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Title: Activation through toll-like receptor 2 on group2 innate lymphoid cells can induce asthmatic characteristics

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Short title: The role of TLR2 expressed on lung ILC2s

Abstract

Background: Type 2 innate lymphoid cells (ILC2s) are one of the sources of IL-5 and IL-13 in allergic airway inflammation. Innate immune receptors such as Toll-like receptors (TLRs) expressed on epithelial cells could contribute to ILC2 activation through IL-33 production, but a direct effect of TLRs on ILC2s remains to be elucidated.

Objectives: We hypothesized that TLRs can directly activate lung ILC2s and participate in the pathogenesis of asthma.

Methods: After intranasal administration of IL-33 to wild type (WT), TLR2KO, and TLR4KO female mice, ILC2s were isolated from harvested lungs. ILC2s were incubated with IL-2 and TLR stimulants (pam3csk4 (PAM), house dust mite extract (HDM)). In some experiments, TLR2 or dectin-1 signaling inhibitors were used. As an in vivo model, the mice were treated with IL-33 and rested until lung recruitment of eosinophils regressed. Then they were treated intranasally with PAM+HDM or vehicle and analyzed.

Results: In vitro stimulation of isolated ILC2s showed that PAM could induce IL-13 and IL-5 production, and HDM had a synergistic effect on this stimulation. Both effects were dependent on TLR2 and NF- κ B signaling. PAM+HDM stimulation of WT mice led to increased ILC2s, airway hyperresponsiveness, and increased levels of both neutrophils and eosinophils in bronchoalveolar lavage fluid. These observations were dependent on TLR2.

Conclusions & Clinical Relevance: TLR2 can directly activate lung ILC2s, an effect that is augmented by HDM. Asthmatic characteristics mediated through the TLR2 pathway were evident in the in vivo mice model. These data implicate a new pathway of ILC2 activation in the pathogenesis of asthma.

Key words

Type 2 innate lymphoid cells, asthma, Toll-like receptor 2, house dust mite extract, mice

Abbreviations

BALF: bronchoalveolar lavage fluid

HDM: house dust mite

ILC: innate lymphoid cell

LPS: lipopolysaccharide

PAM: pam3csk4

TLR: toll-like receptor

WT: wild type

Introduction

Asthma is an allergic disease that affects 1%–21% of adults worldwide ¹ and is characterized by chronic airway inflammation, mucus overproduction, and bronchial hyperresponsiveness. Allergic inflammation, primarily based on an adaptive immune response, is essential for its pathogenesis, and inhaled corticosteroids are often the treatment of choice ². Recently, crosstalk between innate immunity and adaptive immunity has been shown to be important in asthma pathogenesis ².

Toll-like receptors (TLRs)—a family of innate immune receptors that recognize pathogen-associated molecular patterns (PAMPS) and damage-associated molecular patterns (DAMPS)—serve as a host defense ³. In contrast, house dust mite (HDM), a major cause of asthma, activates TLRs on structural cells and contributes to allergic inflammation ⁴.

Type 2 innate lymphoid cells (ILC2s) are recently discovered cell populations that have no antigen-specific receptors but release large amounts of type 2 cytokines such as IL-5 and IL-13 in response to innate cytokines like IL-25, IL-33, and TSLP ^{5 6 7}. ILC2s play a role in the immune response to parasitic infections ⁸ and in tissue repair by releasing amphiregulin after influenza virus infection ⁹, but they are also known to be involved in the pathogenesis of allergic diseases including asthma and atopic dermatitis ¹⁰⁻¹². For example, alarmins, like IL-33, released due to activation of innate immune receptors like TLRs on airway epithelial cells, activate lung ILC2s, leading to the release of type 2 cytokines and contributing to asthma pathogenesis ^{13,14}. However, whether lung ILC2s are directly activated by TLRs and contribute to allergic inflammation remains unknown.

In the present study, we hypothesized that TLRs can directly activate lung ILC2s and contribute to the pathogenesis of asthma. Among TLRs, direct activation of TLR2 by pam3csk4 (PAM) in

ILC2s could produce type 2 cytokines via activation of NF- κ B and AP-1. Moreover, HDM has a synergistic effect on the production of these cytokines in TLR2-stimulated ILCs. To reveal a part of potential contribution regarding the allergic cytokine production in TLR2-activated ILC2s in vivo, we administered intranasal PAM+HDM to IL-33-pretreated mice who had increased numbers of lung ILC2s but in which eosinophilic inflammation was resolved. PAM+HDM induced increased numbers of ILC2s and augmented allergic cytokine expression in vivo, resulting in eosinophilic inflammation and airway hyperresponsiveness, which are key asthmatic characteristics, in a TLR2-dependent manner. These results may indicate a new mechanism of asthma pathogenesis and exacerbation.

Methods

Animals

Specific-pathogen-free C57BL6/N female mice (8–12 weeks old) were purchased from Sankyo Laboratory (Tokyo, Japan), and TLR2- and TLR4-knockout mice were purchased from Oriental Bioservice Inc. (Kyoto, Japan). SCID Beige mice were from Charles River Laboratory (Kanagawa, Japan). The mice were housed in a specific-pathogen-free environment with free access to food and water, under the approval of the Institutional Animal Experiments Committee of Musashino University. The care and use of the animals followed the Principles of Laboratory Animal Care formulated by the National Society for Medical Research of Japan.

Treatment of mice with IL-33, PAM/ HDM

To induce lung ILC2s, 1 µg of recombinant murine IL-33 (eBioscience, San Diego, CA, USA) in 50 µl of PBS was administered intranasally on days 1–3 to the mice, which were anesthetized by using ketamine (90 mg/kg IP) and medetomidine (1 mg/kg IP). On day 8, mice were euthanized and their harvested bilateral lungs were used to isolate ILC2s. In another experiment, 0.5 µg of recombinant murine IL-33 was administered intranasally on days 1–3; then on day 15, PBS or 2.5 µg of pam3csk4 (PAM, Enzo Life Sciences, Farmingdale, NY, USA) with 25 µg of HDM (*Dermatophagoides farinae*) extract (kindly provided by the Institute of Tokyo Environmental Allergy, Japan) was administered in the same manner. In some experiments, PAM or HDM was administered separately. 24 h later (i.e., on day 16), we analyzed the number of ILC2s in the left lung and their RNA was extracted for gene expression analysis. BALF was also collected for cell counts as previously described¹⁵. To measure airway hyperresponsiveness, we used mice 48 h

after treatment (i.e., on day 17).

Isolation and flow cytometric analysis of lung ILC2s

For the experiments using lung ILC2s, lungs were perfused with 3 mL of PBS via the right ventricle, and the lobes were cut into small pieces and digested in HBSS supplemented with 0.2% collagenase (Wako Pure Chemical Industries, Osaka, Japan) and 0.025% DNase (Sigma-Aldrich, St. Louis, MO, USA) for 45 min at 37 °C. This mixture was then processed into single-cell suspensions in sorting buffer, incubated with antibodies to block Fc receptors, and stained with various antibodies as previously described¹⁵. The following antibodies were used: biotin-conjugated antibody mixtures for lineage markers (TER119, CD3e, CD5, CD19, B220, Gr-1, CD11b, CD11c, TCR β , NK1.1, $\gamma\delta$ TCR, and Fc ϵ RI), BV421-conjugated anti-IL-7Ra (CD127), PECy7-conjugated anti-Sca-1, APC-conjugated anti-T1/ST2, PE-conjugated anti-CD25, and FITC-conjugated streptavidin. All antibodies were from eBioscience except for anti-IL-7Ra, which was from BD Pharmingen (San Diego, CA, USA). Lin⁻ Sca-1⁺ CD25⁺ IL-7Ra⁺ ST2⁺ cells were sorted (Cell Sorter SH800, Sony, Tokyo, Japan) as lung ILC2s. Dead cells were identified by use of propidium iodide (Sigma-Aldrich) staining and excluded from our analyses. To examine the expression of CD45.2, TLR2, and TLR4 on ILC2s, FITC-conjugated anti-CD45.2, anti-TLR2 (eBioscience) or anti-TLR4 antibody (Medical and Biological Laboratories, Aichi, Japan) was incubated with ILC2s and analyzed. All data were analyzed by using an SH800 (Sony) cell sorter.

Stimulation of ILC2s

Isolated ILC2s (5,000 /well) were incubated in RPMI with 10% fetal bovine serum and 1%

penicillin streptomycin with 10 ng/ml mouse IL-2 (eBioscience) in 96-well plates. The next day, the ILC2s were stimulated with 5 µg/ml PAM, 50 ng/ml LPS (from *Salmonella abortus equi*¹⁶), or 10 µg/ml HDM alone or in combination (e.g., PAM+LPS or PAM+HDM). IL-33 (10 ng/ml) was also used as a positive control. After 72 h, supernatants were collected for ELISA and RNA was harvested from the cells and used for quantitative PCR analysis. In some experiments, 10 µM Bay 11-7082 (Wako), 10 µM SR11302 (Cayman Chemicals, Ann Arbor, MI, USA), or 1 µg/ml WGP soluble as dectin-1 inhibitor (Invivogen, San Diego, CA, USA) was added 1 h before stimulation.

Isolation of alveolar macrophages

Naive macrophages were isolated from BALF as previously described¹⁵ and used for flow cytometric analysis or mRNA expression analysis.

Lung histopathology

For histopathology, mice were euthanized, a tracheal cannula was inserted, and the lungs were inflated with 4% paraformaldehyde (for fixation), paraffin-embedded, and cut in 5-µm sections. To assess asthmatic characteristics histopathologically, we stained the lung sections with hematoxylin and eosin.

Immunofluorescence staining.

Cytospin preparations of lung ILC2s were fixed with 4% paraformaldehyde. Cells were blocked with 3% FBS in PBS for 1 h, and incubated with FITC-conjugated anti-TLR2 mAb (eBioscience)

for 30 min. Nuclei were stained by using mounting medium containing DAPI (Vectashield, Burlingame, CA) according to the manufacturer's instructions, followed by microscopy (BZ-X-700; KEYENCE, Tokyo, Japan) for analysis.

Assessment of airway hyperresponsiveness

Airway resistance was examined at 48 h after the final administration of PAM+HDM extract by using a flexiVent system (SCIREQ, Montreal, Quebec, Canada). The mice underwent tracheostomy while under anesthesia with ketamine and medetomidine. The animals were ventilated at a tidal volume of 8 mL/kg and a respiratory frequency of 150/min with a positive end-expiratory pressure of 2 cm H₂O. After the mice had been placed on ventilation they were injected with rocuronium bromide. To assess airway responsiveness, methacholine (3.125, 6.25, 12.5, or 25 mg/mL) was administered through nebulization. Airway resistance was measured by using the Snapshot-150 perturbation maneuver.

ELISAs

A mouse IL-13 ELISA Ready-Set-Go kit (eBioscience) was used according to the manufacturer's instructions to measure IL-13 in culture supernatants.

RNA extraction and quantitative real-time PCR of ILC2 mRNA.

RNA extraction from ILC2s was performed with NucleoSpin RNA XS (Takara, Tokyo, Japan) according to the manufacturer's instructions. cDNA synthesis, real-time PCR, and the primers used were described previously ¹⁷.

Statistical analyses

Student's t test was used for comparisons of two groups, whereas ANOVA and Tukey–Kramer's honestly significant difference (HSD) were used for comparisons among multiple groups (JMP Pro11.2.0, SAS Institute, Cary, NC, USA). Statistical significance was set at $p < 0.05$. Data are expressed as the mean $\pm$ SEM.

Results

Direct stimulation of TLR2 on ILC2s induces IL-5 and IL-13.

First, we studied the expression of TLR on ILC2s. According to a public database (GSE73272)¹⁸, some TLRs, including TLR2 and TLR4, are expressed in IL-33-treated ILC2s. We isolated Lin⁻ Sca-1⁺ CD25⁺ IL-7Ra⁺ ST2⁺ lung ILC2s from the lungs of intranasal IL-33-treated WT, TLR2, and TLR4KO mice. All of these cells were confirmed as CD45.2-positive (Fig 1,A). Their expression of TLR2 and TLR4 was identified by using flow cytometry and qPCR (Fig 1, A, B). For these examinations, we included alveolar macrophages as a positive control¹⁹.

Immunofluorescence staining also confirmed the expression of TLR2 in lung ILC2s (Fig 1, D).

Next, we investigated whether these receptors were involved in the production of type 2 cytokines. Stimulation of ILC2s with the TLR2 agonist pam3csk4 (PAM), but not the TLR4 agonist LPS, induced IL-13 production (Fig 1, C). To confirm that this production was TLR2-dependent, we prepared ILC2s from TLR2KO mice in the same manner as we had for WT mice. The number of recruited lung ILC2s, and the level of IL-33–stimulated IL-13 production by those ILC2s were comparable between WT and TLR2KO mice, suggesting that ILC2 function was preserved in TLR2KO mice (Fig 1, E, F). However, IL-13 production by PAM-treated ILC2s was observed only in WT mice, suggesting that the production of IL-13 was TLR2-dependent (Fig 1, G). In addition, the mRNA expression levels of IL-5 and IL-13 were elevated in PAM-treated WT ILC2s but not TLR2KO ILC2s (Fig 1, H).

HDM has a synergistic effect on type 2 cytokine production in PAM-stimulated ILC2s.

HDM is known to activate some innate immune receptors, leading to allergic inflammation. To

clarify the role of HDM in the activation of ILC2s, HDM was added with or without PAM to ILC2s. Although HDM alone could not induce IL-13 production from ILC2s, IL-13 production by ILC2s was induced when HDM was added in combination with PAM (Fig 2, A). This synergistic effect of HDM was also apparent in the mRNA expression of IL-5 and IL-13 in ILC2s (Fig 2, B). These data suggest that TLR2 on lung ILC2s can induce IL-5 and IL-13 production and that HDM has a synergistic effect on type 2 cytokine production.

Next, we investigated which receptor was involved in the synergistic effect of HDM on type 2 cytokine production by PAM-stimulated ILC2s. Our results with the inhibitors of TLR2 signaling suggested that TLR2 itself may be involved in this synergistic effect. However, given that HDM can activate other innate immune receptors such as TLR4 and dectin-1^{14,20}, we examined the role of these receptors on the synergistic effect of HDM on IL-13 production by ILC2s. Lung ILC2s from TLR4KO mice still showed the synergistic of HDM on IL-13 production (Fig 2, C). Dectin-1 inhibitor was used for the assessment of dectin-1. Administration of this inhibitor to WT ILC2s also had no effect on IL-13 production (Fig 2, D). These data demonstrate that the synergistic effect of HDM involves TLR2, whereas TLR4 and dectin-1 are less involved.

NF- κ B and AP-1 are involved in PAM-induced cytokine production by lung ILC2s.

Upon TLR2 signaling, MyD88-mediated IRAK activation results in the activation of the downstream transcription factors NF- κ B and AP-1²¹. To examine whether NF- κ B and AP-1 are involved in PAM- or PAM+HDM-induced IL-13 production by lung ILC2s, we used the NF- κ B inhibitor Bay11-7082 and the AP-1 inhibitor SR11302. Both Bay11-7082 and SR11302 suppressed PAM- and PAM+HDM-induced IL-13 production by lung ILC2s (Fig 3).

These data demonstrate that PAM stimulates TLR2-NF- κ B and AP-1 signaling in ILC2s and that the synergistic effect of HDM involves TLR2, whereas TLR4 and dectin-1 are less involved.

PAM+HDM treatment of ILC2-rich mice induces TLR2-dependent asthmatic characteristics.

To reveal a part of the potential role of PAM+HDM-induced type 2 cytokine production in vivo, we established a new mouse model (Fig 4, A). After IL-33 treatment of WT and TLR2KO mice, inflammation (assessed by examining BALF) was comparable between the two types of mice (Fig 4, B). Then, we let the mice rest for two weeks to allow for resolution of eosinophilic inflammation. After this rest, the mice received a single dose of PAM+HDM intranasally on day 15. PBS-treated WT and TLR2KO mice had a significant number of ILC2s but not eosinophilic cells in their lungs, indicating that the rest for resolution of eosinophilic inflammation was completed but lung ILC2s were still abundant (Fig 4, C, D). PAM+HDM-treated WT mice showed increased neutrophilic and eosinophilic inflammation compared with PBS-treated WT and PAM+HDM-treated TLR2KO mice (Fig 4, D). PAM+HDM treatment augmented the number of lung ILC2s and mRNA expression of IL-5 and IL-13 in the ILC2s of WT but not TLR2KO mice (Fig 4, C, E). PAM+HDM-treated WT mice exhibited significant cell infiltration around the bronchi and veins, as observed histopathologically with hematoxylin and eosin staining; this infiltration was suppressed in similarly treated TLR2KO mice (Fig 4, F).

We then examined airway hyperresponsiveness at 48 h after PAM+HDM treatment, which is a main characteristic of the asthmatic model. Only WT mice treated with PAM and HDM exhibited methacholine-induced airway hyperresponsiveness (i.e., similarly treated TLR2KO mice did not)

(Fig 4, G). PAM+HDM-treated WT mice that were not pretreated with IL-33 did not show this airway hyperresponsiveness (data not shown). To evaluate whether HDM alone could induce increased number of ILC2s and asthmatic characteristics in this model, we also administered HDM or PAM on day 15 instead of PAM+HDM treatment. ILC2 number showed no significant change between PBS group and HDM group (Fig 4, H). These data indicate that a single dose of PAM+HDM administered to mice abundant in lung ILC2s induced accumulation and type 2 cytokine production of ILC2s, and airway hyperresponsiveness. Namely, these mice exhibited the asthmatic characteristics in a TLR2-dependent manner.

Accumulation and type 2 cytokine production of lung ILC2s in PAM+HDM treated

ILC2-rich mice are not dependent on B cells or T cells.

Immune cells like B cells or T cells have an essential role in the pathogenesis of asthma and could also play a role in this PAM+HDM treatment model. To examine this possibility, we performed the same treatment described in Fig 4 A with SCID Beige mice, which have no B cells or T cells. We confirmed these mice have almost no proportion of CD3⁺ or CD19⁺ in CD45⁺ cells but still showed PAM+HDM induced neutrophilic and eosinophilic inflammation revealed by BALF in this model (data not shown). However, in these mice, PAM+HDM treatment still could make increase on lung ILC2s and ILC2 expression of IL-5 and IL-13 were increased (Fig 5 A, B). These data suggest that B cells or T cells did not influence the reactivation of ILC2s induced by PAM+HDM in this model.

Discussion

In this study, we demonstrated a novel role for TLR2 in the activation of lung ILC2s and showed *in vivo* that this role involves direct activation of lung ILC2s by TLR2. A previous study demonstrated that human circulating ILC2s express TLR1, 2, 4, and 6, and mixed TLR ligands can produce IL-5 and IL-13²²; another study showed that CD127⁺ LTi-like cells in human tonsils also produce type 2 cytokines upon TLR2 stimulation²³. In our experiments, murine lung ILC2s produced IL-5 and IL-13 upon TLR2 stimulation by PAM but not in response to TLR4 stimulation by LPS when incubated in the presence of IL-2, whereas TLR2KO ILC2s, which were thought to be at the same level of activation, failed to produce these cytokines. Although it is well-known that PAM can induce production of IL-6 and TNF α from cells like macrophages²⁴, no other type of cells except ILC2s are demonstrated to produce type 2 cytokines upon PAM stimulation.

PAM-induced IL-13 production was dependent on the TLR2 and NF- κ B/AP-1 pathways, as revealed by treatment with inhibitors of these pathways. Our results also demonstrated that HDM could augment PAM-stimulated IL-5 and IL-13 production by ILC2s. This effect was not dependent on TLR4 or dectin-1, which are well-known innate immune receptors activated by HDM. HDM contains over 20 allergens that affect airway structural barriers, inducing innate cytokines such as IL-25, IL-33, and TSLP, which then initiate allergic inflammation. Although TLR2 seems not to be mainly involved in the phenotype of the HDM-induced asthma model²⁵, HDM can activate TLR2 on various cell types and induce release of inflammatory cytokines^{26 27}. Our study implicated HDM activated TLR2 only in PAM-stimulated ILC2s to augment asthmatic phenotype. Among HDM allergens, Der p 13 can bind fatty acid and induce TLR2-associated immune response²⁸. Considering PAM is a synthetic triacylated lipopeptide, part of HDM

allergens like Der p 13 may be capable of combining with PAM and cooperate in activating TLR2.

To exclude the possibility that other innate immune receptors except TLR2, TLR4 and Dectin-1 are involved in these effects, further studies will be necessary.

To elucidate a part of the in vivo role of TLR2-induced allergic cytokine production by ILC2s, IL-33-treated mice were rested so that they had abundant lung ILC2s but exhibited almost no infiltration of inflammatory cells. Single-dose administration of PAM+HDM to these mice led to increased number of lung ILC2s and increased IL-5/ IL-13 expression, neutrophilic and eosinophilic inflammation of lungs, and methacholine-induced airway hyperresponsiveness. These asthmatic characteristics seemed to be dependent on TLR2 and ILC2s, because the results with TLR2KO mice and PAM+HDM-treated mice without IL-33 pretreatment exhibited no airway hyperreactivity. Histopathological findings also revealed inflammatory cell infiltration around the bronchus in a TLR2-dependent manner. It is notable that a single treatment of HDM alone with IL-33-pretreated mice could not result in increased number of ILC2s. This implies mediators release by HDM (for example, from airway epithelial cells) might be less relevant in the reactivation of ILC2s in our models.

To investigate the potential contribution of B cells and T cells in this model, we used SCID Beige mice that have no B cells, T cells, and NK cells. Despite the characteristics, PAM+HDM treatment with IL-33-pretreated mice still could induce the more accumulation of lung ILC2s and increase the expression of IL-5 and IL-13 in ILC2s compared with PBS treatment. These results demonstrate these immune cells might be less relevant in the asthmatic characteristics of our models.

However, we have to note that there are still other types of cells such as macrophages and

epithelial cells that could cause the indirect activation of ILC2s induced by their mediators by PAM+HDM treatment. As IL-33 is the most potent activator of ILC2s^{29 30}, we confirmed intrinsic IL-33 did not influence the increase and allergic cytokine expression of ILC2s by administration of IL-33 neutralizing antibody (data not shown). Even so, we cannot exclude the role of other mediators and the in vivo results might not represent fully what was seen in vitro.

Our model may also offer new insights into asthma exacerbation. As single dose treatment is unlikely to cause IgE-dependent asthma, this model may apply to patients with asthmatic symptom who have apparently non-atopic characteristics. In case of rhinovirus infection, ILC2s play a role in asthma exacerbations^{31 32}. Furthermore, ILC2s can disrupt bronchial epithelial barrier integrity by targeting tight junctions³³, resulting the desquamation of bronchial epithelial cells observed in asthmatic patients³⁴. In these situations, additional infections caused by gram-positive bacteria or *Mycoplasma pneumoniae* that can activate TLR2 may lead to asthmatic characteristics partly through direct activation of ILC2s.

In summary, we here elucidated a novel role for TLR2 in PAM- and PAM+HDM-induced type 2 cytokine production by lung ILC2s and revealed a part of potential contribution in vivo role of allergic cytokine production in TLR2-activated ILC2s. ILC2s, but not TLR2KO ILC2s, produced IL-5 and IL-13 upon PAM stimulation and a synergistic effect of HDM was observed also. Single-dose treatment of IL-33-pretreated mice with PAM+HDM induced asthmatic characteristics in a TLR2-dependent manner. These results provide new insights into the pathogenesis of asthma and its exacerbation.

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Disclosure

The authors declare that they have no relevant conflicts of interest.

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Figure legends

FIG 1. TLR2 expression and function on ILC2s in the lung.

A, Left; Sorting strategy of lung ILC2 is demonstrated. Middle; Histogram of lung ILC2s stained with FITC-conjugated anti-CD45.2 antibody (black solid line). Gray solid line: stained with isotype antibody. Right; Histogram of lung ILC2s (derived from WT, TLR2KO, or TLR4KO) stained with FITC-conjugated anti-TLR2 or anti-TLR4 antibody (black solid line). Gray solid line: stained with isotype antibody. Histogram of WT alveolar macrophages (AM) stained with FITC-conjugated anti-TLR2 antibody is also demonstrated. **B**, mRNA expression levels of TLR2 (left) and TLR4 (right) in ILC2s and alveolar macrophages assessed by use of quantitative PCR analysis. **C**, IL-13 production by purified WT ILC2s (5,000/well) stimulated with PAM or LPS in culture supernatants measured by ELISA. **D**, Immunocytochemistry staining of WT or TLR2KO lung ILC2s with anti-TLR2 antibody. TLR2 (green) and nuclei (blue) are indicated. Original magnification, $\times 400$. **E**, Total number of ILC2s from bilateral lungs in IL-33-treated WT or TLR2KO mice. **F**, IL-13 production by purified WT or TLR2KO ILC2s stimulated with IL-33. The cells were cultured for 72 h and the supernatants were then collected for ELISA. **G**, IL-13 production by purified WT or TLR2KO ILC2s stimulated with PAM or LPS. **H**, IL-5 and IL-13 mRNA expression levels of WT or TLR2KO ILC2s stimulated with PAM or LPS. Data are means $\pm$ SEM. * $p < 0.05$; **A,D**, Representative image of 2 or 3 independent experiments. **B**, **E**, **H**, $n=4-8$ across at least 3 independent experiments. **C**, **F-G**, Combined from triplicate wells of each condition of 3 independent experiments. N.S.: no significant difference.

FIG 2. Augmentation of IL-5 and IL-13 production in PAM-stimulated ILC2s by HDM.

WT or TLR2KO ILC2s were stimulated PAM, HDM, or PAM+HDM in medium containing IL-2.

A, IL-13 levels in supernatants. **B**, IL-5/IL-13 mRNA expression levels assessed by quantitative PCR. **C**, ILC2s from TLR4KO mice. **D**, WT ILC2s incubated with the dectin-1 inhibitor. ILC2s were stimulated with PAM or PAM+HDM and then IL-13 production was measured by ELISA. Data are means $\pm$ SEM. * $p < 0.05$; **A,C,D**, Combined from triplicate wells of each condition of 2-3 independent experiments. **B**, n=6-8 across 3 independent experiments. N.S.: no significant difference.

FIG 3. Involvement of NF- κ B and AP-1 in type 2 cytokine production in ILC2s induced by PAM. WT ILC2s were incubated with the NF- κ B inhibitor Bay 11-7082 or the AP-1 inhibitor SR11302 before stimulation with PAM or PAM+HDM. The amount of IL-13 excreted was examined. Data are means $\pm$ SEM. * $p < 0.05$; Combined from triplicate wells of each condition of 2 independent experiments.

FIG 4. PAM+HDM treatment of mice abundant in lung ILC2s induced asthmatic characteristics in a TLR2-dependent manner in vivo.

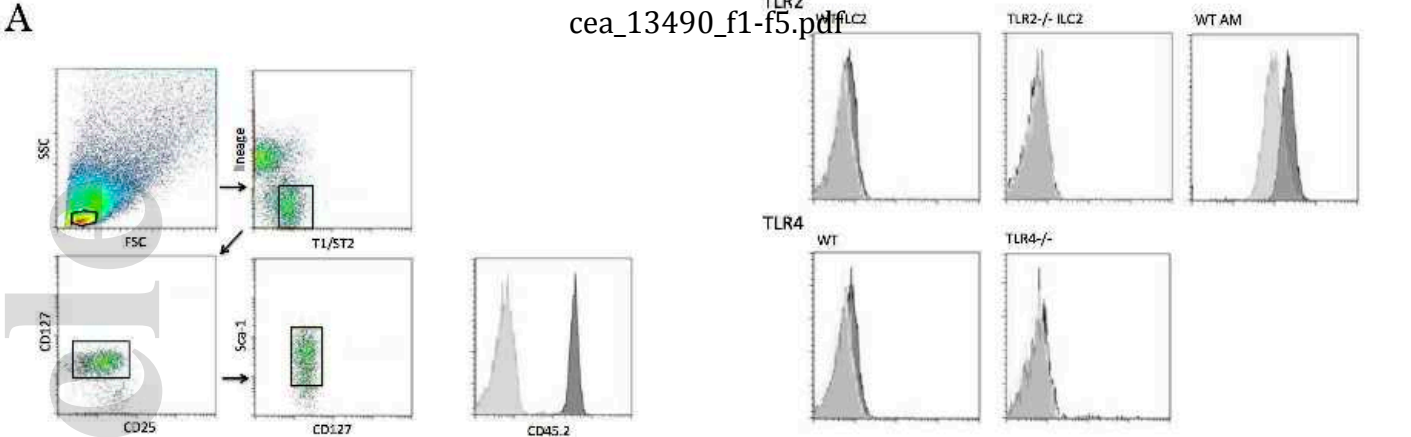
A, Study design to assess the in vivo role of lung ILC2s stimulated by PAM+HDM. WT or TLR2KO mice treated with intranasal IL-33 were rested for 2 weeks. Then, the mice were intranasally treated with PAM+HDM or PBS and analyzed at the indicated times. **B**, Differential cell counts from BALF on day7, after intranasal IL-33 treatments. **C**, Number of ILC2s in the left lung. **D**, Differential cell counts from BALF on day16. **E**, IL-5 and IL-13 expression from isolated ILC2s as measured by qPCR. **F**, Representative lung sections of control WT (upper left),

PAM+HDM-treated WT (upper right), control TLR2KO (lower left), and PAM+HDM-treated TLR2KO (lower right) mice with magnified images (small window). Hematoxylin and eosin staining. Scale bar, 50 μ m; original magnification, $\times 100$. **G**, Total resistance of the respiratory system (Rrs) as dynamic airway resistance was measured using a flexiVent system. **H**, IL-33-pretreated WT mice were subject to PAM or HDM instead of PAM+HDM. the number of ILC2s in the left lung were demonstrated. (PAM or HDM group: n=3-4) Data are means $\pm$ SEM. n = 6–8 in each group; $*p < 0.05$. **G**, * corresponds to the WT PAM+HDM group compared with the other groups.

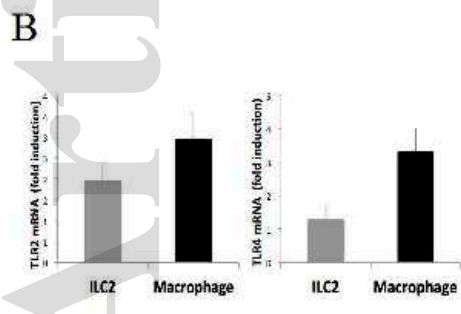
FIG 5. Evaluation of contribution of B cells and T cells to the ILC2 reactivation in PAM+HDM-treated mice pretreated with IL-33.

SCID Beige mice treated as described in **FIG. 4**. **A**, Number of ILC2s in the left lung. **B**, IL-5/IL-13 expression of ILC2s. Data are means $\pm$ SEM. n=4 in each group. $*p < 0.05$.

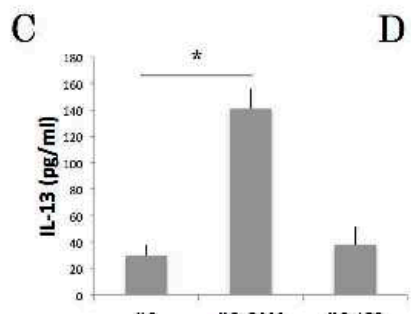
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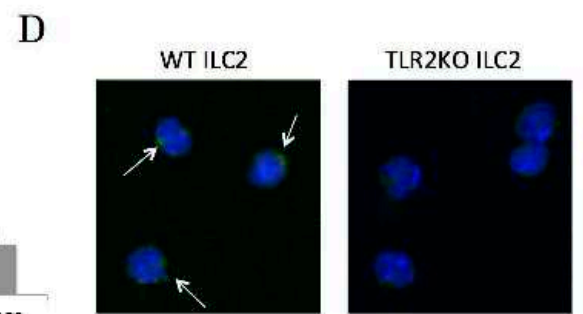
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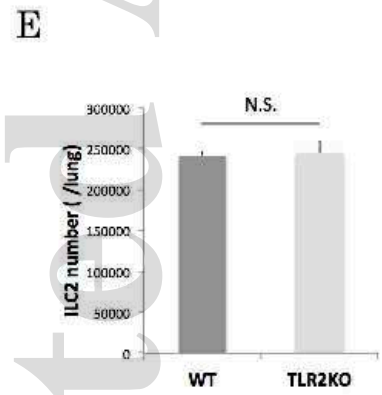
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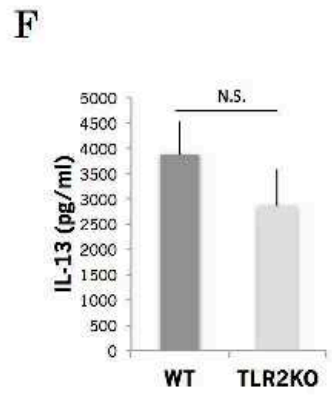
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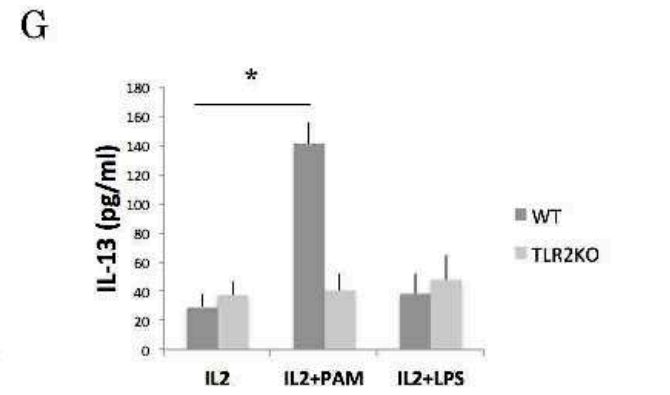
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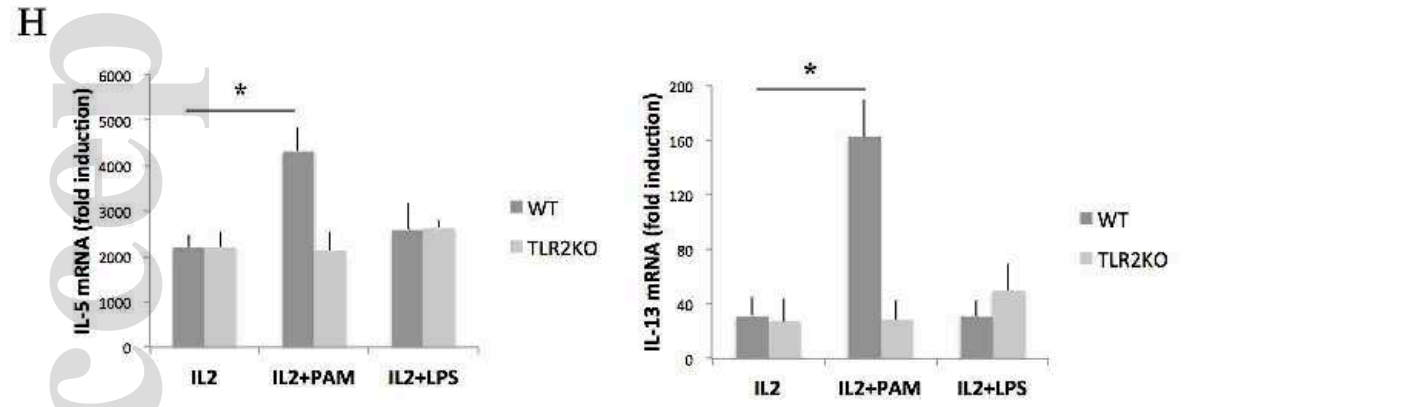
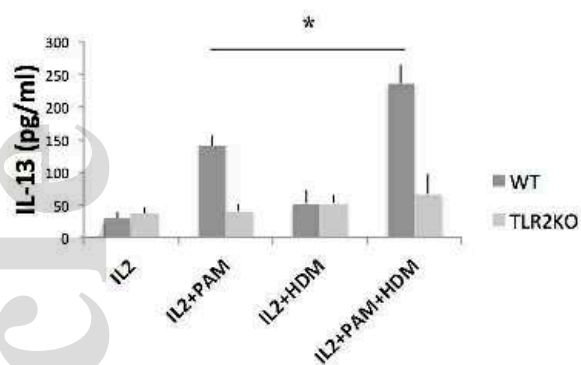
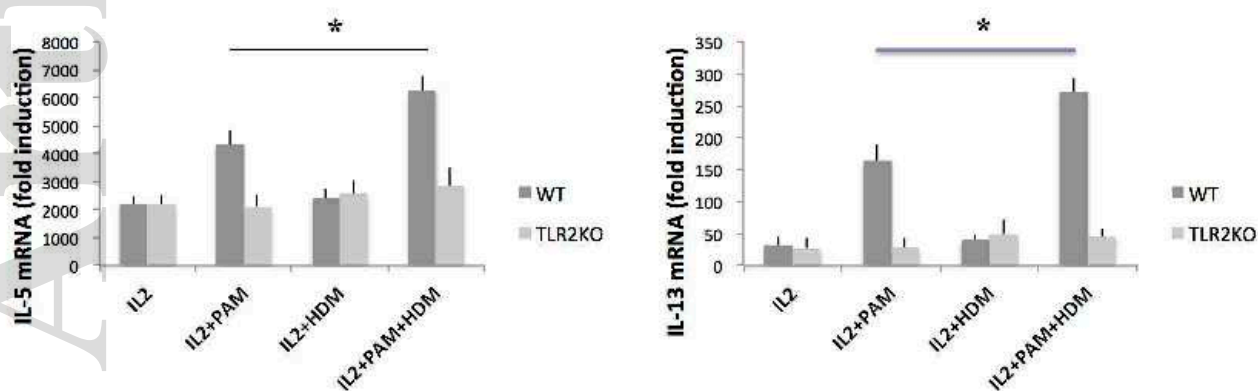


Figure 1

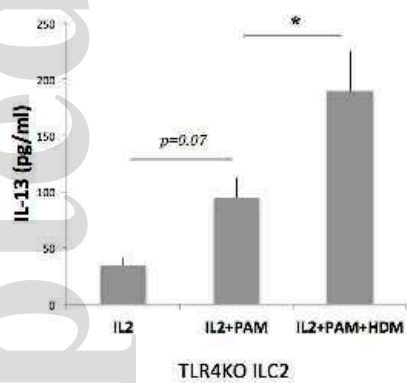
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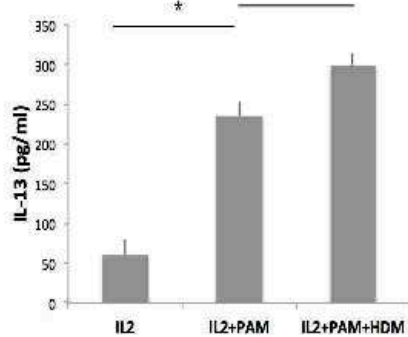


Figure 2

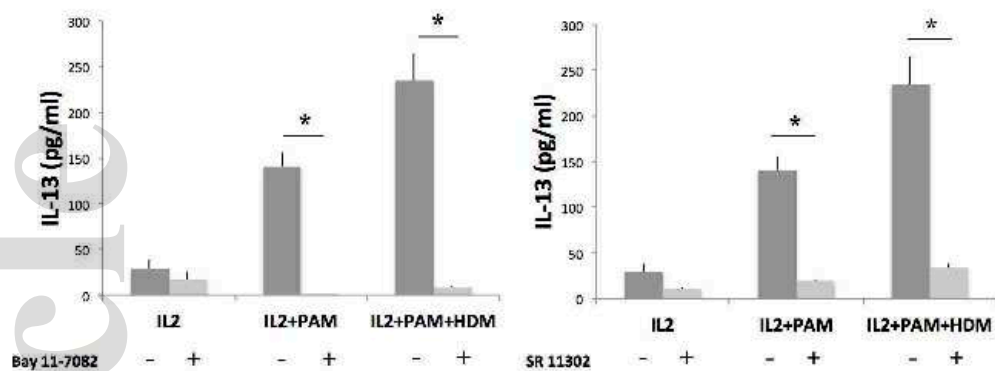


Figure 3

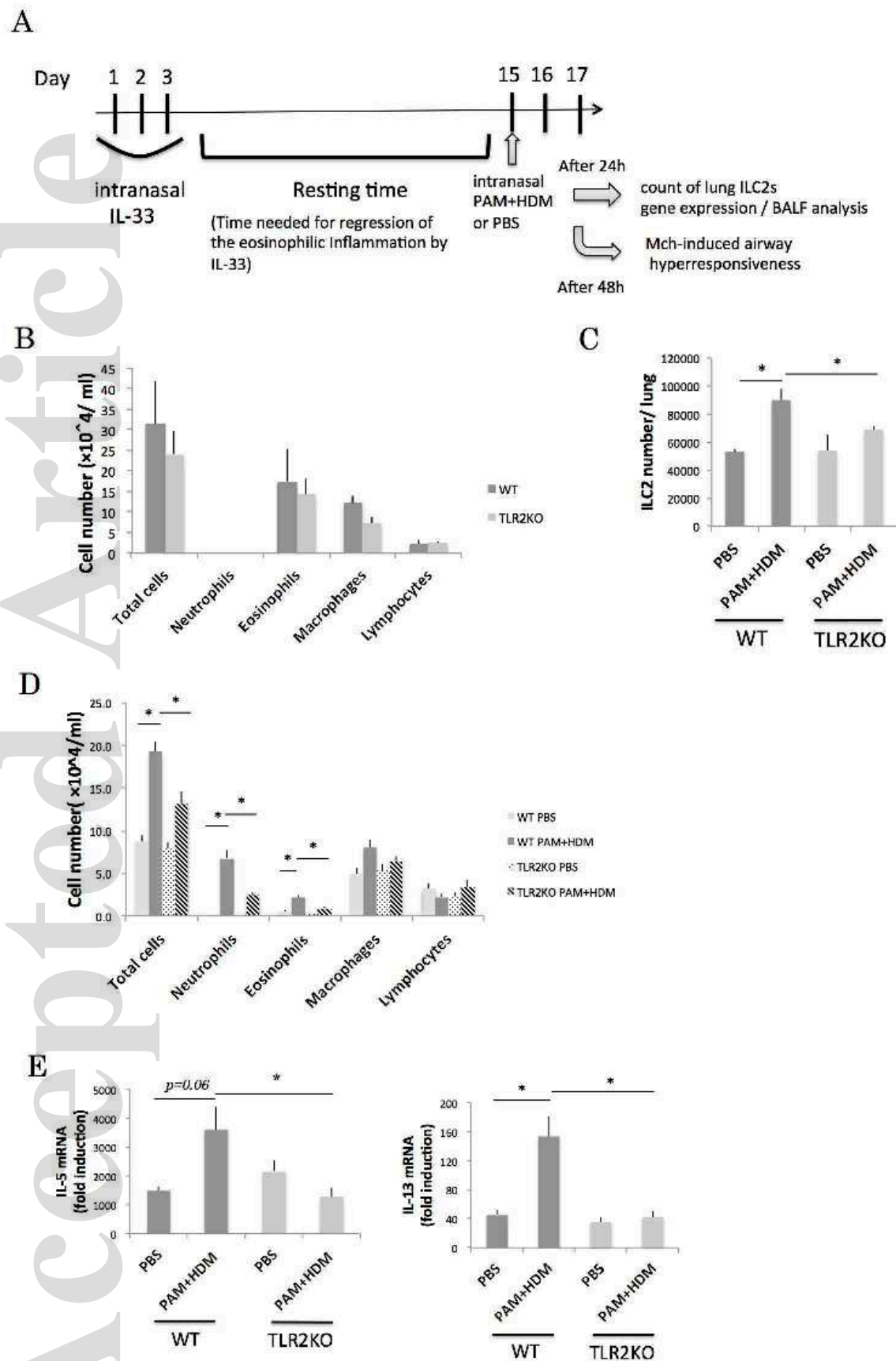


Figure 4

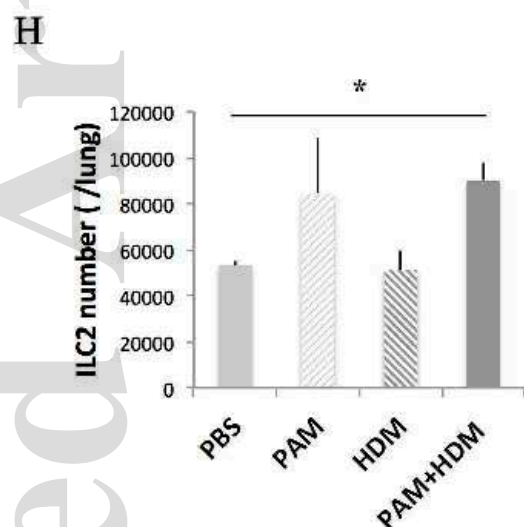
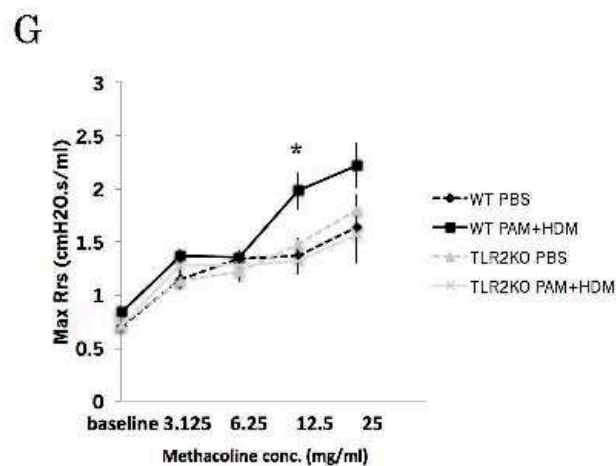
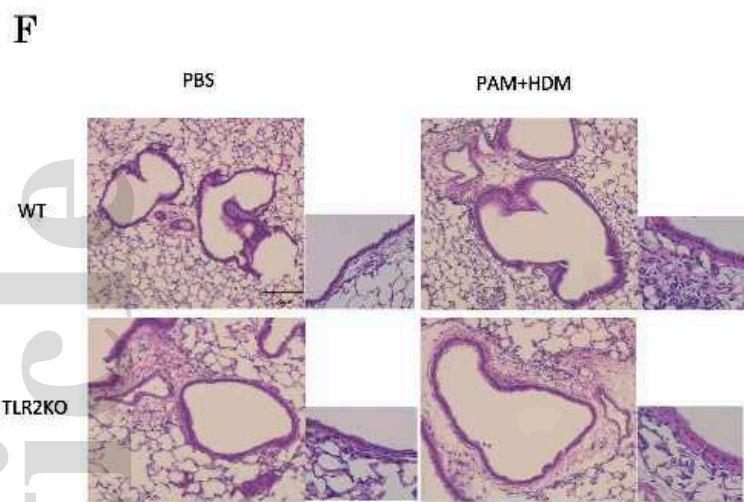
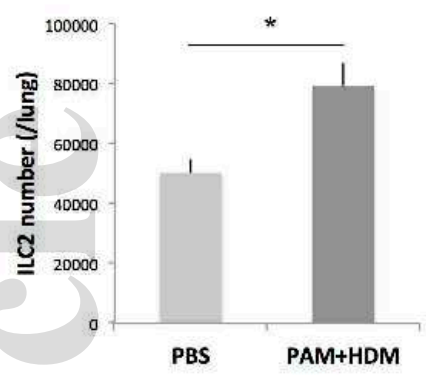


Figure 4

A



B

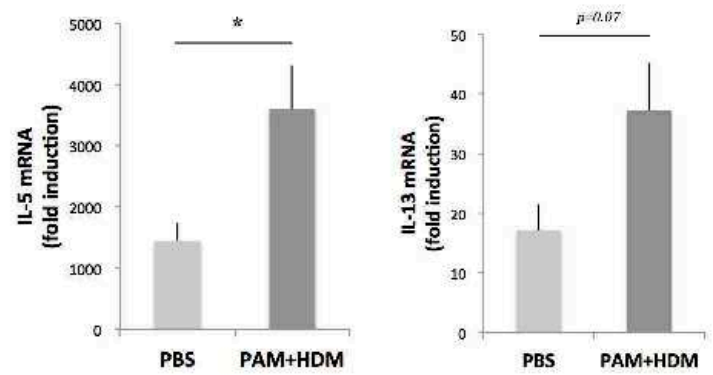


Figure 5