Deletion of PIKfyve alters alveolar macrophage populations and exacerbates allergic inflammation in mice

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Abstract

Alveolar macrophages (AMs) are specialized tissue-resident macrophages that orchestrate the immune responses to inhaled pathogens and maintain organ homeostasis of the lung. Dysregulation of AMs is associated with allergic inflammation and asthma. Here, we examined the role of a phosphoinositide kinase PIKfyve in AMs development and function. Mice with conditionally deleted PIKfyve in macrophages show alteration of AM populations. PIKfyve deficiency results in a loss of AKT activation in response to GM-CSF, a cytokine critical for AM development. These mice display severe lung inflammation and allergic asthma accompanied by infiltration of eosinophils and lymphoid cells after exposure to house dust mite extract. Moreover, they have defects in production of retinoic acid and fail to support Foxp3+ T_reg cells incorporation in lung, resulting in exacerbation of lung inflammation. Thus, PIKfyve plays a role in prevention from excessive lung inflammation through regulating AMs function.
Introduction

Tissue-resident macrophages are heterogeneous cells that have distinct functions in organ homeostasis and innate immune responses to pathogens. In the lung, alveolar macrophages (AMs) reside in the airway lumen or alveolar space, where they encounter and eliminate inhaled pathogens or dust particles. In addition, AMs play a role in maintenance of surfactant homeostasis, which helps to maintain the air-liquid interface of the alveolar wall to prevent alveolar collapse. Ablation of AMs is known to cause pulmonary alveolar proteinosis (PAP), which is characterized by the accumulation of surfactant material and respiratory insufficiency (Martinez-Moczygemba et al, 2008; Suzuki et al, 2008). Therefore, AMs play a central role in host defense and in the maintenance of immune and organ homeostasis of the lung (Hussell & Bell, 2014).

The origin of tissue-resident macrophages has long been debated. Recent fate-mapping studies using genetic markers have demonstrated that macrophage populations, including peritoneal, splenic, lung and liver macrophages, are established from tissue monocytes localized prior to birth, but not from circulating blood monocytes (Ginhoux & Guilliams, 2016; Yona et al, 2013). AMs develop from fetal monocytes that adopt a stable phenotype shortly after birth in the lung (Guilliams et al, 2013; Hoeffel et al, 2015; Schneider et al, 2014), although the underlying mechanisms that regulate development and maturation of AMs are not fully understood. Among the various types of tissue-resident macrophages, AMs have distinctive phenotypes that include high autofluorescence, low CD11b expression, and high CD11c and Siglec-F expression (Guilliams et al, 2013). Furthermore, localized monocytes in the lung predominantly develop to AM in the presence of GM-CSF (Csf2), and therefore mutations in the Csf2 and GM-CSF receptor (Csf2ra and Csf2rb) genes are associated with reduced number and function of AMs, resulting in PAP (Whitsett et al, 2010). Similarly, mice lacking Csf2 and Csf2rb develop PAP and display increased susceptibility to bacterial and

Allergic asthma is a chronic inflammatory disease which is orchestrated by various types of immune cells, including dendritic cells (DCs), T cells, eosinophils, neutrophils, basophils, innate lymphoid cells (ILCs) and AMs (Galli et al, 2008; Lambrecht & Hammad, 2015). Development of allergic asthma is generally controlled by DCs directed toward the adaptive T helper-2 (Th2)-type immune response, which is initially activated by inhaled antigen particles such as house dust mite (HDM), pollen and fungal spores. These allergic antigens are recognized by pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), C-type lectin receptors, NOD-like receptors and DNA sensors (Kawai & Akira, 2011). HDM contains Der p2 antigen, which displays sequence homology with MD-2, a cell surface molecule bridging LPS to TLR4, and triggers cytokine production and airway allergy by stimulating TLR4 on epithelial cells (Hammad et al, 2009). The development of HDM-induced airway allergy in mice is likely to be relevant to human asthma (Gregory et al, 2009). AMs have the anti-inflammatory properties of inhibiting DC antigen presentation (Holt et al, 1993) and producing immunosuppressive mediators such as IL-10 and nitric oxide (NO) (Martinez et al, 1997). These immunological properties suggest that AMs have suppressive roles for asthma development, but the contribution of AMs to asthma has not been clearly demonstrated (Mathie et al, 2015).

We previously showed that the phosphatidylinositol lipid PtdIns5P, which is produced by PIKfyve, is involved in innate immune responses to virus infection (Kawasaki et al, 2013). PIKfyve is a phosphatidylinositol lipid kinase which phosphorylates the phosphatidylinositol ring at the 5' position to produce PtdIns5P or PtdIns3,5P2. PtdIns5P and PtdIns3,5P2 are minor phosphatidylinositol lipid that also contribute to diverse cellular functions including vesicular trafficking (McCartney et al,
2014), ion channel activation (Li et al, 2013) and EGFR signaling (Er et al, 2013). It has been reported that PIKfyve-deficient mice are embryonic lethal (Ikonomov et al, 2011; Takasuga et al, 2013). Using the Cre-loxP system or gene trap mice, the contribution of PIKfyve to various tissue-specific cells such as intestine (Takasuga et al, 2013) and platelets (Min et al, 2014) was investigated, and PIKfyve was found to play essential roles in cellular development and function.

Here, to investigate the functional role of PIKfyve in macrophages, we generated macrophage-specific PIKfyve-deficient mice. We found that $PIKfyve^{flam/flam}$ lsm-cre mice have alteration of AMs population. By contrast, the population of tissue-resident macrophages in spleen, liver, bone or peritoneal cavity was not influenced in $PIKfyve^{flam/flam}$ lsm-cre mice. Macrophage-specific PIKfyve-deficient mice exhibit an increased severity of inflammation and allergic asthma induced by HDM, which is accompanied by enhanced infiltration of eosinophils and lymphoid cells and production of type 2 cytokines. PIKfyve-deficient mice have defects in retinoic acid induction, and fail to recruit $T_{reg}$ cells to the lung. Moreover, AKT activation induced by GM-CSF, a cytokine critical for AM development, was suppressed by PIKfyve-deficiency. Thus, PIKfyve is involved in AM development through regulating AKT activation during GM-CSF receptor signaling, and is required for prevention from allergic responses to HDM.
Results

Loss of PIKfyve causes a reduction in alveolar macrophage number.

To investigate physiological roles of PIKfyve in macrophages, we generated mice lacking PIKfyve in the myeloid lineage. Mice were conditionally targeted with a loxP site flanking exon 5 of the PIKfyve gene (PIKfyvefloxflox) (Fig EV1A), and PIKfyvefloxflox mice were crossed with mice expressing cre recombinase downstream of the lysozyme LysM promoter (lysm-cre). Generation of flox mice was confirmed by Southern blot and PCR analysis (Fig EV1B and EV1C). Immunoblot analysis demonstrated a marked reduction of PIKfyve expression in bone marrow-derived macrophage colony-stimulating factor (M-CSF)-induced macrophages derived from PIKfyvefloxflox lysm-cre mice (Fig EV1D).

To investigate the function and development of AMs in the lung, bronchoalveolar lavage (BAL) fluid and lung were stained with anti-CD11c and anti-Siglec-F. PIKfyvefloxflox lysm-cre mice showed reduced numbers of CD11c\textsuperscript{high} and Siglec-F\textsuperscript{high} AMs in both BAL fluid and lung (Fig 1A and 1B), and reduced expression of the PIKfyve gene in AMs (Fig 1C). We next measured various markers in AMs (Fig 1D). AMs from PIKfyvefloxflox lysm-cre mice had lower autofluorescence (AF), CD64 and CD205 expression and higher CD86 expression than control cells, but AMs from control and PIKfyvefloxflox lysm-cre mice did not have significant differences in CD24, IA/IE, Ly-6C and CD11b expression. (Fig 1D), suggesting that AMs development is partially retarded in PIKfyvefloxflox lysm-cre mice.

Lung is known to contain various myeloid lineage cells such as AMs, neutrophils, eosinophils, CD11b^DCs, CD103^DCs, interstitial macrophages (IMs), Ly-6C^monocytes and Ly-6C^monocytes. To characterize these cells populations, we performed 10 color flow cytometry and sequential gating analysis (Becher et al, 2014; Misharin et al, 2013; Schlitzer et al, 2013). After the exclusion of doublet cells, CD45^
cells were stained with antibodies against various cellular surface markers (Fig 2A and 2B). We first identified CD11b<sup>hi</sup>Siglec-F<sup>+</sup> cells and subsequently separated CD11c<sup>hi</sup>CD64<sup>+</sup> cells and CD11c<sup>lo</sup>CD64<sup>−</sup> cells. CD11b<sup>hi</sup>Siglec-F<sup>+</sup>CD11c<sup>hi</sup>CD64<sup>+</sup> cells were defined as AMs and CD11c<sup>lo</sup>CD64<sup>−</sup>Siglec-F<sup>+</sup> cells were eosinophils. After segregated neutrophils (CD11b<sup>+</sup>Ly-6G<sup>+</sup>) population, CD11c<sup>hi</sup>IA/IE<sup>hi</sup> cells were separated to CD11c<sup>hi</sup>IA/IE<sup>hi</sup>CD11b<sup>+</sup>CD103<sup>+</sup> cells, which were then separated to CD11b<sup>+</sup>DCs (CD11b<sup>+</sup>CD24<sup>+</sup>CD64<sup>+</sup>) and IMs (CD11b<sup>−</sup>CD24<sup>−</sup>CD64<sup>−</sup>). CD11b<sup>+</sup>IA/IE<sup>+</sup> cells were separated to Ly-6C<sup>+</sup>Ly-6G<sup>low</sup> monocytes (CD11b<sup>hi</sup>IA/IE<sup>lo</sup>CD64<sup>−</sup>Ly-6C<sup>+</sup>) and Ly-6C<sup>+</sup>Ly-6G<sup>−</sup> monocytes (CD11b<sup>lo</sup>IA/IE<sup>lo</sup>CD64<sup>−</sup>Ly-6C<sup>−</sup>). Although populations of these cells in the lung were not significantly changed in either control or PIKfyve<sup>lox/lox</sup> lym-cre mice (Fig 2A and 2B), expression of Siglec-F and CD64 in AMs (CD11b<sup>hi</sup>Siglec-F<sup>+</sup>CD11c<sup>hi</sup>CD64<sup>+</sup>) were reduced in PIKfyve<sup>lox/lox</sup> lym-cre mice (Fig 2C), suggesting that PIKfyve<sup>lox/lox</sup> lym-cre mice have altered AMs populations. Furthermore, macrophage populations in other tissues, namely spleen, bone marrow, liver and intraperitoneal fluid, were also investigated by the marker for CD11b and F4/80 (Fig 2D and 2E). However, these cell populations were comparable in PIKfyve<sup>lox/lox</sup> lym-cre mice.

We also investigated the function of neutrophils isolated from bone marrow. PIKfyve protein expression was reduced in neutrophils (Fig EV2A). The Ly-6C<sup>+</sup>Ly-6G<sup>−</sup> monocytes and Ly-6C<sup>hi</sup>Ly-6G<sup>hi</sup> neutrophil populations were unimpaired in PIKfyve<sup>lox/lox</sup> lym-cre mice (Fig EV2B, EV2C and EV2D). Moreover, IL-6 production after LPS stimulation was normal in PIKfyve<sup>lox/lox</sup> lym-cre mice (Fig EV2E), suggesting that PIKfyve deficiency does not influence both development and function of neutrophils. Taken together, these findings indicate that PIKfyve<sup>lox/lox</sup> lym-cre mice have reduced the number of AMs among various myeloid lineage cells.
**PIKfyve deficiency causes alteration in AM populations**

Macrophages derived from monocytes have been shown to localize at particular tissues before birth and to mature at the localized tissue (Guilliams et al, 2013). To address the development of AMs in lung, lungs were isolated at day of birth (DOB), postnatal day 3 (PND3), 3 weeks and 6 weeks, and stained with AM markers (Fig 3A). CD45+ blood cells were gated and stained for expression of CD11b and F4/80, and CD11b+F4/80+ myeloid cell populations were then further gated for CD11c and Siglec-F. Most CD11b+F4/80+ myeloid cells were negative for CD11c and Siglec-F at DOB. At PND3, the CD11c^{high}Siglec-F^{int} cell population was greatly increased in both control and PIKfyve^{floxflo} lsm-cre mice. At 3 weeks and 6 weeks after DOB, CD11c^{high}Siglec-F^{int} cells displayed increased expression of Siglec-F and developed into CD11c^{high}Siglec-F^{high} cells in control mice, but not in PIKfyve^{floxflo} lsm-cre mice (Fig 3A and 3B). These results suggest that the development of AMs in PIKfyve^{floxflo} lsm-cre mice was partially arrested. Therefore, we next investigated whether these cells still have the potential to proliferate. CD11c^{int}Siglec-F^{int} cells or CD11c^{high}Siglec-F^{high} cells in the lung from mice at PND3 or 3 weeks of age, respectively, were stained with DAPI and the cell cycle protein Ki-67 which labels dividing and recently divided daughter cells (Fig 3C and 3D). Ki-67^{+}DAPI^{low} cells were gated as G_{1} stage and Ki-67^{+}DAPI^{high} cells were gated as SG_{2}M stage. At PND3, 30 % of both control and KO cells were G_{1} phase and 5 % of cells were SG_{2}M stage. At 3 weeks after day of birth, 12 % of control cells and 16 % of KO cells were G_{1} phase. In addition, 2 % of control cells and 3.5 % of KO cells were SG_{2}M stage (Fig 3C and 3D). These findings indicated that AMs in PIKfyve^{floxflo} lsm-cre mice have higher growth potency, and were retarded at CD11c^{high}Siglec-F^{int} stage.

**Impaired GM-CSF-induced AKT activation in PIKfyve^{floxflo} lsm-cre cells**
To understand mechanistically why population of AMs is specifically altered among various tissue-resident macrophages in PIKfyve\textsuperscript{flax/flax} lysm-cre mice, we next examined the expression of PIKfyve by real time PCR (rtPCR), and found that PIKfyve was expressed almost ubiquitously (Fig 4A). AMs are the only macrophage type known to require GM-CSF for their development from monocytes loaded in the fetal lung (Guilliams et al, 2013). We therefore hypothesized that alteration of AMs resulting from PIKfyve deficiency occurred downstream of GM-CSF receptor signaling. To test this, M-CSF-derived bone marrow macrophages were stimulated with GM-CSF for 3 days, and CD11b\textsuperscript{+}F4/80\textsuperscript{+} macrophages were further stained with anti-CD11c and anti-Siglec-F (Fig 4B). Although the percentages of CD11c\textsuperscript{+}Siglec-F\textsuperscript{+} cells from control mice and PIKfyve\textsuperscript{flax/flax} lysm-cre mice were equivalent, percentages of the double-positive cells were increased by GM-CSF stimulation in M-CSF-derived control cells but not in PIKfyve\textsuperscript{flax/flax} lysm-cre cells (Fig 4B). GM-CSF association with the GM-CSF receptor \(\alpha\) and \(\beta\) subunit is known to trigger activation of AKT, STAT5, NF-\(\kappa\)B and MAPK (van de Laar et al, 2012). We found that AKT phosphorylation was reduced in M-CSF-induced macrophages from PIKfyve\textsuperscript{flax/flax} lysm-cre mice relative to control cells (Fig 4C). By contrast, phosphorylation of STAT5, p65 (a subunit of NF-\(\kappa\)B), ERK (MAPK), p38 and JNK was comparable in control and PIKfyve\textsuperscript{flax/flax} lysm-cre cells (Fig 4C). These findings suggest that PIKfyve is required for AKT activation during GM-CSF receptor signaling. Next, expression of ligand and receptor for GM-CSF was investigated in AMs. Expression of the GM-CSF gene Csf2, and of the GM-CSF receptor \(\alpha\) and \(\beta\) subunit genes Csf2ra and Csf2rb, was unaltered in AMs or lung tissue in PIKfyve\textsuperscript{flax/flax} lysm-cre cells (Fig 4D). Surface expression of CD131 (GM-CSF receptor \(\beta\) subunit) was also comparable in AM (Fig 4D). To investigate signaling events in AMs, lungs were isolated at PND3 and CD11c\textsuperscript{+}Siglec-F\textsuperscript{int} cells were stained for pAKT, but pAKT-positive cells were not detectable. At PND5,
pAKT-positive cells in CD11c+Siglec-F<sup>int</sup> cells were increased in control cells but reduced in PIKfyve<sup>flax/flax</sup> lym-cre mice (Fig 4F). By contrast, STAT5 phosphorylation was undetectable in both control and PIKfyve<sup>flax/flax</sup> lym-cre cells (Fig 4G). Gene expression for CD11c<sup>high</sup>Siglec-F<sup>high</sup> cells in wild type and CD11c<sup>high</sup>Siglec-F<sup>+</sup> including Siglec-F<sup>int</sup> cells in PIKfyve<sup>flax/flax</sup> lym-cre mice were compared by gene chip analysis, and several genes were upregulated or downregulated (Fig. 4H). Among these, the expression of IRF family genes, encoding transcription factors related to macrophage function and development, was broadly suppressed in PIKfyve<sup>flax/flax</sup> lym-cre cells (Fig 4H). In addition, signature genes expressions for AMs development, Pparg related or lipid metabolite were broadly dysregulated in PIKfyve<sup>flax/flax</sup> lym-cre mice (Fig EV3A, EV3B and EV3C). We also investigated gene expression by rtPCR after separation with Siglec-F<sup>high</sup> and Siglec-F<sup>int</sup> cells in PIKfyve<sup>flax/flax</sup> lym-cre mice and found that expression of PIKfyve, Irf8 and Irf5 was suppressed in the both cells (Fig EV3D and EV3E). As supported, GM-CSF stimulation increased Irf8, Irf5 and Irf1 expression in M-CSF-induced macrophages and these genes expression was suppressed by the AKT inhibitor (GSK690693) (Fig 4I). These results suggest that alteration of AMs in PIKfyve<sup>flax/flax</sup> lym-cre mice is caused by the broad reduction of IRF family genes whose expression is regulated by GM-CSF receptor-AKT signaling axis.

**PIKfyve-deficient mice display increased HDM-induced allergic inflammation**

AMs play fundamental roles in lung homeostasis by performing phagocytosis of surfactant proteins, and abortion of AMs causes PAP (Suzuki et al, 2014). Although AMs in PIKfyve<sup>flax/flax</sup> lym-cre mice show a reduced rate of phagocytosis of OVA proteins (Fig EV4A), expression of surfactant proteins in lung was unimpaired by PIKfyve deficiency and PIKfyve<sup>flax/flax</sup> lym-cre mice did not show PAP-like symptoms (Fig EV4B and EV4C).
Given the association of the allergic response with AMs, mice were challenged with and sensitized by HDM extract derived from *Dermatophagoides pteronyssinus* (Fig 5A). We measured infiltration of neutrophils, eosinophils and CD4\(^+\) T cells in BAL fluid and found that the number of eosinophils and CD4\(^+\) T cells increased in HDM-treated *PIKfyve*\(^{ Pax/lox }\) *lysm-cre* mice compared to control mice (Fig 5B). Hematoxylin and eosin (H/E) and periodic acid Schiff (PAS) staining of lung sections demonstrated that inflammation was exacerbated in HDM-treated *PIKfyve*\(^{ Pax/lox }\) *lysm-cre* mice (Fig 5C). Expression of *Il4*, *Il5*, *Il12* and *Il13* genes was higher in HDM-treated *PIKfyve*\(^{ Pax/lox }\) *lysm-cre* mice than in control mice, whereas *Il10* expression was not altered (Fig 5D). Production of the Th2-related cytokines IL-4 and IL-13 in lung homogenate was also increased in HDM-treated *PIKfyve*\(^{ Pax/lox }\) *lysm-cre* mice (Fig 5E). It has been reported that HDM-induced allergic inflammation is a Th2-type immune response to which ILCs also contribute synergistically (Lambrecht & Hammad, 2015). Lin\(^-\) cells in BAL fluid were further stained by T1/ST2 (IL-1RL1, a subunit of IL-33R) and Sca-1 (Ly-6A/E) and the number of Lin\(^-\)T1/ST2\(^+\)Sca1\(^+\) ILCs was higher in HDM-treated *PIKfyve*\(^{ Pax/lox }\) *lysm-cre* mice (Fig EV5A and EV5B). *Il33* expression was also increased in lung from *PIKfyve*\(^{ Pax/lox }\) *lysm-cre* mice, but *Tslp* expression was not altered (Fig EV5C). We also investigated the number of CD11b\(^+\) DCs and IMs after HDM treatment, and these cells were comparable in both HDM-treated control and *PIKfyve*\(^{ Pax/lox }\) *lysm-cre* mice (Fig EV5D and EV5E). These results strongly suggest that allergic inflammation in *PIKfyve*\(^{ Pax/lox }\) *lysm-cre* mice is mediated through a Th2-type response.

**Egress of allergic asthma in PIKfyve-deficient mice is caused by a reduction of Foxp3\(^+\) T\(_{reg}\) cells.**

To investigate whether cells from *PIKfyve*\(^{ Pax/lox }\) *lysm-cre* mice are inflammatory or not,
we measured inflammatory cytokines in CD11c^{high}Siglec-F^{+} cells prepared from mice challenged with HDM (Fig EV6A). Induction of Tnfa, Il6 and Il1b expression was significantly reduced in HDM-challenged PIKfyve-deficient cells, suggesting that CD11c^{high}Siglec-F^{+} cells from PIKfyve^{loxd/lox} lym-cre mice are not highly inflammatory. In addition, we measured inflammatory cytokine production by M-CSF-induced bone marrow-derived macrophages (M-BMMs) stimulated with LPS or HDM in the presence or absence of GM-CSF (Fig EV6B). The levels of TNFα, IL-6 and IL-1β after LPS or HDM stimulation was comparable between control and PIKfyve-deficient cells, suggesting that PIKfyve-deficiency does not influence macrophage capacities of inflammatory cytokine production in response to LPS or HDM.

Inflammation by Th2 type response is controlled by regulatory T (T_{reg}) cells, and T_{reg} cells development is regulated by retinoic acid and TGFβ. Retinoic acid is produced by aldehyde dehydrogenase 1a, which oxidizes retinal and comprises three genes, Aldh1a1, Aldh1a2 and Aldh1a3. Gene expression of Aldh1a1, Aldh1a2, Aldh1a3 and Tgfβ was measured in AMs in BAL, and in AMs or DCs in lung, by rtPCR (Fig 6A and 6B). Aldh1a1 expression was higher in AMs in BAL, but Tgfβ was not (Fig 6A). Although Tgfβ expression was comparable in control and PIKfyve^{loxd/lox} lym-cre, Aldh1a1 expression was higher than Aldh1a2 and Aldh1a3 in AMs, and was suppressed in AMs from PIKfyve^{loxd/lox} lym-cre mice (Fig 6B), suggesting that T_{reg} cells development is supported by AMs. To examine Foxp3^{+} T_{reg} cell incorporation in lung, lungs were isolated from HDM-treated mice and stained for CD4, CD25 and Foxp3 (Fig 6C and 6D). The percentage of Foxp3^{+} T_{reg} cells in lung was reduced in PIKfyve^{loxd/lox} lym-cre mice, whereas CD25^{+}Foxp3^{+} T_{reg} cells in spleen and lymph node was comparable in both control and PIKfyve^{loxd/lox} lym-cre mice (Fig 6C and 6D). To investigate whether AMs directly regulate Foxp3^{+} T_{reg} cell development, we used an in
vitro assay to generate T_{reg} cells from naïve T cells along with AMs. CD4^{+}CD25^{-} naïve T cells isolated from spleen were cocultured with AMs in BAL from control or PIKfyve^{fl/fl} lsm-cre mice (Fig 6E). Foxp3^{+} T_{reg} cells constituted 30% of CD4^{+} cells in the presence of AMs from control mice, but only 10% in the presence of AMs from PIKfyve^{fl/fl} lsm-cre mice. This reduction in the percentage of Foxp3^{+} T_{reg} cells by AMs from PIKfyve^{fl/fl} lsm-cre mice was rescued by adding retinoic acid to the culture medium. In addition, intranasal administration of retinoic acid rescued the number of T_{reg} cells in PIKfyve^{fl/fl} lsm-cre mice (Fig 6F). Consistent with the rescue by retinoic acid of Foxp3^{+} T_{reg} cell development in vitro and in vivo, administration of retinoic acid suppressed HDM-induced inflammation in PIKfyve^{fl/fl} lsm-cre mice (Fig 6G and EV6C). These results suggest that reduced production of retinoic acid and subsequent failure of Foxp3^{+} T_{reg} cells incorporation in lung is responsible for exacerbation of HDM-induced allergic inflammation in PIKfyve^{fl/fl} lsm-cre mice.
Discussion

Tissue-resident macrophages including AMs are originally derived from fetal monocytes, which are locally loaded before birth and develop to mature macrophages (Guilliams et al, 2013). Among the several tissue-resident macrophage types, AMs require GM-CSF for development. In Csf2-deficient mice, early AM commitment from fetal monocytes is impaired. Although short-term perinatal intrapulmonary GM-CSF administration rescues AM development for several weeks, the resulting AMs display immature phenotypes (Guilliams et al, 2013). These results suggest that GM-CSF is required at two stages for AM development: early stage development from fetal monocytes to CD11c\textsuperscript{high}Siglec-F\textsuperscript{int} stage, and late stage development from CD11c\textsuperscript{high}Siglec-F\textsuperscript{int} to mature CD11c\textsuperscript{high}Siglec-F\textsuperscript{high} stage. The PIKfyve\textsuperscript{flax/flax} lsm-cre mice generated in this study show impaired AM development that is retarded at CD11c\textsuperscript{high}Siglec-F\textsuperscript{int} stage (Fig 1 and 2). At DOB, CD11b\textsuperscript{+}F4/80\textsuperscript{+} myeloid cells contain small number of CD11c\textsuperscript{high}Siglec-F\textsuperscript{int} cells, which increased markedly at PND3 in both control and PIKfyve\textsuperscript{flax/flax} lsm-cre mice. At 3 and 6 weeks after DOB, CD11c\textsuperscript{high}Siglec-F\textsuperscript{int} cells display an increase in the expression of Siglec-F and develop into CD11c\textsuperscript{high}Siglec-F\textsuperscript{high} cells in control mice, but not in PIKfyve\textsuperscript{flax/flax} lsm-cre mice. This finding suggests that AM development in PIKfyve\textsuperscript{flax/flax} lsm-cre mice is retarded at the CD11c\textsuperscript{high}Siglec-F\textsuperscript{int} stage that is located at the intermediate stage between fetal monocytes and mature AMs (Fig 3). However, we could not clearly characterize CD11c\textsuperscript{high}Siglec-F\textsuperscript{int} cell population in PIKfyve\textsuperscript{flax/flax} lsm-cre mice. Further analyses will be required to determine these cell types.

It is possible that alteration of AMs population in PIKfyve\textsuperscript{flax/flax} lsm-cre mice is caused by impaired GM-CSF receptor signaling. Whereas expression of csf2, csf2ra, csf2rb and CD131 is unimpaired, phosphorylation of AKT, a downstream molecule of GM-CSF receptor signaling, is decreased in AMs from PIKfyve\textsuperscript{flax/flax} lsm-cre mice (Fig
4). By contrast, PIKfyve deficiency does not abrogate activation of NF-κB and MAP kinases. These findings suggest that PIKfyve regulates AKT activation during GM-CSF receptor signaling. PIKfyve is a lipid kinase that directly produces PtdIns5P or PtdIns3,5P₂ via phosphorylation of the 5 position of the inositol ring. A previous study indicated that AKT is activated by PtdIns3,4,5P₃ or PtdIns3,4P₂ (Martelli et al, 2006). PIKfyve may indirectly regulate the production of PtdIns3,4,5P₃ or PtdIns3,4P₂, since inositol lipid production is balanced in cells by several kinases and phosphatases. Alternatively, activation of PI3K, which produces PtdIns3,4,5P₃, may be regulated by PIKfyve.

Comparative analysis of transcription factor expression indicated that mRNA expression of the IRF family members was broadly decreased in PIKfyve-deficient AMs (Fig. 4H). Whereas PPARγ is a transcription factor that reportedly regulates AM development (Schneider et al, 2014), Pparg expression was comparable in control and PIKfyve-deficient AMs. AMs in PPARγ-deficient mice show disruption of lipid homeostasis and accumulation of lipid droplets. AMs in PIKfyve-deficient mice, however, showed normal morphology and were not stained with Oil Red O (data not shown). Comparison of Pparg related genes and lipid metabolite genes expression suggested that PIKfyve may also be involved in PPARγ-mediated AM development (Fig EV3B and EV3C). GM-CSF stimulation induced expression of Irf8, Irf5 and Irf1 and AKT inhibitor suppressed expression of these genes (Fig 4I). These results indicate that members of the IRF family that are induced by AKT activation during GM-CSF receptor signaling are involved in AM development. Although defective AM development has not been reported, Irf genes are required for development and function of various types of myeloid cell linages (Zhao et al, 2015). Therefore, it may be possible that multiple IRF family members coordinately regulate AM development. This point should be clarified in the future.
Phagocytosis of surfactant proteins by AMs is crucial for lung homeostasis to prevent PAP disease. Disruption of AMs causes PAP disease, and PPARγ- or CsF2-deficient mice show an accumulation of surfactant proteins and PAP-like symptoms. Phagocytosis of AMs is partially impaired in PIKfyve\textsubscript{flox/flox} lysm-cre mice, but these mice did not develop PAP-like symptoms (Fig EV3). One possible explanation for this relatively minor phenotype in PIKfyve-deficient mice is the expression level of the cre gene in AMs. AMs express both LysM and CD11c, and PPARγ deficiency after crossing with lysm-cre mice does not lead to a disease phenotype (Gautier et al, 2012) whereas PPARγ deficiency after crossing with CD11c-cre mice leads to ablation of AMs and progression to PAP-like symptoms (Schneider et al, 2014). Therefore, further analysis using PIKfyve\textsubscript{flox/flox} mice crossed with CD11c-cre mice will be required in the future.

PIKfyve ablation causes alteration of AMs population, suggesting the possibility that AM development is arrested at the CD11c\textsuperscript{high}Siglec-F\textsuperscript{int} stage in PIKfyve-deficient mice. PIKfyve-deficient mice are highly sensitive to HDM treatment and show increases in infiltration of eosinophils and lymphoid cells into the BAL space through a Th2-type immune response (Fig 5). Although the contribution of AMs to allergic asthma is controversial (Balhara & Gounni, 2012), our results are consistent with the finding that AM depletion by clodronate liposomes causes egress of HDM-induced asthma (Mathie et al, 2015). During the inflammatory response, macrophages switch their phenotype from M1 to M2, which drives the Th2-type immune response (Martinez & Gordon, 2014). However, PIKfyve deficiency did not influence the tendency for M1/M2 polarization (data not shown). In addition, expression of inflammatory cytokines such as Tnfa, Il-6 and Il-1b was unimpaired in PIKfyve-deficient mice (Fig EV6A and EV6B), suggesting that increased allergic responses in PIKfyve-deficient mice are not due to aberrant induction of these
inflammatory cytokines. AMs are known to produce TGFβ and retinoic acid, which are likely responsible for induction of Foxp3+ Treg cells in the lung (Soroosh et al, 2013). The presence of Foxp3+ Treg cells inhibits the development of allergic asthma by suppressing the Th2-type immune response (Kim et al, 2010; Soroosh et al, 2013). Our results suggest that Th2-type immune responses are suppressed by Foxp3+ Treg cells that are induced by AMs in control mice, and this suppression is abrogated in PIKfyve<sup>flox/flox</sup> lysm-cre mice. Our results also suggest that premature AMs do not induce sufficient retinoic acid, and therefore the number of Foxp3+ Treg cells in lung is reduced in PIKfyve<sup>flox/flox</sup> lysm-cre mice (Fig 6).

Notably, we found that PIKfyve is dispensable for the population of several tissue-resident macrophage types other than AMs. Although macrophage population in other tissue was unimpaired in PIKfyve-deficient mice, a previous study indicated that not all macrophages are equal in terms of LysM targeting (Schneider et al, 2014) and more careful analyses are required to understand the role of PIKfyve in tissue-resident macrophage development. So far, csf2 (Paine et al, 2001; Yoshida et al, 2001), csfrb (Tanaka et al, 2011), bach2 (Nakamura et al, 2013) and pparg (Schneider et al, 2014) have been shown to regulate AM development and function. PIKfyve-deficient mice show altered AM population and defective AKT activation in response to GM-CSF. PIKfyve-deficient mice are highly sensitive to HDM-induced asthma due to a lack of induction of Foxp3+ Treg cells and increased infiltration of eosinophils and lymphoid cells, suggesting that AM maturation in the lung is a crucial step for preventing asthma following antigen inhalation. We propose that PIKfyve represents a therapeutically important target for the suppression of asthma and lung inflammation in which AMs are involved.
AUTHOR CONTRIBUTIONS
T. Kawasaki performed experiments, analyzed data and wrote the manuscript. K.I. helped with lung sectioning. H.M. performed ES injection. S.A. provided material. T. Kawai wrote the manuscript and supervised research.

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COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.
FIGURE LEGENDS

Figure 1 AMs in BAL fluid and lung were reduced in PIKfyve^{ flox/flox } lysm-cre mice. (A) Flow cytometry of AMs in BAL fluid and lung stained with anti-CD11c and anti-Siglec-F. (B) Number of AMs in BAL fluid and percentage of AMs in lung are shown in bar graphs. (C) PIKfyve gene expression in AMs was measured by rtPCR. (D) SSC, FSC and autofluoresces (AF), and expression of CD64, CD86, IA/IE, Ly-6C, CD24, and CD 205 in AMs. Data are represented as mean +/- SD. *P<0.05 (Student’s t-test).

Figure 2 Macrophages and other myeloid lineage cells population in various tissues. (A) Gating to separate myeloid lineage cells in lung. Doublet cells were excluded and CD45^{+} cells were separated with distinct cell surface markers; alveolar macrophages (AMs) (CD11b^{int}Siglec-F^{+}CD11c^{high}CD64^{+}), eosinophils (CD11c^{−}Siglec-F^{+}), neutrophils (CD11b^{−}Ly-6G^{+}), CD103^{+}DCs (CD11c^{high}IA/IE^{high}CD11b^{−}CD103^{+}), CD11b^{+}DCs (CD11c^{high}IA/IE^{high}CD11b^{+}CD103^{−}CD24^{−}CD64^{+}), interstitial macrophages (IMs) (CD11c^{high}IA/IE^{high}CD11b^{+}CD103^{−}CD24^{−}CD64^{+}), Ly-6C^{+}monocytes (CD11b^{+}IA/IE^{CD64^{+}/Ly-6C^{+}}) and Ly-6C^{−}monocytes (CD11b^{+}IA/IE^{CD64^{−}/Ly-6C^{−}}). (B) Number of indicated cells in lungs. (C) Expression of surface makers in AMs (D) Flow cytometry of spleen, bone marrow, liver and intraperitoneal cells stained with anti-F4/80 and anti-CD11b. (E) Percentage of F4/80^{+}CD11b^{+} macrophages in each tissue. Data are represented as mean +/- SD. *P<0.05 (Student’s t-test).

Figure 3 AMs development in PIKfyve^{ flox/flox } lysm-cre mice is retarded at CD11^{high}Siglec-F^{int} premature stage. (A) Lungs were harvested at the indicated times after birth, and analyzed by flow cytometry. CD45^{+} blood cells were gated for CD11b
and F4/80, and CD11b\(^{+}\)F4/80\(^{+}\) cells were stained with anti-CD11c and anti-Siglec-F. (B) Percentages of CD11c\(^{\text{high}}\)Siglec-F\(^{\text{high}}\) mature AMs (upper) and of CD11c\(^{\text{high}}\)Siglec-F\(^{\text{int}}\) premature AMs (lower) in CD45\(^{+}\) cells are plotted as bar graphs. (C) CD11c\(^{\text{high}}\)Siglec-F\(^{\text{high}}\) mature AMs in lung were stained with DAPI and anti-Ki-67 or isotype control. Ki-67\(^{+}\)DAPI\(^{\text{low}}\) cells were gated as G\(_1\) phase and Ki-67\(^{+}\)DAPI\(^{\text{high}}\) cells were gated as SG\(_2\)M phase. (D) The percentage of G\(_1\) and SG\(_2\)M phase cells are plotted as a bar graph. Data are represented as mean +/- SD. *P<0.05 (Student’s t-test).

**Figure 4** GM-CSF receptor signaling is impaired in PIKfyve\(^{\text{floox}/\text{floox}}\) lym-cre mice during AMs development. (A) AMs and IMs in lung, spleen, bone marrow, liver and intraperitoneal cavity were isolated and PIKfyve expression was measured by rtPCR. (B) M-CSF-induced macrophages were cultured with GM-CSF for 3 days and CD11b\(^{+}\)F4/80\(^{+}\) cells were stained with anti-CD11c and anti-Siglec-F. Percentages of CD11c\(^{+}\)Siglec-F\(^{+}\) cells in CD11b\(^{+}\)F4/80\(^{+}\) cells are plotted as a bar graph. (C) M-CSF-induced macrophages were stimulated with GM-CSF and the time course of phosphorylation level of AKT, STAT5, NF-κB p65 and ERK was investigated by Western blot analysis using the indicated antibodies. (D) AMs or lungs were isolated and the expression of csf2, csfra and csfrb was measured by rtPCR. (E) Expression of CD131 (GM-CSF receptor β) in AM was measured by FACS. (F, G) Lungs were isolated at PND5. CD45\(^{+}\) cells were stained with anti-CD11c and anti-Siglec-F, and CD45\(^{+}\)CD11c\(^{+}\)Siglec-F\(^{+}\) cells were stained with anti-pAKT (F) and anti-pSTAT5 (G). Percentages of pAKT-positive cells are plotted in a bar graph. (h) CD11c\(^{\text{high}}\)Siglec-F\(^{\text{high}}\) cells were isolated from control and CD11c\(^{\text{high}}\)Siglec-F\(^{+}\) cells including Siglec-F\(^{\text{int}}\) cells were isolated from PIKfyve\(^{\text{floox}/\text{floox}}\) lym-cre mice. Gene expression in AMs was measured by gene chip analysis and the expression of transcription factors involved in macrophage development and function is shown. (I) M-CSF-induced macrophages were

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**Figure 4** GM-CSF receptor signaling is impaired in PIKfyve\(^{\text{floox}/\text{floox}}\) lym-cre mice during AMs development. (A) AMs and IMs in lung, spleen, bone marrow, liver and intraperitoneal cavity were isolated and PIKfyve expression was measured by rtPCR. (B) M-CSF-induced macrophages were cultured with GM-CSF for 3 days and CD11b\(^{+}\)F4/80\(^{+}\) cells were stained with anti-CD11c and anti-Siglec-F. Percentages of CD11c\(^{+}\)Siglec-F\(^{+}\) cells in CD11b\(^{+}\)F4/80\(^{+}\) cells are plotted as a bar graph. (C) M-CSF-induced macrophages were stimulated with GM-CSF and the time course of phosphorylation level of AKT, STAT5, NF-κB p65 and ERK was investigated by Western blot analysis using the indicated antibodies. (D) AMs or lungs were isolated and the expression of csf2, csfra and csfrb was measured by rtPCR. (E) Expression of CD131 (GM-CSF receptor β) in AM was measured by FACS. (F, G) Lungs were isolated at PND5. CD45\(^{+}\) cells were stained with anti-CD11c and anti-Siglec-F, and CD45\(^{+}\)CD11c\(^{+}\)Siglec-F\(^{+}\) cells were stained with anti-pAKT (F) and anti-pSTAT5 (G). Percentages of pAKT-positive cells are plotted in a bar graph. (h) CD11c\(^{\text{high}}\)Siglec-F\(^{\text{high}}\) cells were isolated from control and CD11c\(^{\text{high}}\)Siglec-F\(^{+}\) cells including Siglec-F\(^{\text{int}}\) cells were isolated from PIKfyve\(^{\text{floox}/\text{floox}}\) lym-cre mice. Gene expression in AMs was measured by gene chip analysis and the expression of transcription factors involved in macrophage development and function is shown. (I) M-CSF-induced macrophages were
stimulated with GM-CSF in the presence and the absence of GSK690693, and the expression of Irf8, Ifr5 and Irf1 was measured by rtPCR. Data are represented as mean +/- SD. *P<0.05 (Student’s t-test).

**Figure 5** Allergic inflammation in PIKfyve\textsuperscript{flax/flax} lym-cre mice is mediated through a Th2-type response. (A) Protocol for inducing airway inflammation by HDM intranasal administration. (B) Numbers of infiltrating neutrophils, eosinophils and CD4\textsuperscript{+} T cells in BAL fluid are plotted as a bar graph. (C) Representative hematoxylin and eosin (H/E) and periodic acid Schiff (PAS) staining of lung sections. Scale bar : 20 µm (upper) 5 µm (lower) (D) Cytokine gene expression in lung tissue was determined by rtPCR. (E) IL-4 and IL-13 production in lung homogenates was investigated by ELISA. Data are represented as mean +/- SD. *P<0.05 (Student’s t-test).

**Figure 6** Reduced production of retinoic acid and subsequent failure of Foxp3\textsuperscript{+} T\textsubscript{reg} cells incorporation in lung is responsible for exacerbation of HDM-induced allergic inflammation in PIKfyve\textsuperscript{flax/flax} lym-cre mice. (A) Aldh1a1 and TGF\textbeta expression in BAL AMs, lung AMs and CD11c\textsuperscript{+} DCs was measured by rtPCR. (B) Comparison of Aldh1a1 and TGF\textbeta expression in BAL AMs from control and PIKfyve\textsuperscript{flax/flax} lym-cre mice. (C) CD4\textsuperscript{+} T cells in lung were gated for Foxp3 and CD25. (D) Percentages of Foxp3-positive T cells in lung, and of Foxp3- and CD25-positive T cells in spleen and lymph node (LN) are plotted. (E) Splenic CD4\textsuperscript{+}CD25\textsuperscript{-} T cells were isolated and co-cultured with AMs from BAL fluid for 5 days with and without retinoic acid. Total cells were stained with anti-CD4 and anti-Foxp3 and the percentages of Foxp3\textsuperscript{+} T\textsubscript{reg} cells in CD4 T cells are plotted as a bar graph. (F) Percentages of Foxp3\textsuperscript{+} T\textsubscript{reg} cells in lung after administration of retinoic acid. (G) Numbers of neutrophils and eosinophils in BAL fluid after administration of HDM in the presence or absence of retinoic acid. Data
are represented as mean +/- SD. *P<0.05 (Student’s t-test).

Figure EV1 Generation of macrophage-specific PIKfyve knockout mice. (A) Design of PIKfyve knockout mouse generation. (B) Southern blot analysis using genomes from wild type and PIKfyve Neo mice having a LacZ and neomycin cassette in the target allele. The LacZ and neomycin cassette was deleted using an frt site by crossing with FLP recombinase-expressing transgenic mice. (C) PCR genotyping of mice using a primer pair for the loxP site. (D) PIKfyve\textsuperscript{flox/flox} mice were crossed with mice expressing cre recombinase downstream of the lysozyme LysM promoter (lysm-cre). Protein expression of PIKfyve and β-actin (Actin) in bone marrow-derived M-CSF-induced macrophages was investigated by Western blot analysis.

Figure EV2 Population and functional analysis of neutrophils in bone marrow. (A) Protein expression of PIKfyve and β-actin in neutrophils was investigated by Western blot analysis. (B) Flow cytometry of neutrophils and monocytes in bone marrow. CD11b\textsuperscript{+} cells were gated and analyzed for Ly-6C and Ly-6G. Percentages of CD11b\textsuperscript{+} cells (C), Ly-6C\textsuperscript{int}Ly-6G\textsuperscript{high} neutrophils (D), and Ly-6C\textsuperscript{int}Ly-6G\textsuperscript{high} monocytes (E) are shown as bar graphs. (f) Ly-6C\textsuperscript{int}Ly-6G\textsuperscript{high} neutrophils in bone marrow were sorted and stimulated with LPS. IL-6 production was analyzed by ELISA. Data are represented as mean +/- SD.

Figure EV3 Gene expression in AMs. CD11c\textsuperscript{high}Siglec-F\textsuperscript{high} cells were isolated from control and CD11c\textsuperscript{high}Siglec-F\textsuperscript{+} cells including Siglec-F\textsuperscript{int} cells were isolated from PIKfyve\textsuperscript{flox/flox} lym-cre mice. (A, B, C) Gene expression in these cells was measured by gene chip analysis. The expression of signature genes for AMs development and
function (A), Pparγ regulated gene (B) and lipid metabolisms related genes (C) are shown as bar graph. Gene expression in AMs. CD11c<sup>high</sup>Siglec-F<sup>high</sup> cells were isolated from control and PIKfyve<sup>flax/flax</sup> lysm-cre mice, and CD11c<sup>high</sup>Siglec-F<sup>int</sup> cells were isolated from PIKfyve<sup>flax/flax</sup> lysm-cre mice. Gene expression for PIKfyve (D), and Irf8, 5 and I (E) were measured by qPCR.

**Figure EV4** Maintenance of lung homeostasis by AMs. (A) Mice were intranasally administered Alexa633-labeled OVA protein. AMs in BAL were isolated and stained with anti-CD11c. Histogram (left) and percentage (right) of OVA-loaded cells are shown. (B) Lung sections of 8-month-old mice were stained with H/E. Scale bar : 500 µm (C) Surfactant gene expression in lung was measured by rtPCR. Data are represented as mean +/- SD.

**Figure EV5** ILCs, CD11b<sup>+</sup> DCs and IMs contribution to the HDM-induced allergic inflammation. (A) BAL fluid cells were stained with lineage markers and were further stained with anti-T1/ST2 and anti-Sca-1. (B) Percentages of ILCs in BAL fluid are plotted as a bar graph. (C) Il33 and Tslp gene expression in lung tissue was determined by rtPCR. (D, E) Number of CD11b<sup>+</sup>DCs (D) amd IMs (E) was measured by multi FACS analysis. Data are represented as mean +/- SD. *P<0.05 (Student’s t-test).

**Figure EV6** Mechanism of HDM-induced asthma in PIKfyve-deficient mice. (A) Mice were intranasaly administrated HDM and CD11c<sup>high</sup>Siglec-F<sup>high</sup> cells were isolated from control and CD11c<sup>high</sup>Siglec-F<sup>+</sup> cells including Siglec-F<sup>int</sup> cells were isolated from PIKfyve<sup>flax/flax</sup> lysm-cre mice. Expression of Tnfα, Il6 and Il1b was measured by qPCR. (B) M-CSF-induced macrophages were stimulated with LPS or HDM in the presence or absence of GM-CSF. TNFα, IL-6, and IL-1β productions were measured by ELISA. (C)
Mice were treated with HDM, with or without retinoic acid, and lung sections were stained with H/E. Scale bar : 10 µm
Materials and Methods

**Generation of PIKfyve flox mice.** PIKfyve-targeted embryonic stem (ES) cells were obtained from the European Mouse Mutant Cell Repository (EuMMCR). The ES cells were generated from JM8/A3, having the C57BL/6 background. Insertion of the targeting cassette at PIKfyve exon 5 was confirmed by Southern blot. ES cells were injected into 8 cells stage embryo from ICR female mice, and heterozygous F1 progeny were obtained by crossing with C57BL/6 female mice. F1 mice were crossed with Flp mice to extract the β-geo neomycin cassette. The resultant PIKfyve\(^{flox/+}\) mice were crossed with lysm-cre mice to generate PIKfyve\(^{flox/flox}\) lysm-cre mice. All animal maintenance and experiments were performed in accordance with the guidelines of the Committee on Animal Research at Nara Institute of Science and Technology and the Research Institute for Microbial Diseases at Osaka University.

**Western blot analysis, Cells and reagents.** M-CSF-induced macrophages were obtained from mouse bone marrow cells cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS, Life Technologies), 100 µM 2-ME and 10 ng/ml murine M-CSF (ProteoTech) for 5–7 days in a 5% CO\(_2\) atmosphere. Cells were stimulated with 10 ng/ml murine GM-CSF (ProteoTech) and lysed in 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 2.5 mM EGTA, 0.2 % Triton 100. Samples were separated by SDS page and immunoblotted with following antibodies: anti-PIKfyve (P0054) (Sigma), anti-pAKT (17-9715) (eBioscience), anti-pSTAT5 (17-9010) (Cell Signaling), anti-pp65 (#3033S), anti-p65 (#8242S), anti-ERK (#4695P), anti-pERK (#9101S), anti-pp38 (#4511S) anti-p38 (#9212S) and anti-actin (sc-1616) (Santa Cruz Biotechnology). mIL-4 (DY404), mIL-13 (DY413), mTNFα (DY410), mIL-1β (DY401) and mIL-6 (DY406) ELISAs (R&D Systems) were performed according to the manufacturer’s instructions. LPS was purchased from Invivogen.
**FACS analysis.** BAL fluid cells or lung cells were stained with antibody for anti-CD45 (560695, 558702, 560510) (BD Biosciences), anti-CD86 (560582) anti-CD3ε (561108), anti-CD64 (558539) anti-CD4 (553046), anti-CD11c (550261, 562454), anti-Siglec-F (552126) anti-CD24 (560510) anti-CD64 (558539) anti-CD4 (553046), anti-CD11c (550261, 562454), anti-Siglec-F (552126) anti-CD24 (560536) anti-Ly-6C (560596) anti-Ly-6G (560600), anti-Sca-1 (553108), anti-CD11b (69-0112) (eBioscience), anti-c-Kit (25-1171) anti-ST2 (17-9335) anti-CD103 (48-1031) anti-CD205 (25-2051) and anti-F4/80 (123128) (BioLegend). Intracellular staining was performed after surface marker staining, and cells were fixed and permeabilized by Transcription Factor Buffer Set (BD Biosciences) and further stained with anti-Foxp3 (12-5773) (eBioscience), anti-Ki-67 (25-5698), anti-pAKT (17-9715) and anti-pSTAT5 (17-9010). Data were collected by FACS Aria or Accuri C6 (BD Biosciences), and analyzed by FlowJo software (TreeStar).

**Histological analysis.** Lungs were infiltrated with 0.8 ml PBS: O.C.T. Compound (Tissue-Tek), mounted in O.C.T. Compound, and frozen and stored at -80°C. Sections of 10 mm obtained using a cryostat (Thermo Scientific) were collected on glass slides and stained by H/E or PAS.

**Quantitative-rtPCR and microarray.** Total RNA was isolated using Trizol reagent (Life Technologies) and reverse transcribed by ReverTraAce (Toyobo) according to the manufacturer’s instructions. rtPCR was performed using the following primers: mSPA, sense 5’-taaatttgccatggaaga-3’, reverse 5’-atgagctgcagaagaaggt-3’; mSPB, sense 5’-ctgcttcctaccctctgc-3’, reverse 5’-cttggccacaggttactagc-3’; mSPC, sense 5’-ataggacatgtagaagctcggagttt-3’, reverse 5’-cagcatgagaagccgttggag-3’; mSPD, sense 5’-caaagggagaagcttggaacta-3’, reverse 5’-cttttgcacctttagatcct-3’; mGAPDH, sense 5’-tgaacctgccgcttggaaga-3’, reverse 5’-agttagcccaatggcccttcag-3’; mCsf2ra, sense 5’-
cctgctttctcaacgtactg-3\', reverse 5\'-gagactgcgcatgttac-3\'; mCsf2 rb, sense 5\'-gtggagcgaagagactcttg-3\', reverse 5\'-ccaaagggaaggattcaggag-3\'; mCsf2, sense 5\'-acatgacagccacatctac-3\', reverse 5\'-cttaagggtatcagctcag-3\'; mAldhla1, sense 5\'-gacaggtcttccagattgtgct-3\', reverse 5\'-aagactccctccaccattc gagtagtgc-3\'; mAldhla2, sense 5\'-tgccagaactcagagatgg-3\', reverse 5\'-ccaccttgtctctcttga-3\'; mAldhla3, sense 5\'-gacaggtcttccagattgtcag-3\', reverse 5\'-aagactccctccaccattcagtagtgc-3\'; mAldhla3, sense 5\'-tgccagaactcagagatgg-3\', reverse 5\'-ccaccttgtctctcttga-3\'; mAldhla3, sense 5\'-gacaggtcttccagattgtcag-3\', reverse 5\'-aagactccctccaccattcagtagtgc-3\'; mAldhla3, sense 5\'-tgccagaactcagagatgg-3\', reverse 5\'-ccaccttgtctctcttga-3\'; mAldhla3, sense 5\'-gacaggtcttccagattgtcag-3\', reverse 5\'-aagactccctccaccattcagtagtgc-3\'; mAldhla3, sense 5\'-tgccagaactcagagatgg-3\', reverse 5\'-ccaccttgtctctcttga-3\'; mAldhla3, sense 5\'-gacaggtcttccagattgtcag-3\', reverse 5\'-aagactccctccaccattcagtagtgc-3\'; mAldhla3, sense 5\'-tgccagaactcagagatgg-3\', reverse 5\'-ccaccttgtctctcttga-3\'; mAldhla3, sense 5\'-gacaggtcttccagattgtcag-3\', reverse 5\'-aagactccctccaccattcagtagtgc-3\'; mAldhla3, sense 5\'-tgccagaactcagagatgg-3\', reverse 5\'-ccaccttgtctctcttga-3\'; mAldhla3, sense 5\'-gacaggtcttccagattgtcag-3\', reverse 5\'-aagactccctccaccattcagtagtgc-3\'; mAldhla3, sense 5\'-tgccagaactcagagatgg-3\', reverse 5\'-ccaccttgtctctcttga-3\'; mAldhla3, sense 5\'-gacaggtcttccagattgtcag-3\', reverse 5\'-aagactccctccaccattcagtagtgc-3\'; mAldhla3, sense 5\'-tgccagaactcagagatgg-3\', reverse 5\'-ccaccttgtctctcttga-3\'; mAldhla3, sense 5\'-gacaggtcttccagattgtcag-3\', reverse 5\'-aagactccctccaccattcagtagtgc-3\'; mAldhla3, sense 5\'-tgccagaactcagagatgg-3\', reverse 5\'-ccaccttgtctctcttga-3\'; mAldhla3, sense 5\'-gacaggtcttccagattgtcag-3\', reverse 5\'-aagactccctccaccattcagtagtgc-3\'; mAldhla3, sense 5\'-tgccagaactcagagatgg-3\', reverse 5\'-ccaccttgtctctcttga-3\'; mAldhla3, sense 5\'-gacaggtcttccagattgtcag-3\', reverse 5\'-aagactccctccaccattcagtagtgc-3\'; mAldhla3, sense 5\'-tgccagaactcagagatgg-3\', reverse 5\'-ccaccttgtctctcttga-3\'; mAldhla3, sense 5\'-gacaggtcttccagattgtcag-3\', reverse 5\'-aagactccctccaccattcagtagtgc-3\'; mAldhla3, sense 5\'-tgccagaactcagagatgg-3\', reverse 5\'-ccaccttgtctctcttga-3\'; mAldhla3, sense 5\'-gacaggtcttccagattgtcag-3\', reverse 5\'-aagactccctccaccattcagtagtgc-3\'; mAldhla3, sense 5\'-tgccagaactcagagatgg-3\', reverse 5\'-ccaccttgtctctcttga-3\'; mAldhla3, sense 5\'-gacaggtcttccagattgtcag-3\', reverse 5\'-aagactccctccaccattcagtagtgc-3\'; mAldhla3, sense 5\'-tgccagaactcagagatgg-3\', reverse 5\'-ccaccttgtctctcttga-3\'; mAldhla3, sense 5\'-gacaggtcttccagattgtcag-3\', reverse 5\'-aagactccctccaccattcagtagtgc-3\'; mAldhla3, sense

For microarray analysis, RNA was isolated by spin column (Zymo research) and amplified by Ovation\textsuperscript{TM} RAN amplification system V2. Microarray gene expression data are available from the Gene Expression Omnibus (GEO), accession number GSE93849.

**HDM-induced mouse asthma model.** Female mice from 8-12 weeks of ages were intranasally administered 0.2 μg/20 μl DerP1 containing *Dermatophagoides pteronyssinus* extract (Institute of Tokyo Environmental Allergy) at indicated time points, and historical and FACS analyses were then performed.

**Statistics.** Statistical significance was determined by Student’s *t*-test. A *P* value of < 0.05 was considered significant.
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