

# Polymorphonuclear myeloid-derived suppressor cells attenuate allergic airway inflammation by negatively regulating group 2 innate lymphoid cells

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doi:10.1111/imm.13040

Received 31 July 2018; revised 20 November 2018; accepted 6 December 2018.

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## Summary

Hyperactivation of the type 2 immune response is the major mechanism of allergic asthma, in which both group 2 innate lymphoid cells (ILC2s) and type 2 helper T (Th2) cells participate. Myeloid-derived suppressor cells (MDSCs) alleviate asthma by suppressing Th2 cells. However, the potential effects of MDSCs on the biological functions of ILC2s remain largely unknown. Here, we examined the roles of MDSCs (MDSCs) in the modulation of ILC2 function. Our results showed that polymorphonuclear (PMN)-MDSCs, but not monocytic (M-) MDSCs, effectively suppressed the cytokine production of ILC2s both *in vitro* and *in vivo*, thereby alleviating airway inflammation. Further analyses showed that cyclo-oxygenase-1 may mediate the suppressive effects of PMN-MDSCs on ILC2 responses. Our findings demonstrated that PMN-MDSCs may serve as a potent therapeutic target for the treatment of ILC2-driven allergic asthma.

**Keywords:** allergic airway inflammation; group 2 innate lymphoid cells; polymorphonuclear myeloid-derived suppressor cells.

## Introduction

Allergic asthma is a common inflammatory disease of the airways characterized by airway hyper-reactivity (AHR) and reversible airflow obstruction, a typical type 2 inflammatory reaction to an allergen resulting from a complex interplay between genetic and environmental factors.<sup>1,2</sup> A complex system of local immune pathways maintains homeostasis within the lungs, and group 2 innate lymphoid cells (ILC2s) and adaptive type 2 helper T (Th2) cells are an integral part of this. Asthma was initially believed to be an allergic, eosinophilic, Th2-mediated disease. Th2 cells and type 2 cytokines, including interleukin-4 (IL-4), IL-5 and IL-13, were identified as deleterious immune pathway elements in the pathogenesis of asthma.<sup>3</sup> Recently, ILC2s have been shown to have a functional diversity similar to that of their

adaptive counterparts, Th2 cells,<sup>4–7</sup> producing large amounts of type 2 cytokines in pulmonary mucosa, and thereby playing vital roles in the pathogenesis of AHR.<sup>8–10</sup> Previous studies have shown that ILC2s interact with regulatory T cells,<sup>11</sup> natural killer cells,<sup>12</sup> and plasmacytoid dendritic cells<sup>13</sup> to set the immunological tone of allergic lung inflammation.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of pathologically activated myeloid precursors and relatively immature myeloid cells that have been implicated in the immunological regulation of many pathological conditions.<sup>14,15</sup> According to phenotypic and morphological features, MDSCs are divided into two subpopulations: polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs).<sup>16,17</sup> Our previous study reported that PMN-MDSCs have suppressive roles in ovalbumin (OVA)-induced and Th2 cell-mediated AHR.<sup>18</sup>

Abbreviations: BALF, bronchoalveolar lavage fluid; H&E, haematoxylin & eosin; ILC, innate lymphoid cell; IL, interleukin; MDSC, myeloid-derived suppressor cell; M-MDSC, monocytic myeloid-derived suppressor cell; OVA, ovalbumin; PMN, polymorphonuclear; Th2, T helper type 2

However, the dedicated roles of MDSCs (PMN-MDSCs or M-MDSCs) in the pathogenesis of ILC2-induced inflammatory lung diseases, such as asthma, remain largely unknown.

In this study, we examined the roles of MDSCs in the suppression of ILC2 function in a papain- and IL-33-mediated allergic lung inflammation mouse model. Our findings provide important insights into the roles of PMN-MDSCs in preventing allergic lung inflammation by regulating ILC2 function.

## Materials and methods

### *Mice*

Experiments performed in mice were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. C57BL/6 and BALB/c mice were purchased from the Laboratory Animal Centre of Sun Yat-sen University, and OT-1 transgenic mice were kindly provided by Hui Zhang (Sun Yat-sen University). *Rag1*<sup>-/-</sup> mice (B6.129S7-Rag1tm1Mom/JNju) were purchased from Nanjing Biomedical Research Institute of Nanjing University. *Cox-1*<sup>-/-</sup> mice were generated as described previously.<sup>18</sup> All mice were bred in pathogen-free facilities, and age-matched littermates were used as controls.

### *Isolation of single cells from tissues*

Bone marrow cells were obtained by flushing femurs and tibias with a 20-ml syringe containing RPMI-1640 medium followed by lysis of red blood cells by Ammonium-Chloride-Potassium (ACK) buffer. To isolate cells from bronchoalveolar lavage fluid (BALF), 0.8 ml of cold phosphate-buffered saline (PBS) was flushed into the lungs twice via a thin tube inserted into a cut made in the trachea as described previously.<sup>15</sup> To obtain lung cells, lungs were perfused with 10 ml cold PBS through the right ventricle of the heart before removal, cut into pieces on ice and then incubated with 0.5 mg/ml collagenase type I (Invitrogen, Carlsbad, California, USA) in RPMI-1640 medium (Life Technologies, Grand Island, NY) at 37° with continuous agitation. The crude suspensions were further filtered through 70-µm cell strainers to obtain single-cell suspensions. Single-cell suspensions were then fractionated by Percoll (GE Healthcare, Uppsala, Sweden) density-gradient centrifugation. Spleens were passed through 70-µm cell strainers to obtain single-cell suspension and red blood cells were lysed with ACK buffer.

### *Human peripheral blood mononuclear cell isolation*

Mononuclear cells from the peripheral blood of adults or cord blood were isolated by Ficoll centrifugation, and red

blood cells were lysed by ACK buffer and resuspended in PBS.

### *Co-culture MDSCs with ILC2s*

Sorted bone marrow ILC2s (10<sup>4</sup> cells/well) were cultured in the presence of IL-33 (100 ng/ml), IL-2 (20 ng/ml) and IL-7 (20 ng/ml). Media were half changed every 3 days, M-MDSCs, PMN-MDSCs or neutrophils were added on day 6 at the indicated ratio. After 48 hr, the culture supernatants were collected and the cytokines were measured by enzyme-linked immunosorbent assay (ELISA).

### *Transwell migration assay*

ILC2s (5 × 10<sup>4</sup> cells in 300 µl RPMI-1640 medium containing 10% fetal bovine serum) were cultured in the presence of IL-33 (100 ng/ml), IL-2 (20 ng/ml) and IL-7 (20 ng/ml) and seeded onto the top wells of 96-well transwell plates (5-µm pore size; Corning, Union City, CA). PMN-MDSCs (5 × 10<sup>3</sup> cells in 700 µl RPMI-1640 medium containing 10% fetal bovine serum) were loaded on the bottom of the filter membranes of the transwell plates. After 48 hr of incubation at 37° with 5% CO<sub>2</sub>, the culture supernatants were collected and the amounts of cytokines were measured by ELISA.

### *Allergy-induced airway inflammation models in mice*

For induction of papain-induced acute type 2 airway inflammation,<sup>15</sup> mice were anaesthetized with isoflurane, followed by intranasal administration of papain or PBS for 5 consecutive days (20 µg papain in 40 µl PBS). Bronchoalveolar lavage fluid and lungs were harvested for analysis on day 6. For IL-33-induced airway inflammation, recombinant mouse IL-33 (BioLegend, San Diego, California, USA) (500 ng/mouse) or PBS was administered intranasally for 3 days.<sup>19</sup> Twenty-four hours after the final challenge, mice were killed and samples were collected.

### *Flow cytometric analysis and sorting*

Before staining with fluorochrome-conjugated antibodies, single-cell suspensions were incubated with an unlabelled purified anti-Fc receptor blocking antibody (anti-CD16/CD32) and cell viability dye. The live/dead<sup>TM</sup> fixable far-red dead cell stain kit, Invitrogen<sup>TM</sup> was used to exclude dead cells for subsequent analysis. The cell phenotype was evaluated with an LSRII flow cytometer (BD Biosciences, San Jose, CA), and data were analysed in FLOWJo V10.0.7 (FlowJo, TreeStar, Ashland, Oregon, USA). The fraction of labelled cells was analysed with a minimum of 50 000

events. For measurement of intracellular cytokine expression, cells were isolated *ex vivo* and stimulated in complete RPMI-640 medium + 10% fetal bovine serum with 50 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich, St Louis, USA), 1 µg/ml ionomycin (Sigma-Aldrich), and 1 µg/ml brefeldin A (eBioscience, San Diego, CA) for 4 hr. Cells were subsequently surface-stained, fixed and permeabilized using an intracellular fixation and permeability kit (eBioscience), and stained with the indicated cytokines. For the flow cytometric sorting, a BD FACS Aria cell sorter (BD Biosciences) was used. The antibodies used are listed in the Supplementary material (Table S1). For mouse ILC2 sorting, cells were initially depleted of T, B, myeloid and erythroid lineages by labelling with biotin-conjugated anti-CD3, anti-CD45R/B220, anti-CD11b, anti-Ly6G and anti-erythroid marker (TER-119), followed by streptavidin-paramagnetic particles (BD Biosciences) according to the manufacturer's instructions. The remaining cells were stained with the specific fluorochrome-conjugated antibodies (bone marrow immature ILC2s: Lin<sup>−</sup> CD45<sup>+</sup> Sca-1<sup>+</sup> CD25<sup>+</sup> CD127<sup>+</sup>; Lung ILC2s: Lin<sup>−</sup> CD45<sup>+</sup> KlrG1<sup>+</sup> CD25<sup>+</sup> CD90.2<sup>+</sup> ST2<sup>+</sup>) and sorted with Aria III (BD Biosciences). For human ILC2 sorting, human peripheral blood mononuclear cells were first depleted of T cells, B cells, natural killer cells, myeloid cells, granulocytes and red blood cells by labelling with biotin-conjugated anti-CD2, anti-CD3, anti-CD10, anti-CD11b, anti-CD14, anti-CD16 and anti-CD19, then with magnetic beads that, when placed in a magnetic field, leave lineage-negative cells untouched and free in solution. The collected lineage-negative cells were then stained with the specific fluorochrome-conjugated antibodies for ILC2s (Lin<sup>−</sup> CD45<sup>+</sup> CD127<sup>+</sup> CRTH2<sup>+</sup>). For mouse MDSC, the sorting strategy was CD11b<sup>+</sup> Ly6C<sup>lo</sup> Ly6G<sup>+</sup> for PMN-MDSCs and CD11b<sup>+</sup> Ly6C<sup>hi</sup> Ly6G<sup>−</sup> for M-MDSCs. For human MDSC, the sorting strategy was CD14<sup>−</sup> CD11b<sup>+</sup> CD15<sup>+</sup> for PMN-MDSCs and CD11b<sup>+</sup> CD14<sup>+</sup> HLA-DR<sup>−/low</sup> CD15<sup>−</sup> for M-MDSCs. Antibodies are listed in the Supplementary material (Table S1).

#### PMN-MDSC depletion

In the PMN-MDSC depletion experiment, 40 µg/g of anti-DR5 (BioXcell, West Lebanon, New Hampshire, USA) was administered to mice intraperitoneally 24 hr before IL-33 challenge, rat IgG isotype was used as control.

#### PMN-MDSC suppressive assay

The PMN-MDSCs from mice with airway inflammation were sorted by flow cytometry and plated on U-bottomed 96-well plates, followed by co-culture with CD8 T cells from OT-I mice at different ratios in the presence of cognate peptides: OT-1, SIINFEKL. Cells were incubated for

3 days and T-cell proliferation was analysed by flow cytometry.

#### PMN-MDSC transfer

The PMN-MDSCs were purified from spleens of newborn mice (7–9 days old). In the papain model,  $2 \times 10^6$  isolated PMN-MDSCs were injected through the tail vein 1 day before papain treatment and on the 3rd day of papain treatment. For adoptive transfer in the IL-33 model, 2 million PMN-MDSCs were injected through the tail vein 1 day before IL-33 treatment.

#### Statistical analysis

*P* values were calculated using unpaired two-tailed Student's *t*-tests in GRAPH PAD PRISM v6 (GraphPad, San Diego, CA). *P* < 0.05 was considered statistically significant.

## Results

### PMN-MDSCs, but not M-MDSCs, suppressed IL-5 and IL-13 production by ILC2s

To explore the effects of MDSCs on ILC2s, we performed *in vitro* suppression assays. First, PMN-MDSCs (CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>lo</sup>) and M-MDSCs (CD11b<sup>+</sup> Ly6G<sup>−</sup> Ly6C<sup>hi</sup>) were purified from newborn mice, which we had previously shown to display suppressive activity,<sup>20</sup> and neutrophils (CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>lo</sup>) were sorted from spleens of naive adult mice (6–8 weeks of age) (see Supplementary material, Fig. S1a). Additionally, CD115 (Macrophage Colony-stimulating Factor Receptor) and CD244 were used to identify PMN-MDSCs in this study (see Supplementary material, Fig. S1b).<sup>21</sup> Subsequently, these cells were co-cultured with ILC2s (CD45<sup>+</sup> Lin<sup>−</sup> CD90.2<sup>+</sup> CD127<sup>+</sup> ST2<sup>+</sup> cells) at different ratios *in vitro*. After 3 days in culture, we measured the production of type 2 cytokines using ELISA. As expected, ILC2s secreted large amounts of IL-5 and IL-13, whereas their production was obviously decreased as the numbers of PMN-MDSCs increased (Fig. 1a). Neither M-MDSCs or neutrophils, however, failed to display any effects (Fig. 1b,c). The inhibitory effects of PMN-MDSCs on the cytokine secretion of ILC2 were further confirmed upon stimulation with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (1 µg/ml) (Fig. 1d). However, co-culture in the presence of transwells completely cancelled this suppressive effect (Fig. 1e). Consistently, co-culture of human ILC2s with PMN-MDSCs from human cord blood displayed similar results (Fig. 1f). Neutrophils from the peripheral blood of adults, however, failed to show any noticeable effects on human ILC2 (Fig. 1g). These results indicated that PMN-MDSCs efficiently suppressed the cytokine production of ILC2 in a cell–cell contact-dependent manner.

Next, bone marrow cells were cultured to induce the differentiation of ILC2s *in vitro* (see Supplementary material, Fig. S2a), followed by co-culture with PMN-MDSCs. Results showed that the frequency of ILC2s

(Lin<sup>-</sup> CD45<sup>+</sup> CD25<sup>+</sup> Sca-1<sup>+</sup> cells) did not differ in the presence or absence of PMN-MDSCs; however, the proportion of IL-5<sup>+</sup> IL-13<sup>+</sup> ILC2s (see Supplementary material, Fig. S2b,c), as well as the concentrations of IL-5 and IL-13

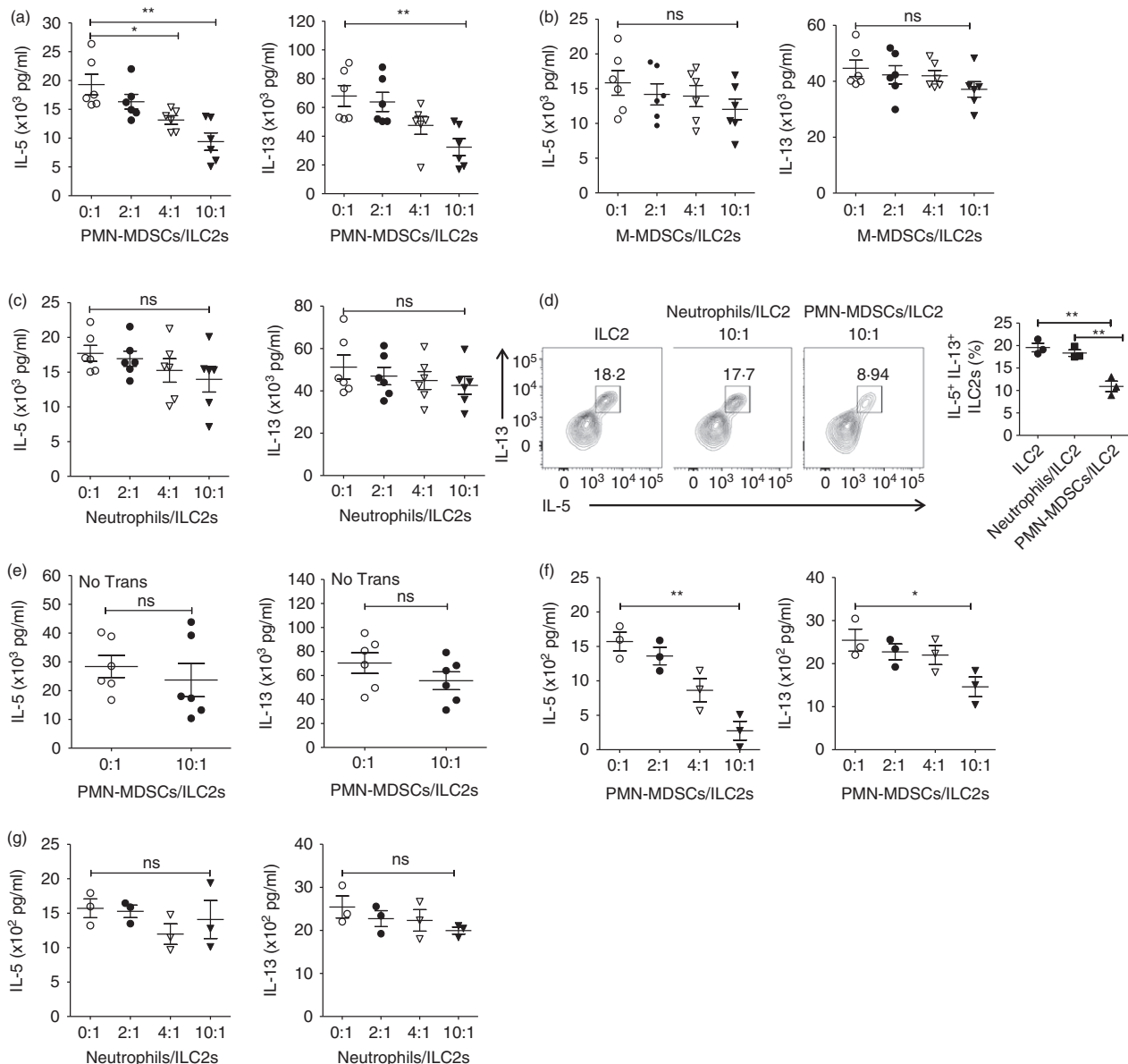


**Figure 1.** Polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) attenuate group 2 innate lymphoid cell (ILC2) cytokine production *in vitro*. Purified iILC2 cells from bone marrow (BM) were cultured with interleukin-2 (IL-2) (20 ng/ml), IL-7 (20 ng/ml) and IL-33 (100 ng/ml) for 5 days, followed by co-culture with PMN-MDSCs (CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>lo</sup>) (a), monocyte (M-) MDSCs (CD11b<sup>+</sup> Ly6G<sup>-</sup> Ly6C<sup>hi</sup>) (b) or neutrophils (CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>lo</sup>) (c) at the indicated ratios for 48 hr. The amounts of IL-5 and IL-13 were measured by ELISA. PMN-MDSCs and M-MDSCs were purified from spleens of neonatal mice; neutrophils were sorted from spleens of adult mice. (d) For the secretion of cytokines from ILC2s, ILC2s were stimulated for 4 hr with phorbol 12-myristate 13-acetate (PMA) and ionomycin. Flow cytometric analysis of IL-5<sup>+</sup> IL-13<sup>+</sup> ILC2s was performed. (e) ILC2s were co-cultured with PMN-MDSCs in the presence or absence of transwells and the cytokine production was determined by ELISA. (f, g) Human ILC2s were co-cultured with human PMN-MDSCs (f) or neutrophils (g) at the indicated ratios, then IL-5 and IL-13 levels were measured using ELISA. Human ILC2s (CD45<sup>+</sup> Lin<sup>-</sup> CD127<sup>+</sup> CRTH2<sup>+</sup>) and neutrophils (CD14<sup>-</sup> CD11b<sup>+</sup> CD15<sup>+</sup>) were purified from peripheral blood mononuclear cells (PBMCs) of healthy adults, human PMN-MDSCs (CD14<sup>-</sup> CD11b<sup>+</sup> CD15<sup>+</sup>) were purified from cord blood. In all plots, mean  $\pm$  SEMs are shown. ns, no statistical significance; \*P < 0.05; \*\*P < 0.01 in two-tailed Student's *t*-test. Data are representative of three independent experiments.

in the supernatants (see Supplementary material, Fig. S2d), were dramatically decreased after co-culture with PMN-MDSCs. The proliferation or apoptosis of ILC2 was not affected by PMN-MDSCs, as revealed by Ki-67 staining (see Supplementary material, Fig. S2e) or Annexin V staining (see Supplementary material, Fig. S2f).

### PMN-MDSCs alleviated ILC2-induced allergic lung inflammation *in vivo*

To evaluate the effect of PMN-MDSCs on ILC2 function *in vivo*, PMN-MDSCs were intravenously transferred to C57BL/6 mice, and papain was intranasally administered to induce allergic lung inflammation (schema in Fig. 2a). In papain-treated animals, transfer of PMN-MDSCs resulted in clear attenuation of lung inflammation compared with the PBS control, as shown by haematoxylin & eosin (H&E) staining (Fig. 2b). The infiltration of eosinophils (Fig. 2c), and the amounts of IL-5 and IL-13 (Fig. 2d), in the BALF were significantly reduced upon PMN-MDSC transfer. Consistently, the frequencies of IL-5<sup>+</sup> IL-13<sup>+</sup> ILC2s (Lin<sup>-</sup> CD45<sup>+</sup> CD90.2<sup>+</sup> CD25<sup>+</sup> IL-5<sup>+</sup> IL-13<sup>+</sup> cells) were dramatically decreased, although the total number of lung ILC2s (Lin<sup>-</sup> CD45<sup>+</sup> CD90.2<sup>+</sup> Klr1<sup>+</sup> ST2<sup>+</sup> GATA3<sup>+</sup> cells) did not show noticeable differences (Fig. 2e,f). The flow gating strategies of lung ILC2s and their cytokine production are presented in the Supplementary material (Fig. S3a, b). The numbers of PMN-MDSCs, but not M-MDSCs, were elevated in the papain model (see Supplementary material, Fig. S4). These results suggested that PMN-MDSCs suppressed the function of ILC2s *in vivo* and attenuated ILC2-dependent lung inflammation.

We next performed IL-33 injection to induce allergic inflammation in which ILC2s were thought to play a key role (schema shown in the Supplementary material, Fig. S5a). Consistent with the observations from the papain model, PMN-MDSC transfer obviously alleviated lung inflammation (see Supplementary material, Fig. S5b), eosinophil infiltration and type 2 cytokine production, when compared with the neutrophil control group (see Supplementary material, Fig. S5c,d). The frequencies of IL-5<sup>+</sup> IL-13<sup>+</sup> ILC2s, not total lung ILC2, were decreased as expected (see Supplementary material, Fig. S5e,f). Expansion of PMN-MDSCs was also observed in the IL-33-induced allergic model (see Supplementary material, Fig. S5g). Collectively, these results showed that PMN-MDSCs effectively attenuated lung inflammation by suppressing ILC2 responses.

### Depletion of PMN-MDSCs aggravated ILC2-induced allergic lung inflammation

We next depleted PMN-MDSCs by intraperitoneal injection of antibody against TRAIL-R (DR5ab)<sup>22–24</sup> 24 hr before IL-33 challenge (schema in Fig. 3a). As expected, a dramatic decrease in the number of PMN-MDSCs was

observed in the spleens of mice after DR5ab injection (Fig. 3b). Furthermore, PMN-MDSC depletion aggravated lung inflammation, as indicated by H&E staining (Fig. 3c), the levels of eosinophils (Fig. 3d) and type 2 cytokine production (Fig. 3e) in the BALF. Flow cytometric analysis showed that the frequencies and number of lung ILC2 did not change (Fig. 3f), whereas their secretion of IL-5 and IL-13 was obviously increased upon DR5 antibody injection (Fig. 3g). These data indicated that depletion of PMN-MDSCs aggravated ILC2-induced airway inflammation.

### PMN-MDSCs abrogated allergic lung inflammation in a T-cell-independent manner

To exclude the effects of bystander T cells, which were previously reported to be suppressed by PMN-MDSCs in OVA-induced AHR,<sup>18</sup> *Rag1*<sup>-/-</sup> mice lacking T cells and B cells were used in the papain model (schema in Fig. 4a). Results showed that transfer of PMN-MDSCs obviously ameliorated papain-induced AHR in *Rag1*<sup>-/-</sup> mice, when compared with mice receiving neutrophils, including lung histology staining by H&E, the infiltration of eosinophils in BALF, and the amounts of effector cytokines in BALF (Fig. 4b–d). The cytokine production of ILC2s was also impaired by PMN-MDSCs, although no differences in ILC2 numbers were observed (Fig. 4e,f). These results suggested that the suppressive effect of PMN-MDSCs on ILC2 responses were independent of T cells.

### PMN-MDSCs alleviated ILC2-induced allergic lung inflammation through COX-1

Next, we explored how PMN-MDSCs exerted suppressive effects on ILC2s to alleviate allergic lung inflammation. We have shown that deficiency of cyclo-oxygenase (COX)-1 impaired the immunosuppressive function of PMN-MDSCs, which enhanced Th2 responses and resulted in aggravation of OVA-induced allergic lung inflammation.<sup>18</sup> To determine whether COX-1 mediates the effect of PMN-MDSCs on ILC2 during allergic lung inflammation, we first evaluated the role of COX-1 in papain-induced AHR in mice (schema in Fig. 5a). As expected, genetic ablation of COX-1 (*Cox-1*<sup>-/-</sup>) in mice worsened lung inflammation, including lung histology staining, infiltration of eosinophils and the amounts of effector cytokines in BALF (Fig. 5b–d). Flow cytometric analysis revealed that the frequency of lung ILC2s failed to show differences between *Cox-1*<sup>-/-</sup> mice and wild-type controls; however, *Cox-1*<sup>-/-</sup> lung ILC2s secreted much more IL-5 and IL-13 (Fig. 5e,f). Accompanied by the aggravation of lung inflammation and enhanced ILC2 responses, the levels and immunosuppressive functions of PMN-MDSCs, not M-MDSCs, were significantly impaired in *Cox-1*<sup>-/-</sup> mice in the papain model (see

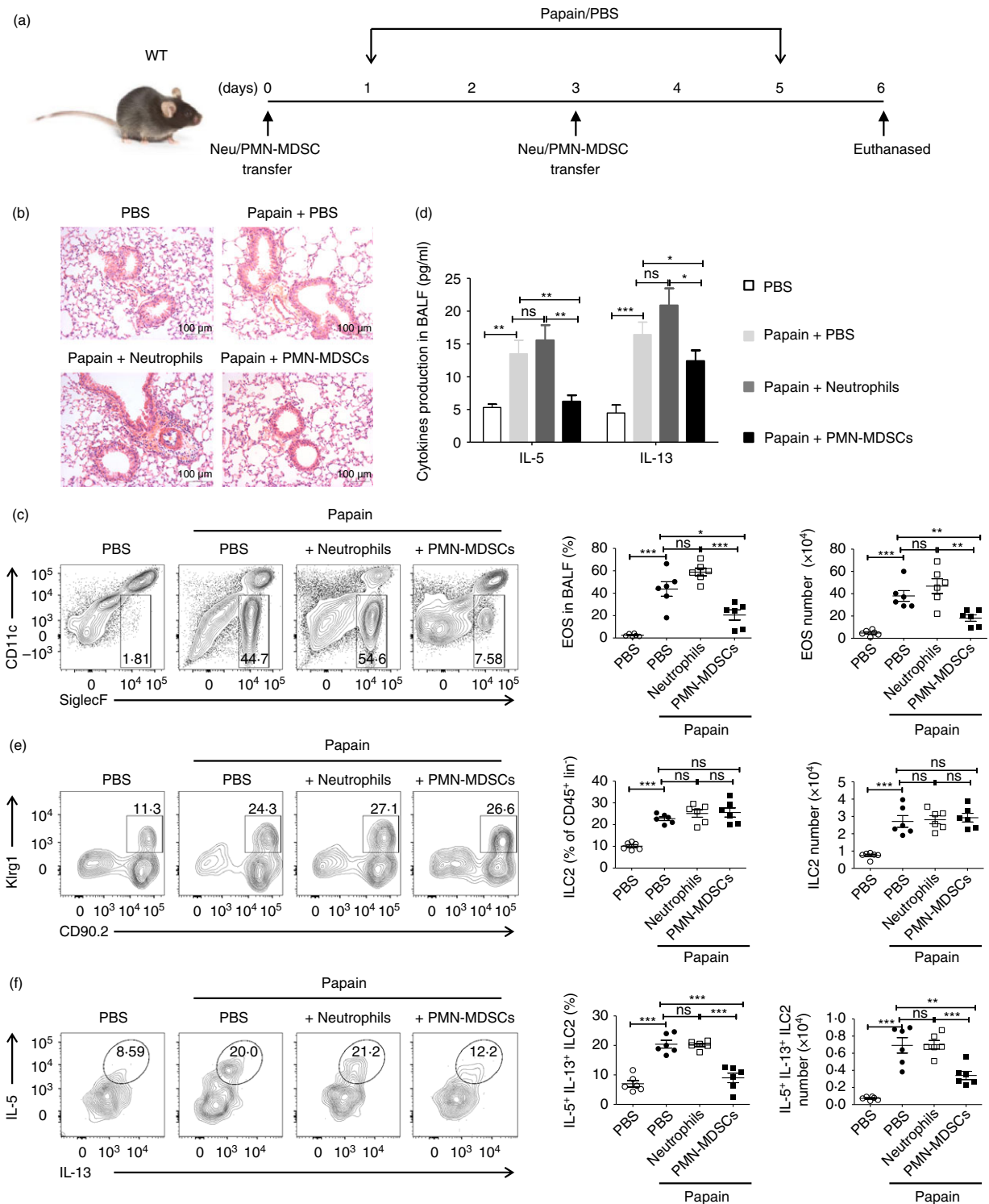


**Figure 2.** Polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) alleviate papain-induced allergic lung inflammation *in vivo*. (a) PMN-MDSCs or neutrophils were injected into wild-type (WT) mice intravenously, followed by intranasal challenges with papain or phosphate-buffered saline (PBS) for 5 consecutive days. One day after the last challenge, mice were killed ( $n = 6$ /group). (b) Lung histology ( $\times 200$  magnification) by H&E staining. (c) Flow cytometric analysis of eosinophils in bronchoalveolar lavage fluid (BALF). (d) The concentrations of interleukin-5 (IL-5) and IL-13 in the BALF were determined by ELISA. (e) Representative flow cytometric and statistical results of total group 2 innate lymphoid cells (ILC2s) ( $CD45^+ Lin^- CD90.2^+ Klr1^+$  cells) in lung. (f) Representative flow cytometric and statistical results of IL-5<sup>+</sup> IL-13<sup>+</sup> ILC2s ( $CD45^+ Lin^- CD90.2^+ CD25^+ IL-5^+ IL-13^+$ ) in lung. In all plots, mean  $\pm$  SEM are shown. ns, no statistical significance; \* $P$  < 0.05; \*\* $P$  < 0.01; \*\*\* $P$  < 0.001 in two-tailed Student's *t*-test. Data are representative of three independent experiments.

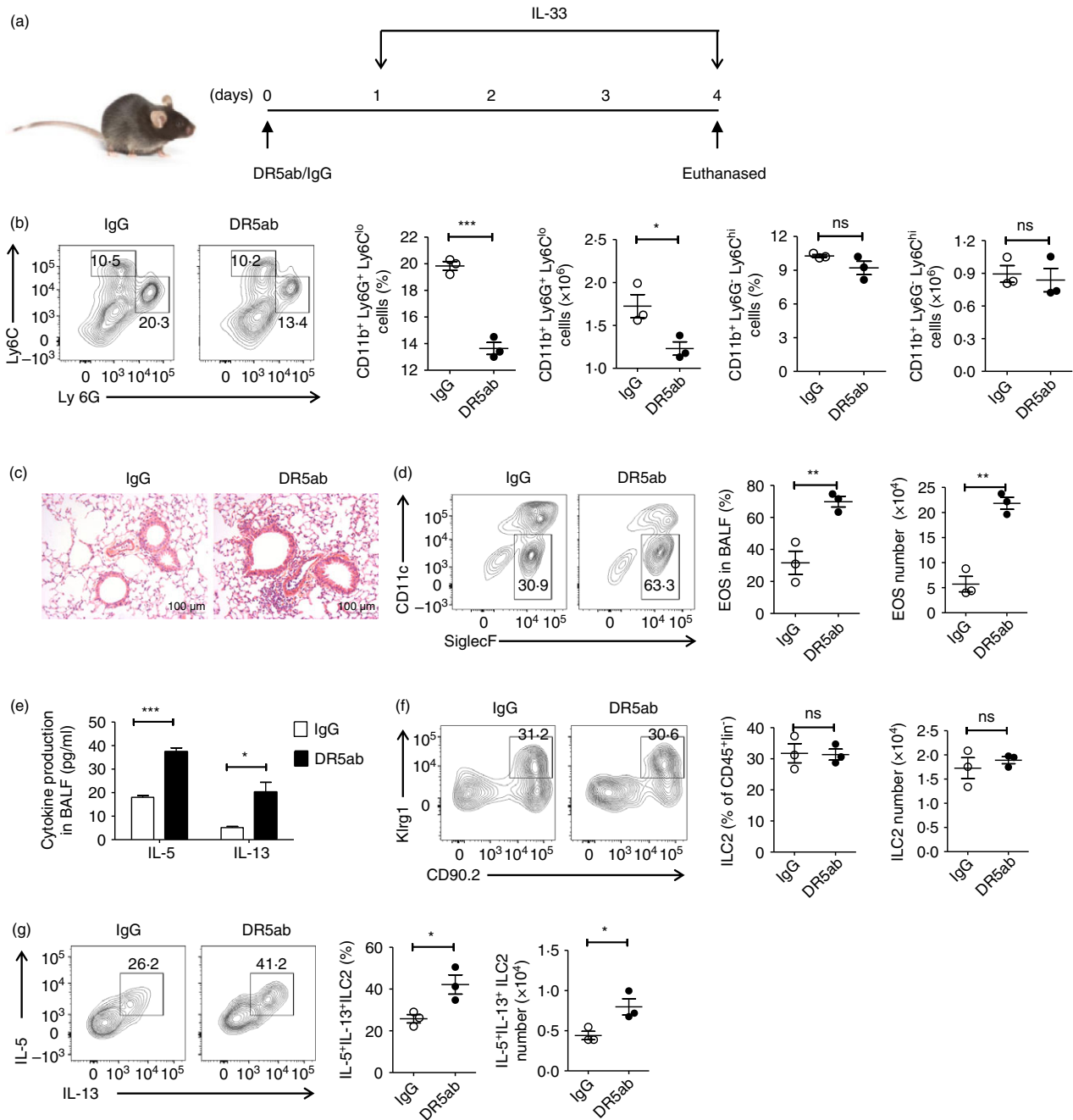


**Figure 3.** Depletion of polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) aggravated group 2 innate lymphoid cell (ILC2) - induced lung inflammation *in vivo*. (a) Mice were injected with DR5ab antibody or anti-IgG control intraperitoneally, followed by intranasal challenge with interleukin-33 (IL-33) or phosphate-buffered saline (PBS) for 3 consecutive days. One day after the last challenge, mice were killed ( $n = 3/\text{group}$ ). (b) The levels of MDSCs in spleen. (c) Lung histology ( $\times 200$  magnification) after anti-DR5 or anti-IgG control injection. (d) Flow cytometric analysis of eosinophils in the bronchoalveolar lavage fluid (BALF). (e) The concentrations of IL-5 and IL-13 in the BALF were evaluated by ELISA. (f) Representative flow cytometric and statistical results of total lung ILC2s (Lin<sup>-</sup> CD45<sup>+</sup> CD90.2<sup>+</sup> Klr1<sup>+</sup> cells). (g) Representative flow cytometric and statistical results of IL-5<sup>+</sup> IL-13<sup>+</sup> ILC2s (CD45<sup>+</sup> Lin<sup>-</sup> CD90.2<sup>+</sup> CD25<sup>+</sup> IL-5<sup>+</sup> IL-13<sup>+</sup>) in lung. In all plots, mean  $\pm$  SEM are shown. ns, no statistical significance; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  in two-tailed Student's *t*-test. Data are representative of three independent experiments.

Supplementary material, Fig. S6a,b). *Cox-1*<sup>-/-</sup> lung ILC2s failed to show any defect in their production of IL-5 and IL-13 during *in vitro* culture (Fig. 5g), suggesting that

COX-1 deficiency did not impair ILC2 function directly. However, when PMN-MDSCs from COX-1-deficient newborn mice were co-cultured with ILC2s at a ratio of

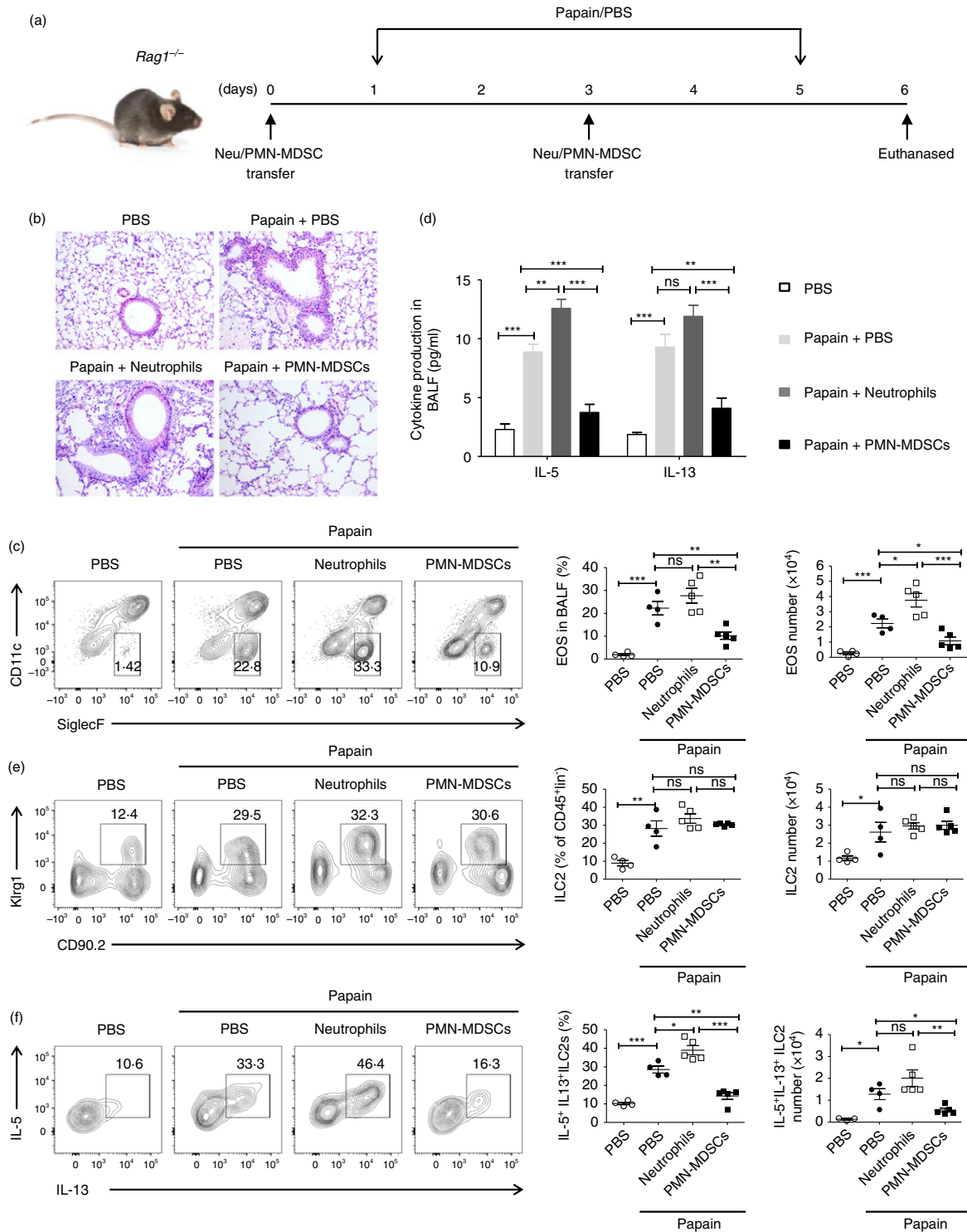


**Figure 4.** Polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) alleviate papain-induced allergic lung inflammation in *Rag1*<sup>-/-</sup> mice. (a) PMN-MDSCs or neutrophils were injected into *Rag1*<sup>-/-</sup> mice intravenously, followed by intranasal challenge with papain or phosphate-buffered saline (PBS) for 5 consecutive days. One day after the last challenge, mice were killed ( $n = 4$  or 5/group). (b) Lung histology ( $\times 200$  magnification) by H&E staining. (c) Flow cytometric analysis of eosinophils in the bronchoalveolar lavage fluid (BALF). (d) The concentrations of interleukin-5 (IL-5) and IL-13 in the BALF were evaluated by ELISA. (e) Representative flow cytometric and statistical results of total group 2 innate lymphoid cells (ILC2s) ( $\text{Lin}^- \text{CD45}^+ \text{CD90.2}^+ \text{Klr1g1}^+$  cells) in lung. (f) Representative flow cytometric and statistical results of IL-5<sup>+</sup> IL-13<sup>+</sup> ILC2s ( $\text{CD45}^+ \text{Lin}^- \text{CD90.2}^+ \text{CD25}^+ \text{IL-5}^+ \text{IL-13}^+$ ) in lung. In all plots, mean  $\pm$  SEM are shown. ns, no statistical significance; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  in two-tailed Student's *t*-test. Data are representative of three independent experiments.

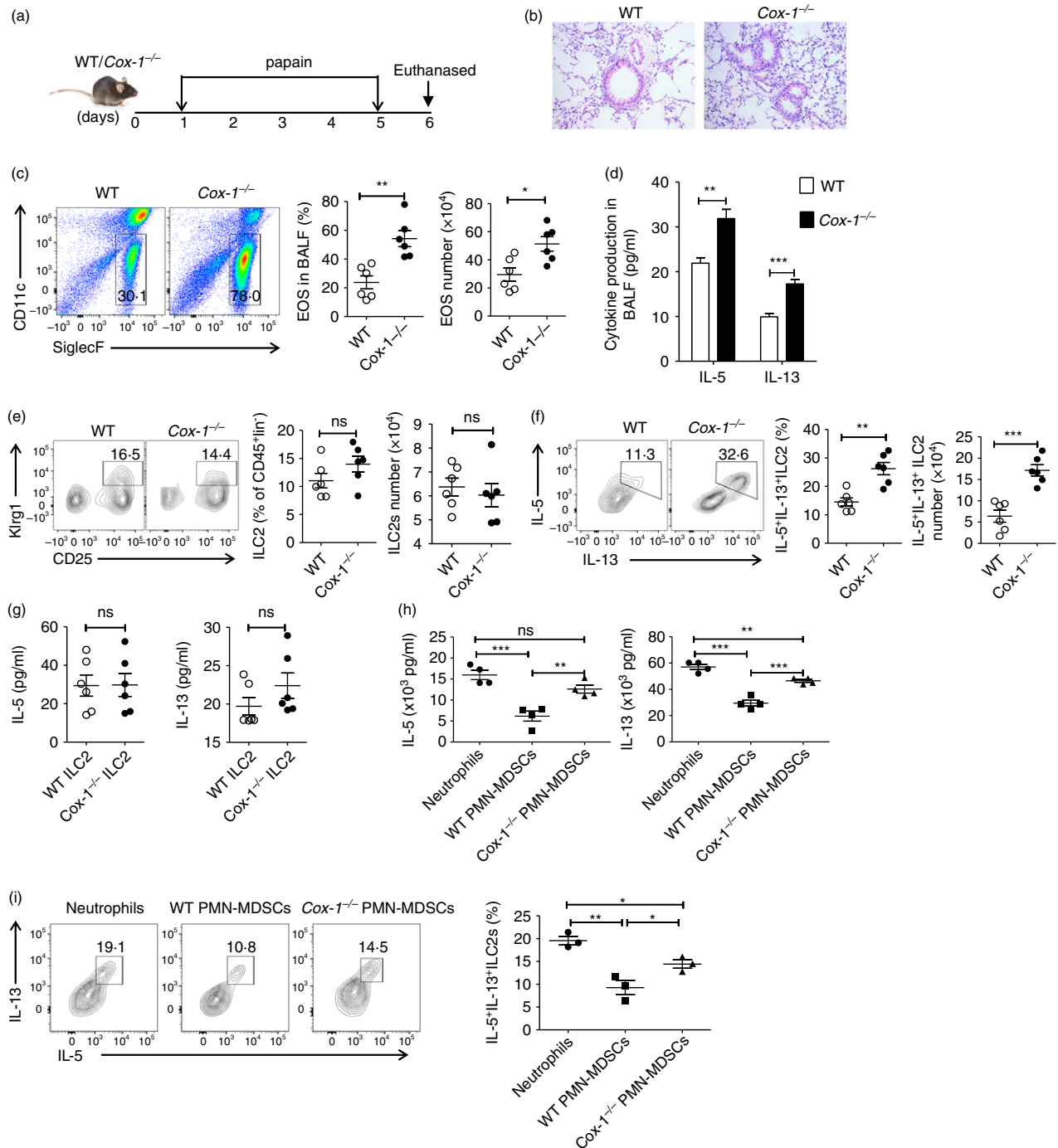


**Figure 5.** Cyclo-oxygenase 1 (COX-1) deficiency aggravates papain-induced allergic lung inflammation. (a) *Cox1*<sup>-/-</sup> mice and their wild-type (WT) littermate controls were given papain or phosphate-buffered saline (PBS) intranasally for 5 consecutive days. One day after the last challenge, lung inflammation was measured and samples were collected ( $n = 6/\text{group}$ ). (b) Lung histology (×200 magnification) by H&E staining. (c) Flow cytometric analysis of eosinophils in the bronchoalveolar lavage fluid (BALF). (d) The concentrations of interleukin-5 (IL-5) and IL-13 in the BALF were evaluated by ELISA. (e) Representative flow cytometric and statistical results of total group 2 innate lymphoid cells (ILC2s) (Lin<sup>-</sup> CD45<sup>+</sup> CD90.2<sup>+</sup> Klr1<sup>+</sup> cells) in lung. (f) Representative flow cytometric and statistical results of IL-5<sup>+</sup> IL-13<sup>+</sup> ILC2s (CD45<sup>+</sup> Lin<sup>-</sup> CD90.2<sup>+</sup> CD25<sup>+</sup> IL-5<sup>+</sup> IL-13<sup>+</sup>). (g) Equal numbers of purified lung ILC2s from WT and *Cox1*<sup>-/-</sup> mice were cultured in the presence of 20 ng/ml of IL-2, IL-7 and 100 ng/ml IL-33 for 3 days. The amounts of IL-5 and IL-13 in supernatants were evaluated by ELISA. (h-i) Purified iILC2 cells from bone marrow (BM) were cultured with interleukin-2 (IL-2) (20 ng/ml), IL-7 (20 ng/ml) and IL-33 (100 ng/ml) for 5 days, followed by co-culture with PMN-MDSCs (CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>lo</sup>) from spleens of neonatal WT or *Cox-1*<sup>-/-</sup> mice respectively. Neutrophils (CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>lo</sup>) from adult mice were used as control. (h) The amounts of IL-5 and IL-13 were measured by ELISA. (i) Flow cytometric analysis of IL-5<sup>+</sup> IL-13<sup>+</sup> ILC2s was performed. In all plots, mean ± SEM are shown. ns, no statistical significance; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  in two-tailed Student's test. Data are representative of three independent experiments.

10 : 1, the cytokine production of ILC2s was significantly enhanced (Fig. 5h–i), indicating that *Cox-1*<sup>−/−</sup> PMN-MDSCs displayed much weaker suppressive effects on ILC2. Collectively, these data indicated that COX-1 may mediate the interaction between PMN-MDSC and ILC2 during allergic inflammation.

## Discussion

Accumulation of PMN-MDSCs was previously reported in OVA- and Th2 cell-induced allergic airway inflammation, and adoptive transfer of PMN-MDSCs clearly suppresses airway inflammation. However, the role of MDSCs in innate immunity during airway inflammation remains largely unknown. In this study, we demonstrated a novel role of PMN-MDSCs in the suppression of ILC2 function both *in vitro* and *in vivo*.

Unlike helper T lymphocytes in adaptive immunity, ILCs do not express antigen receptors that recognize ‘non-self’ structures, but they secrete similar cytokines with the corresponding helper T cells.<sup>4,6</sup> ILC2s are widely recognized for their rapid responses to the epithelial-cell-derived cytokines IL-33, IL-25 and thymic stromal lymphopoietin, upon allergen stimulation.<sup>25</sup> ILC2s then secrete type 2 cytokines (e.g. IL-5 and IL-13) and facilitate eosinophilic inflammatory responses in mouse models of asthma.<sup>8,26</sup> Both MDSCs and ILC2s participate in the allergic inflammation, but their potential interaction remains unclear. In patients with asthma, higher levels of circulating ILC2s are accompanied by increased numbers of MDSCs, and the levels of ILC2 signature cytokines (IL-5 and IL-13) in asthma were positively correlated with MDSC-related enzymes (e.g. arginase-1 and inducible nitric oxide synthase).<sup>27</sup> It was also reported that IL-13 secretion by ILC2s is correlated with increased numbers of M-MDSCs in multiple types of tumours.<sup>28,29</sup> In this study, we demonstrated that PMN-MDSCs, but not M-MDSCs, suppress the function of ILC2s in a cell–cell contact-dependent manner.

By performing both *in vitro* and *in vivo* assays, we demonstrated that PMN-MDSCs could efficiently suppress the cytokine production of lung ILC2s. Deficiency of COX-1, the enzyme responsible for synthesis of prostaglandins, significantly impaired the effect of PMN-MDSCs on ILC2s. We have reported that MDSCs with deletion of COX-1 display lower levels of prostaglandin E<sub>2</sub>, which caused defects in their immunosuppressive activity toward Th2 responses in asthma.<sup>18</sup> Therefore, it is possible that MDSCs use a similar mechanism in the suppression of both ILC2 and Th2. The detailed mechanism of PMN-MDSC-mediated ILC2 responses, however, deserves to be further investigated. The clinical significance of the interaction between MDSC and ILC2 in patients with allergic airway inflammation, should also be evaluated.

## Acknowledgements

This work was supported by the following grants: National Natural Science Foundation of China (grants 91542112; 81571520, 81771665 and 81742002; grants 31700061), National Natural Science Foundation of Guangdong (2017B030311014), Science and Technology Programme of Guangzhou (201605122045238).

## Disclosures

The authors declare having no competing interests.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Flow cytometric analysis of neutrophils and MDSC subsets.

**Figure S2.** Effect of PMN-MDSCs on cytokine production of ILC2s *in vitro*.

**Figure S3.** Gating Strategy of ILC2 and ILC2-secreting cytokines in Lung.

**Figure S4.** Expansion of PMN-MDSCs in papain model.

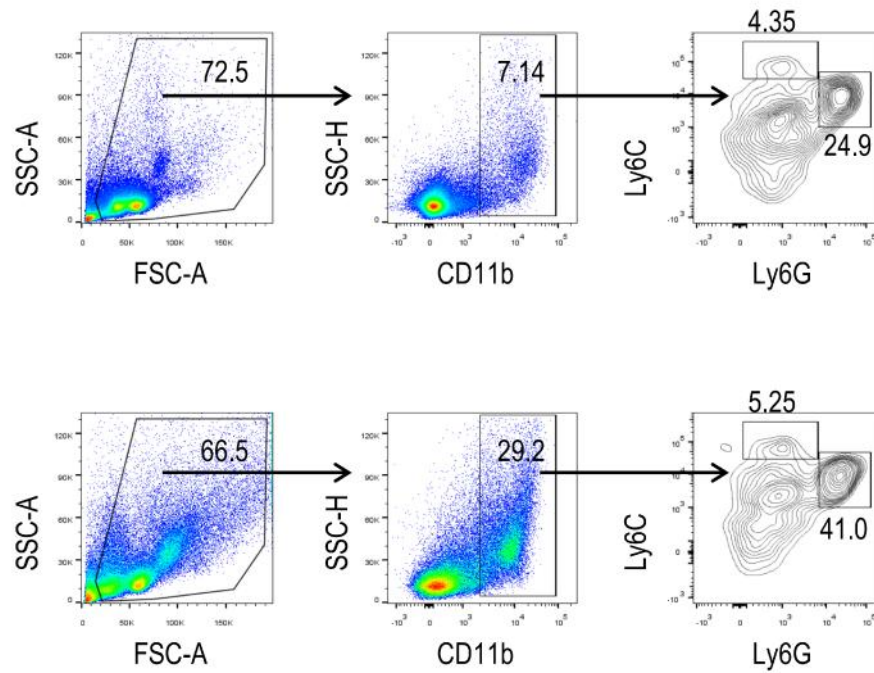
**Figure S5.** PMN-MDSCs alleviate IL-33-induced allergic lung inflammation *in vivo*.

**Figure S6.** The function of PMN-MDSCs was impaired in *Cox1*<sup>−/−</sup> mice in papain model.

**Table S1.** Antibodies used for flow.

## Supplemental Figure

**A**



**B**

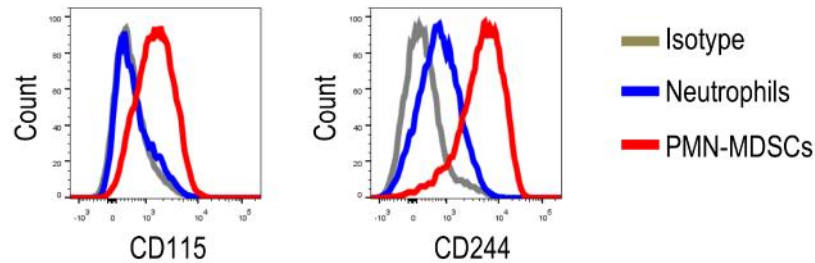


**Figure S1** Flow cytometric analysis of neutrophils and MDSC subsets

(a) Representative flow cytometric analysis of neutrophils (CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>lo</sup>) from spleen of adult mice (upper); Or PMN-MDSCs (CD11<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>lo</sup>) and M-MDSCs (CD11b<sup>+</sup> Ly6G<sup>-</sup> Ly6C<sup>hi</sup>) from 2-week newborn mice (lower). (b) CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>lo</sup> cells were gated as neutrophils and PMN-MDSC. The expression of CD115 and CD244 in neutrophils and PMN-MDSCs were evaluated by flow cytometry. Data are representative of three independent experiments.

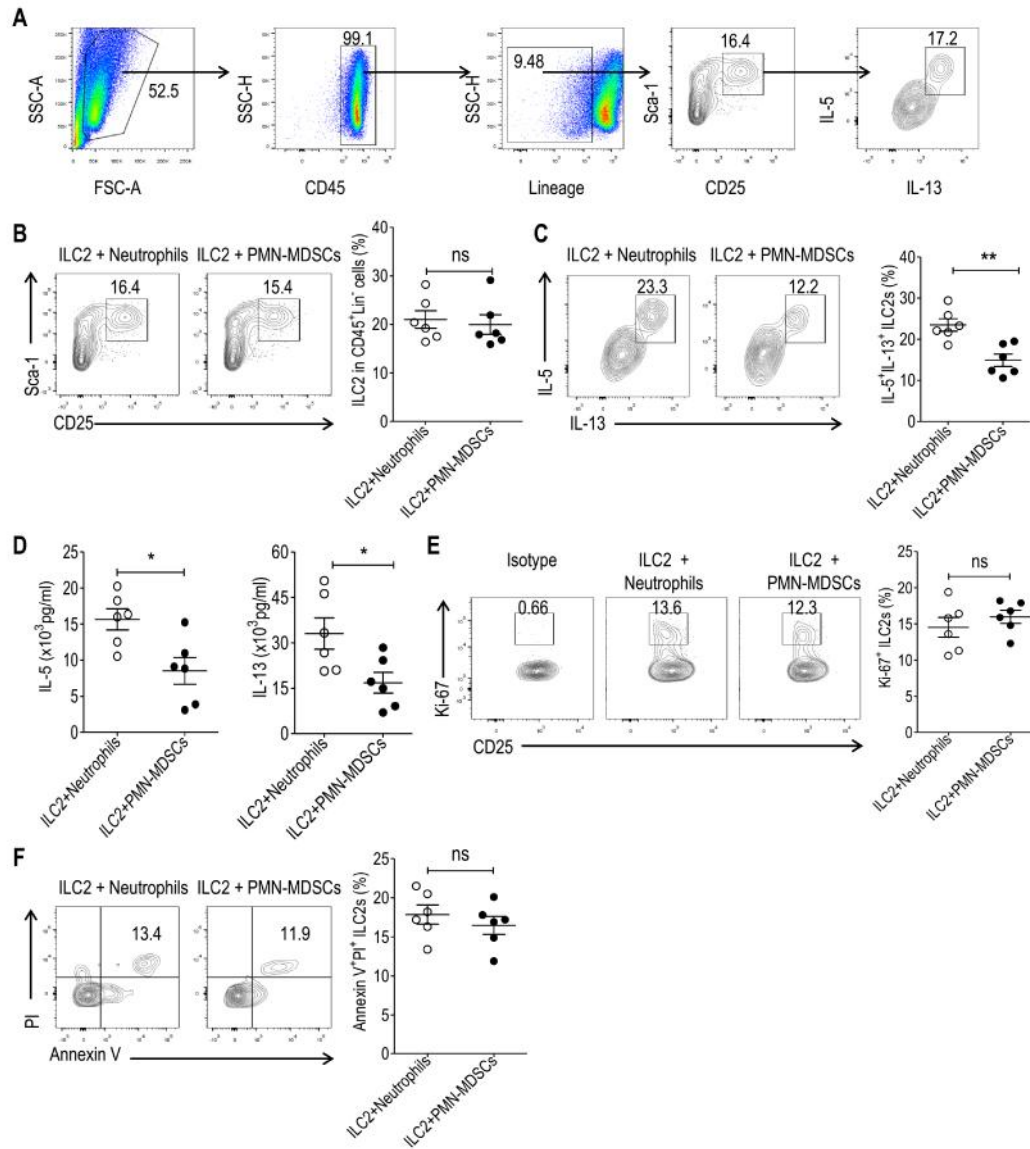


**Figure S2** Effect of PMN-MDSCs on cytokine production of ILC2s *in vitro*.

ILC2s were generated by culturing bone marrow cells in IL-7 (10 ng/ml) and IL-33 (10 ng/ml) for 5 days. After *in vitro* expansion (ILC2 level is about 15%), cells were co-cultured with PMN-MDSCs or neutrophils (10 times cell number of ILC2) in the presence of IL-2 (10 ng/ml), IL-7 (10 ng/ml). After 48 hours, flow cytometric analysis of ILC2 was performed, including gating strategy of ILC2 and ILC2-secreting cytokines (a), the levels of ILC2 (b), intracellular IL-5 and IL-13 production of ILC2

(c), proliferation of ILC2 (Ki-67 staining) (e), apoptosis of ILC2 (Annexin V and PI staining) (f). (c) The amounts of IL-5 and IL-13 in supernatants were measured by ELISA. In all plots, mean  $\pm$  SEM are shown. ns: no statistical significance; \*  $P < 0.05$ , \*\*  $P < 0.01$ , in two-tailed Student's test. Data are representative of three independent experiments.

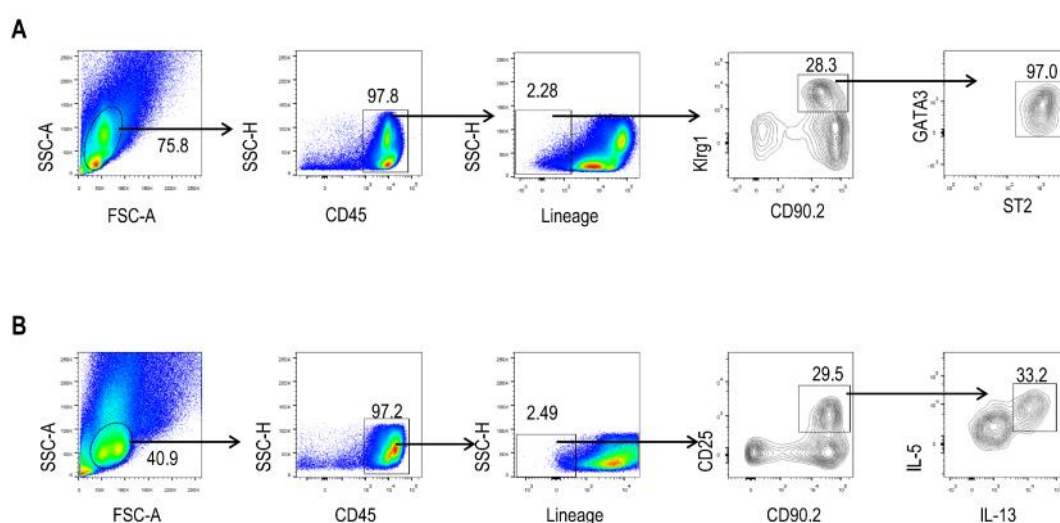


**Figure S3** Gating Strategy of ILC2 and ILC2-secreting cytokines in Lung

(a) For lung ILC2, cells were pre-gated on  $CD45^{+} Lin^{-}$ , and then  $CD90.2^{+} Klr1^{+}$  cells were analyzed, which are  $GATA3^{+} ST2^{+}$  cells. We used  $CD45^{+} Lin^{-} CD90.2^{+} Klr1^{+}$  cells as lung ILC2. (b) For ILC2-secreting cytokines, cells were stimulated for 4 h with PMA (phorbol 12-myristate 13-acetate; 50 ng/ml) and ionomycin (1  $\mu$ g/ml), then stained with ILC2 markers and antibodies against IL-5 and IL-13. Data are representative of three independent experiments.

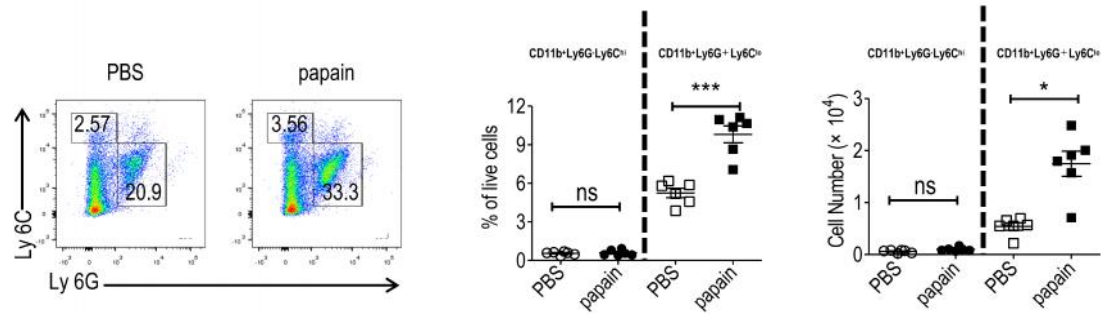


**Figure S4** Expansion of PMN-MDSCs in papain model

WT mice were intranasally administered with papain or PBS for 5 d ( $n = 6$  for each group). Mice were sacrificed 24 h after the last treatment.  $CD11b^{+}Ly6G^{+}Ly6C^{lo}$  cells were gated as PMN-MDSCs;  $CD11b^{+}Ly6G^{-}Ly6C^{hi}$  cells were gated as M-MDSC in lung. ns: no statistical significance; \*  $P < 0.05$ , \*\*\*  $P < 0.001$  in two-tailed Student's test. Data are representative of three independent experiments.

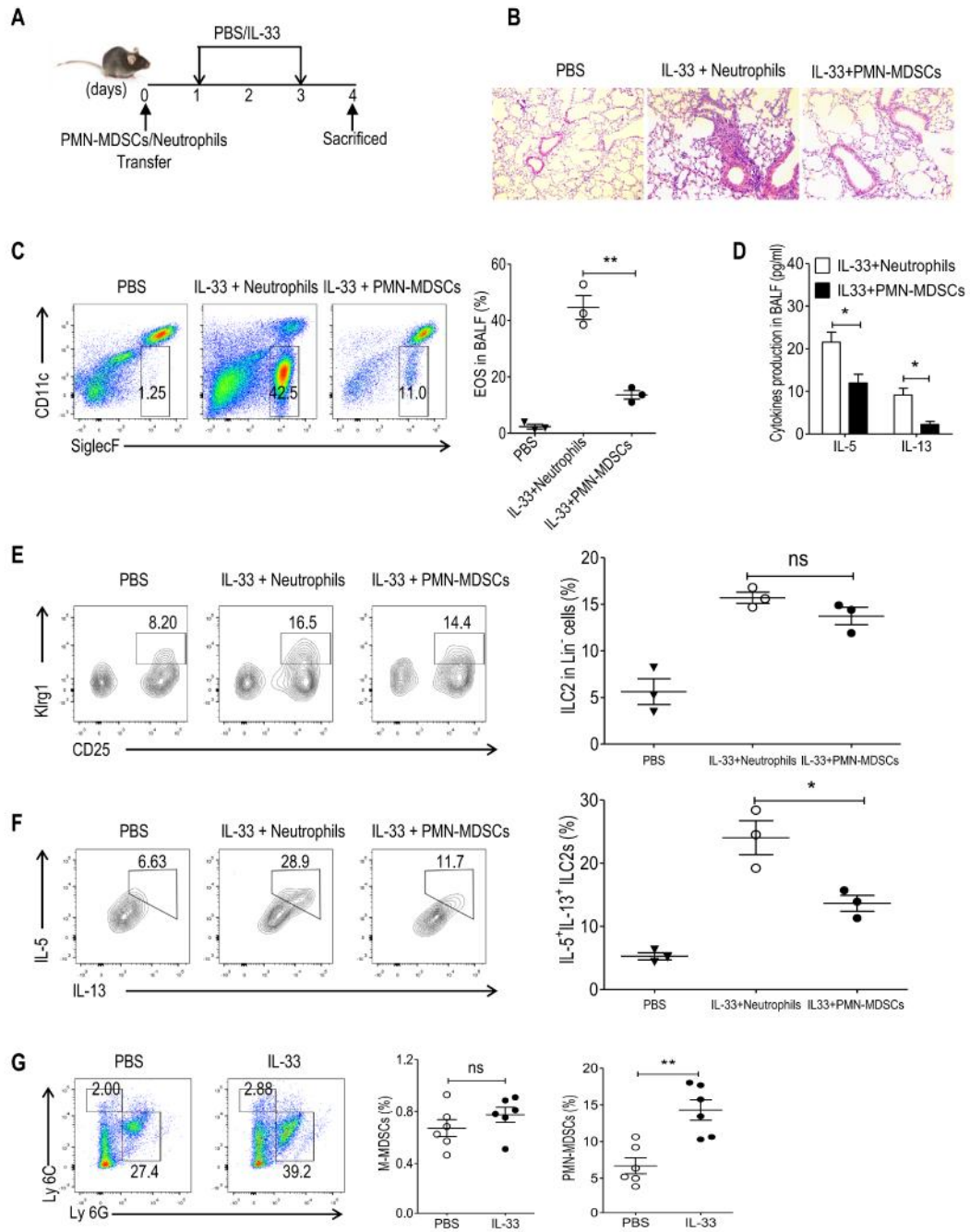


**Figure S5** PMN-MDSCs alleviate IL-33-induced allergic lung inflammation *in vivo*

(a) PMN-MDSCs or neutrophils were injected into WT mice intravenously one day before initiation of 3-day IL-33 model. One day after the last IL-33 challenge, lung inflammation was measured and samples were collected (n=3/group). (b) Lung histology (200×magnification) by H&E staining. (c) Flow cytometric analysis of total

eosinophils in the BALF. (d) The concentrations of IL-5 and IL-13 in the BALF were evaluated by ELISA. (e) Representative flow cytometric and statistical results of total ILC2s ( $\text{Lin}^- \text{CD45}^+ \text{CD90.2}^+ \text{Klrg1}^+$ ) in lung. (f) Representative flow cytometric and statistical results of IL-5 $^+$  IL-13 $^+$  ILC2s in lung. (g) Flow cytometric analysis of MDSCs in lung in PBS- or IL-33-treated mice. In all plots, mean  $\pm$  SEM are shown. ns: no statistical significance; \*  $P < 0.05$ ; \*\*  $P < 0.01$  in two-tailed Student's test. Data are representative of three independent experiments.

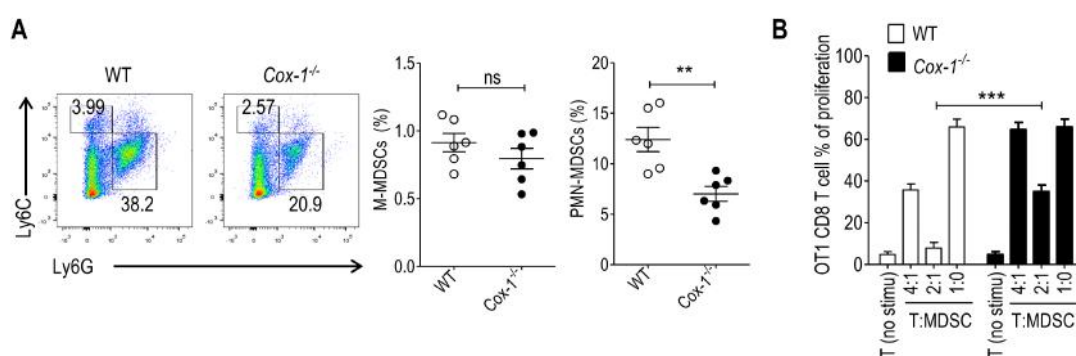


**Figure S6** The function of PMN-MDSCs was impaired in *Cox1*<sup>-/-</sup> mice in papain model

(a) Flow cytometric analysis of MDSCs in lung of *Cox1*<sup>-/-</sup> mice and their littermate WT controls after papain treatment. (b) The functional assay of PMN-MDSCs in lung of *Cox1*<sup>-/-</sup> mice and their littermate WT controls after papain treatment. In all plots, mean  $\pm$  SEM are shown. ns: no statistical significance; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$  in two-tailed Student's test. Data are representative of three independent experiments.

**Supplemental Table Antibodies used for flow.**

| Antibody                           | Clone    | Manufacturer |
|------------------------------------|----------|--------------|
| Lineage cocktail                   | —        | eBioscience  |
| Streptavidin PE-Cyanine7           | —        | eBioscience  |
| Streptavidin APC                   | —        | eBioscience  |
| Streptavidin FITC                  | —        | eBioscience  |
| CD45                               | 30-F11   | eBioscience  |
| SCA1                               | D7       | eBioscience  |
| CD117/c-Kit                        | 2B8      | eBioscience  |
| KLRG1                              | 2F1      | eBioscience  |
| Ki-67                              | SolA15   | eBioscience  |
| CD25                               | PC61.5   | eBioscience  |
| CD170/Siglec F                     | 1RNM44N  | eBioscience  |
| CD11c                              | N418     | eBioscience  |
| IL-33R/ST2                         | RMST2-33 | eBioscience  |
| IL-13                              | eBio13A  | eBioscience  |
| CD90.2                             | 53-2.1   | eBioscience  |
| CD127                              | A7R34    | BioLegend    |
| CD11b                              | M1/70    | eBioscience  |
| Ly6G                               | 1A8      | eBioscience  |
| Ly6C                               | HK1.4    | eBioscience  |
| CD8                                | 53-6.7   | eBioscience  |
| IL-5                               | TRFK5    | eBioscience  |
| Anti-human Lineage Cocktail        | —        | eBioscience  |
| Anti-human CD45                    | HI30     | eBioscience  |
| Anti-human CD127                   | EBioRDR5 | eBioscience  |
| Anti-human CRTH2                   | BM16     | eBioscience  |
| Anti-human CD161                   | HP-3G10  | eBioscience  |
| Anti-human CD11b                   | ICRF44   | eBioscience  |
| Anti-human HLA-DR <sup>-/low</sup> | LN3      | eBioscience  |
| Anti-human CD14                    | 61D3     | eBioscience  |
| Anti-human CD15                    | HI98     | eBioscience  |