$ cells from homogenate lungs of Wt and *Pla2g5*-null mice naive or administered r-IL-33 for 10 days. Values are mean ± SEM of two (B) or three (A) independent experiments with 7–15 mice per group. **P< 0.005, *P< 0.05.

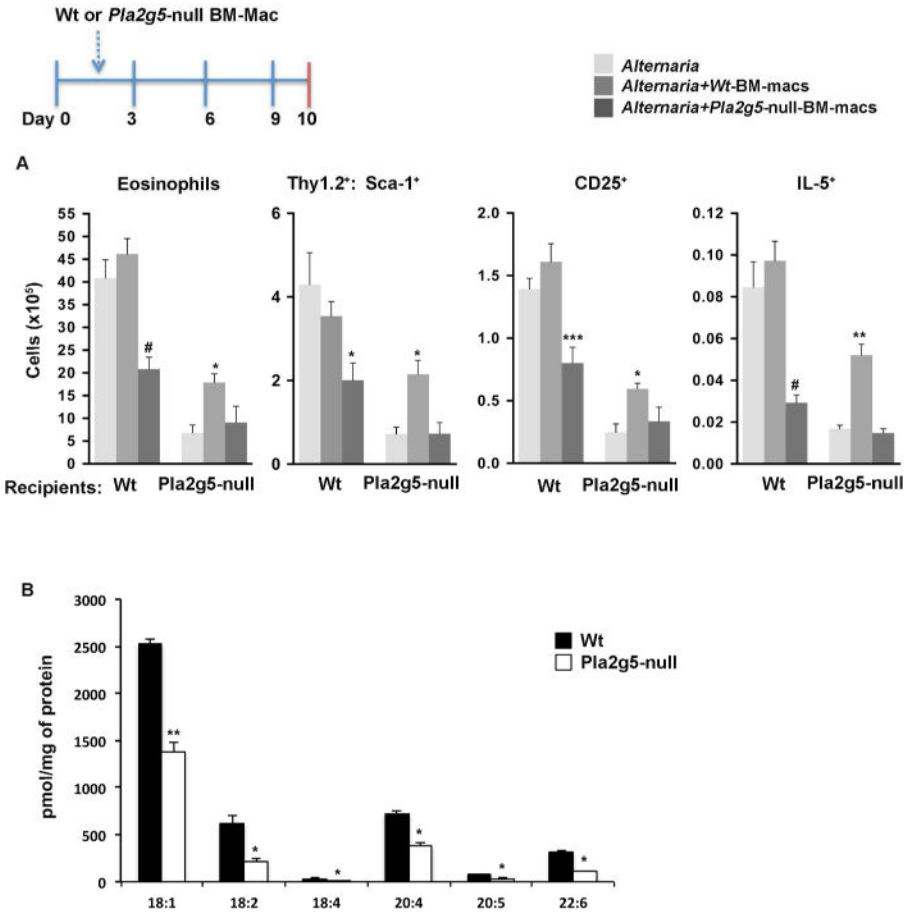

Figure 4. Transfers of Wt and *Pla2g5*-null BM-macrophages regulate *Alternaria*-induced pulmonary inflammation

A) *Pla2g5*-null and Wt recipient mice received Wt (medium grey bars) or *Pla2g5*-null (dark gray bars) BM-macrophages intratracheally at day 2, followed by *Alternaria* i.n. at day 3, 6 and 9 or only *Alternaria* (light grey bars). Mice were euthanized 18 h after the last dose. Analysis by flow cytometry of eosinophils gated as $CD45^+$ $CD11c^-$ $SiglecF^+$ lung cells and expression of CD25, Sca-1 and intracellular IL-5 on $Thy1.2^+$ ILC2s. B) Production of FFAs measured by mass spectrometry in Wt and *Pla2g5*-null BM-Macrophages. (A) Values are mean ± SEM of 2 independent experiments with 10–12 mice per group. (B) Data are from 3 independent experiments. # P<0.0001, *** P<0.0005, **P< 0.005, *P< 0.05.

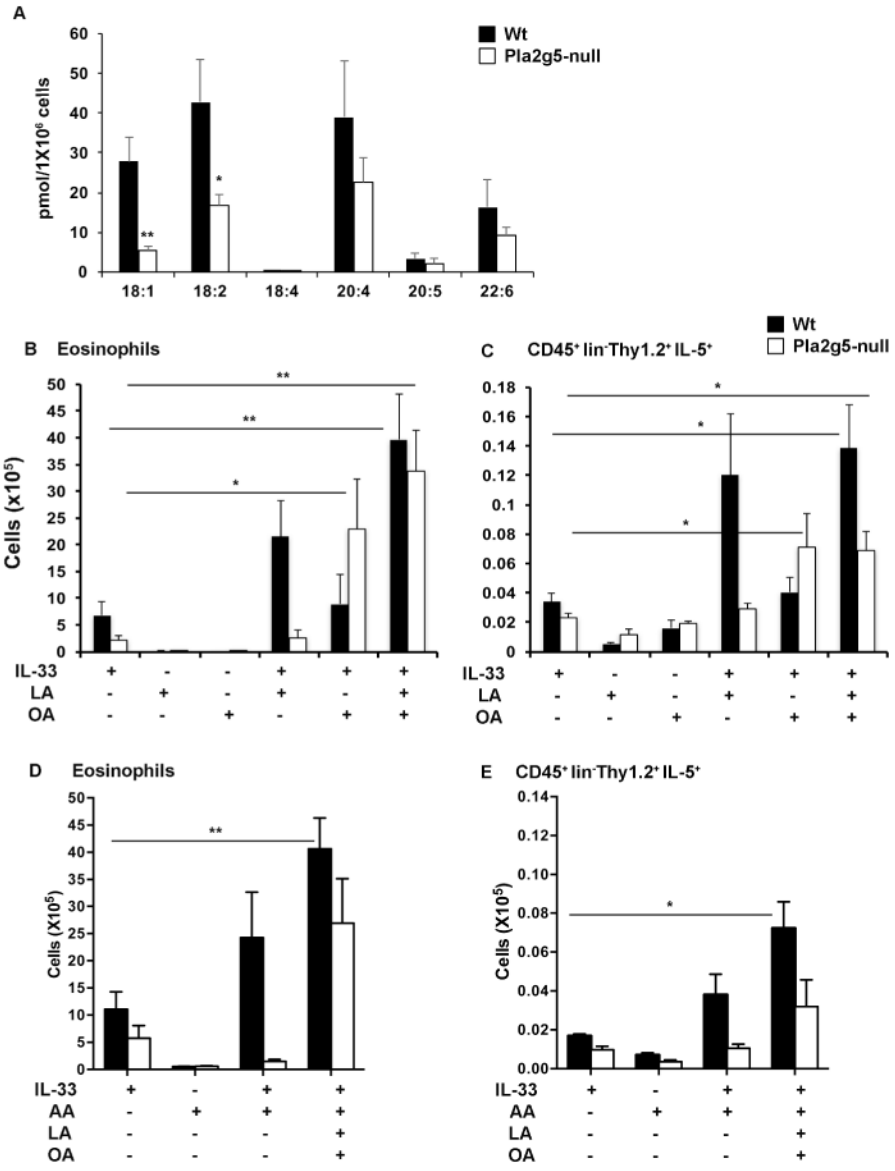

Figure 5. Intranasal administration of LA and OA in combination with r-IL-33 increased eosinophilia and ILC2 activation in Wt and *Pla2g5*-null mice

(A) Production of FFAs measured by mass spectrometry in Wt and *Pla2g5*-null lung macrophages enriched by Percoll gradients of lung homogenates pulled from 5–8 mice. Flow cytometry analysis of (B, D) numbers of eosinophils and (C, E) Thy1.2$^+$ ILC2s expressing intracellular IL-5 in homogenate lungs of Wt (black bars) and *Pla2g5*-null mice (white bars) treated i.n. with IL-33, LA or OA (B, C), or IL-33, LA, OA and AA (D, E) as indicated. Values are mean ± SEM of two independent experiments with 5 samples (A), two-four independent experiments (B, C, D) or one representative experiment (E) with 4–12 mice per group, and were compared by t-test (A) or One-way ANOVA with Sidak's correction for multiple comparisons. **P< 0.005, *P< 0.05.

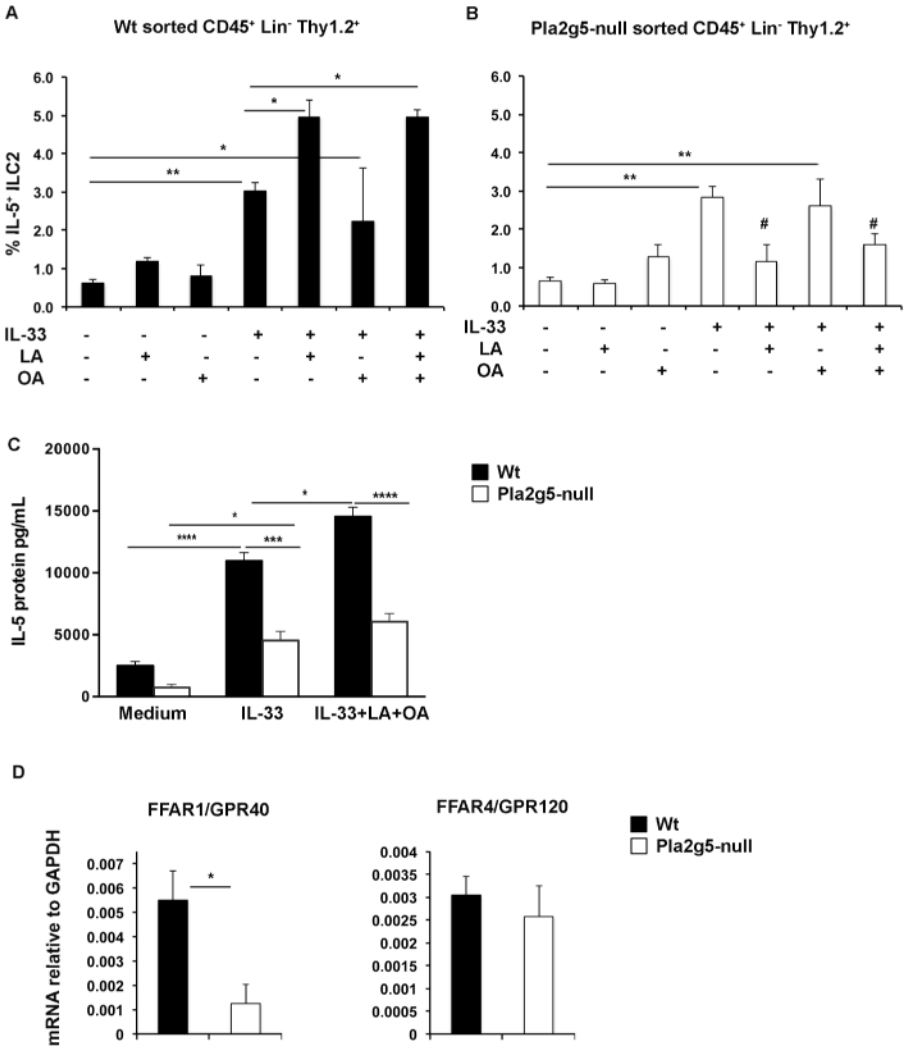

Figure 6. Differential LA- and OA-induced activation of sorted Wt and *Pla2g5*-null ILC2s and FFAR1 expression

ILC2s were expanded in-vivo by 4 *Alternaria* challenges for 10 days. CD45$^+$ lin-, Thy1.2$^+$ cells were FACS sorted from 3–4 pulled lung homogenates, and rested for 40 hours prior to in-vitro stimulation with LA (200 μM), OA (200 μM), IL-33 (30ng/ml) or all together for 8 hours then analyzed by flow cytometry for percentage of IL-5 positive Wt (A) and *Pla2g5*-null (B) ILC2s. Unstimulated cells were used as controls. (C) IL-5 levels were measured by ELISA in the supernatants of sorted Wt and *Pla2g5*-null Thy1.2$^+$ ILC2s stimulated as indicated. (D) Expression of FFAR1 and FFAR4 mRNA relative to GAPDH measured by qPCR in sorted ILC2s from *Alternaria*-treated Wt and *Pla2g5*-null mice. Data are from at least 3 independent experiments. Values are expressed as means ± SEM and were compared by One-way ANOVA (A–B), Two-way ANOVA (C) with Sidak's correction for multiple comparisons, or t-test (D). ****P< 0.0001, *** P<0.0005, **P<0.005, *P< 0.05, # P<0.05 vs. IL-33 alone.