

**Activation of group 2 innate lymphoid cells exacerbates and confers corticosteroid-resistance
to mouse nasal type 2 inflammation**

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Abstract

Both T_h2 cells and group 2 innate lymphoid cells (ILC2s) contribute to allergic diseases. However, their exact role and relationship in nasal allergic disorders is unclear. In this study, we investigated the cooperation of T_h2 cells and ILC2s in a mouse model of nasal allergic disorder. To differentially activate T_h2 cells and/or ILC2s in nasal mucosa, mice were intranasally administered ovalbumin (OVA) antigen, papain, an ILC2-activator, or both for 2 weeks. Epithelial thickness and number of eosinophils in the nasal mucosa were evaluated at 24 hours after the final challenge. Intranasal administration of OVA and papain preferentially activated T_h2 cells and ILC2s, respectively, in the nose. Both OVA and papain increased the nasal epithelial thickness and number of eosinophils, and their coadministration significantly enhanced the symptoms. Although T/B-cell-deficient mice showed severely decreased nasal symptoms induced by OVA or OVA-plus-papain, the mice still showed slight papain-induced nasal symptoms. In ILC2-deficient mice, OVA-plus-papain-induced nasal symptoms were suppressed to the same level as OVA-alone. Similarly, IL-33- and ST2-deficient mice showed decreased OVA-plus-papain-induced nasal symptoms. IL-5 induced eosinophilia only, but IL-13 contributed to both nasal epithelial thickening and eosinophilia induced by OVA-plus-papain. Dexamethasone ameliorated OVA-alone-induced nasal epithelial thickening. However,

OVA-plus-papain-induced nasal epithelial thickening was only partially controlled by dexamethasone. These results demonstrate that IL-33/ST2-pathway-mediated ILC2 activation exacerbated T_H2-cell-induced nasal inflammation by producing IL-13. Although T_H2-cell-alone-induced nasal inflammation was controlled by corticosteroid treatment, the activation of ILC2s conferred treatment resistance. Therefore, ILC2s and its activators could be therapeutic targets for treatment-refractory nasal allergic disorders.

Introduction

Although type 2 immunity is essential for protection against parasite infection and venom, the aberrant activation of type 2 immunity to innocuous antigens elicits allergic diseases (1). In the nose, allergic rhinitis (AR) and chronic rhinosinusitis with nasal polyps (CRSwNP) are the two major type 2 immunity associated diseases. The symptoms of AR are characterized by early-phase responses (sneezing and watery runny nose) caused by IgE-mediated mast cell degranulation and late-phase responses (nasal congestion) caused by infiltrated inflammatory cells and tissue damage (2-4). CRSwNP is a T_H2-dominated subtype of CRS characterized by the presence of nasal polyps and intensive eosinophil infiltration (5). T_H2 cells play a central role in the pathogenesis of disease by producing type-2 cytokines such as IL-4, IL-5, and IL-13 (2-4). IL-5 increases the migration, adhesion, and survival of eosinophils, which promotes tissue eosinophilia. IL-13 also contributes to eosinophilia and promotes goblet-cell and epithelial cell hyperplasia (5). In addition, type-2 cytokines reduce tissue plasminogen activator (t-PA) levels in nasal epithelial cells, which promote fibrinogenesis, resulting in nasal polyp generation in CRSwNP (6).

Group 2 innate lymphoid cells (ILC2s) were recently identified as a novel cell population that produces large amounts of type-2 cytokines in response to epithelium-derived

IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) (7-10). Lung inhalation of allergens such as papain, *Alternaria alternata* and house dust mite (HDM), induces IL-33-dependent ILC2 activation resulting in the development of allergic pulmonary inflammation independent of adaptive immunity (11-13). In addition, ILC2s promote the proliferation of T_H2 cells directly, which is cell contact and MHC class II-dependent (14-17) and indirectly, which is IL-13-mediated dendritic-cell-dependent (18). Therefore, ILC2s contribute to the exacerbation of allergic pulmonary inflammation by producing type-2 cytokines and activating T_H2 cells.

ILC2s have also been implicated in nasal allergic diseases. Circulating ILC2s are increased in patients with seasonal AR during the allergen season (19,20) and in patients with cat allergy after nasal provocation (21). In addition, ILC2s were observed in nasal polyps from CRSwNP patients (22-26). Therefore, ILC2s may have a role in promoting nasal type 2 inflammation. However, because of the lack of experimental studies focusing on nasal ILC2s, their exact role in nasal allergic diseases is poorly understood. Furthermore, the interrelationship between T_H2 cells and ILC2s in the nose has not been examined.

Here, we investigated whether T_H2 cells and ILC2s cooperate in nasal type-2 inflammation by using ovalbumin (OVA) and papain as a T_H2 cell-inducing antigen and an ILC2 activator, respectively. We revealed that the simultaneous activation of T_H2 cells and ILC2s in

the nose additively exacerbated nasal epithelial thickening and eosinophilia. In addition, ILC2 activation mediated via the IL-33/ST2-pathway might confer corticosteroid resistance to nasal epithelial thickening.

Methods

Mice

BALB/c and C57BL/6 mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). BALB/c-background *Il33*^{-/-} and *Il1rl1*^{-/-} mice were generated as previously described (27-29). BALB/c-background *Rag2*^{-/-} mice were purchased from Taconic (Germantown, NY, USA). C57BL/6-background *Rora*^{sg/sg}, Δ dblGATA and *Il13*^{-/-} mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were maintained under specific pathogen-free conditions. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care Committee of the Hyogo College of Medicine (NO. 14-007 and NO. 16-065).

Mouse model

Mice were intranasally (i.n.) administered PBS (20 μ L), 5% OVA (20 μ L in PBS), papain (10 μ g/20 μ L in PBS containing 2.5 mM L-cysteine), or OVA plus papain without alum on days 1, 3, 6, 8, 10, and 13. To examine the effect of *Alternaria Alternata* extract in nasal mucosa, mice were i.n. administered *Alternaria Alternata* extract (100 μ g/20 μ L in PBS) together with OVA using

the same time course as for the administration of OVA plus papain. At 24 hours after the last challenge, mice were euthanized for analysis. To deplete eosinophils, anti-IL-5 antibody (100 µg/body) was intraperitoneally (i.p.) administered on days 1, 5, 8, and 12. In some experiments, mice were i.p. administered dexamethasone (2.5 or 5 mg/kg) at 24 and 2 hours before each nasal challenge.

Bone marrow transplantation (BMT)

Recipient 7-week-old C57BL/6 wild-type mice were lethally irradiated (11 Gy). Donor Bone marrow (BM) cells were prepared from 3- to 4-week-old wild-type or *Rora*^{sg/sg} mice. BM cells (10⁷ cells/body) were suspended in 100 µl PBS and transferred intravenously into recipients. Mice were used for experiments from 8 weeks after transplantation.

Reagents

Phycoerythrin (PE)-conjugated antibodies against CD3 (145-2C11), CD8a (53-6.7), and fluorescein isothiocyanate (FITC)-conjugated antibody against CD90.2 (Thy1.2) (53-2.1) were purchased from eBioscience (San Diego, CA, USA). PE-conjugated antibodies against CD4

(RM4-5), CD49b (DX-5), and Siglec-F (E50-2440) were purchased from BD Biosciences (San Diego, CA, USA). PE-conjugated antibodies against CD11b (M1/70), CD11c (N418), CD19 (6D5), Ly-6G/Ly-6c (Gr-1) (RB6-8C5), IL-5 (TRFK5), allophycocyanin (APC)-conjugated Streptavidin, and Pacific Blue-conjugated antibody against CD45 (30-F11) were purchased from BioLegend (San Diego, CA, USA). APC-conjugated antibody against IL-13 (eBio13A) and PE-conjugated antibody against IgE was purchased from eBioscience and Southern Biotech (Birmingham, AL, USA), respectively. Biotin-conjugated antibody against T1/ST2 (DJ8) was purchased from MD Bioproducts (St Paul, MN, USA). Papain from papaya latex, OVA (grade V), and L-cysteine were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Dexamethasone was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *Alternaria Alternate* extract was purchased from ITEA Inc. (Tokyo, Japan). The neutralizing monoclonal anti-IL-5 antibody was described previously (30).

Measurement of papain- and OVA-specific IgG1

Serum were collected at days 0 and 14. Serum papain-specific IgG1 levels were measured by ELISA, as described previously (31). Plates were coated with papain (10 µg/ml), and blocked with PBS containing 1% BSA. The coated plates were incubated with serum (1/50000 dilution)

for overnight at 4 °C. After that, the plates were incubated with biotin-conjugated goat anti-mouse IgG1 mAb (Southern Biotech) for 1.5 hours at 37 °C. HRP-conjugated streptavidin (R&D Systems, Inc., MN, USA) and TMB Microwell Peroxidase Substrate System (SeraCare Life Sciences, MA, USA) were used for detecting papain-specific IgG1. OVA-specific IgG1 levels were measured by Mouse OVA-IgG₁ ELISA KIT according to the manufacturer's direction (AKRIE-040, Shibayagi, Gunma, Japan).

Histological analysis

Nasal tissue specimens were generated as previously described (32). Briefly, nasal tissues were fixed in 4% paraformaldehyde at 4 °C for 3 days and decalcified in 0.12 mol/L EDTA solution (pH 7.4) for 7 days at room temperature. Tissues were embedded in paraffin, sectioned coronally (4-µm thickness), and then stained with hematoxylin and eosin. To evaluate the nasal epithelial thickness, bilateral nasal mucosae on posterior lateral meatus were measured at unilateral 3 random points using cellSens imaging software and AX80/DP72 (OLYMPUS, Tokyo, Japan), and the mean values of the mucosal thickness were calculated. To assess the eosinophil infiltration into nasal mucosa, eosinophils, which were stained with eosin and had two-lobed nuclei, were counted in bilateral nasal anterior lateral mucosae. For IL-33 staining in

nasal tissue, deparaffinized sections were heated in citrated buffer (pH 6.0) for antigen retrieval. After blocking with 1% BSA in PBS for 1 hour, sections were incubated with rabbit anti-mouse Biotinylated IL-33 antibody (R&D Systems), followed by Streptavidin, Alexa Fluor 488 conjugate (Invitrogen, MA, USA). Stained sections were mounted with Prolong Antifade Gold with DAPI (Invitrogen) and evaluated under a microscope Zeiss LSM 780 (Carl Zeiss). Computer software, Zen 2012 (Carl Zeiss), was used for image processing and analysis.

Flow Cytometry

To count the number of ILC2s in nasal mucosa, whole nasal cells were isolated as previously described (33). Briefly, noses were minced and digested for 50 minutes at 37 °C with collagenase (150 U/mL) and DNase I (10 µg/mL). Subsequently, cell suspensions were filtered using a cell strainer and red blood cells were lysed. ILC2s in nasal mucosa were stained with antibodies against CD45, lineage markers (CD3, CD4, CD8, CD11b, CD11c, CD19, DX-5, Gr-1, Siglec F, and IgE), Thy1.2, and ST2. Intracellular cytokine staining was performed using BD Cytofix/Cytoperm™ Plus Fixation/permeabilization Kit (BD biosciences). Stained cells were analyzed by a FACSCanto II flow cytometer (BD Biosciences) and FlowJo software (version

7.6.1, Tree Star Inc., Ashland, OR, USA). Lineage⁻CD45⁺ST2⁺Thy1.2⁺ cells were defined as nasal ILC2s (Fig. 1C).

Stimulation of nasal cells in vitro for intracellular cytokine staining

Isolated whole nasal cells were stimulated with PMA (30 ng/ml, Sigma Aldrich, Japan) and ionomycin (A23187) (500 ng/ml, Sigma Aldrich) for 4 hours in the presence of Golgi-Stop.

Quantitative RT-PCR

CD4⁺ T cells in nasal mucosa were isolated by an Auto MACS Separator using antimouse-CD4 MicroBeads (L3T4) (Miltenyi Biotec, Bergisch Gladbach, Germany). Total RNAs from CD4⁺ T cells were isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan). Gene expression levels were quantified using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) and the Thermal cycler dice RT-PCR system (Takara Bio Inc., Otsu, Japan). The results are shown as the relative expression standardized to eukaryotic 18S rRNA levels. The specific

primers and probes used for quantitative RT-PCR were TaqMan probes for *114*, *115*, *113*, and 18S rRNA (Applied Biosystems).

Statistical analysis

Representative data or pooled data were presented as the means $\pm$ SEMs of more than 2 independent experiments. Two-tailed Student *t*-test and 1-way ANOVA with Tukey test were used to determine the statistical significance between 2 groups and between more than 2 groups, respectively. *P* values of less than 0.05 were considered significant.

Results

Nasal administration of OVA and papain activates T_H2 cells and ILC2s, respectively, to cooperatively elicit nasal type 2 inflammation.

To investigate the contribution of T_H2 cells and ILC2s in nasal allergy, we used a model antigen OVA and a well-characterized ILC2-activator, papain, which activates ILC2s through the IL-33/ST2-pathway (13,31) and is a cause of occupational rhinoconjunctivitis (34,35). As shown in Fig. 1A, wild-type mice were i.n. administered 20 µl of PBS, 5% OVA, papain (10 µg/body), or OVA plus papain on days 1, 3, 6, 8, 10, and 13, and then analyzed on day 14. Similar to previous reports that the serial nasal administration of an antigen activated T_H2 immunity (27,36), in this study OVA administration elevated the expression of type 2 cytokine mRNAs, *Il4*, *Il5*, and *Il13*, in nasal CD4⁺ T cells (Fig. 1B). However, OVA-alone administration did not expand the ILC2 population, identified as Lin⁻CD45⁺ST2⁺Thy1.2⁺ cells in the nose (Fig. 1C and D). Because papain is also an antigen with adjuvant activity (37), we used a low dose of papain (10 µg/dose) to minimize the effect on T_H2 cell activation. In this protocol, papain significantly expanded the nasal ILC2 population (Fig. 1D). Although papain induced papain-specific IgG1 production (Fig. 1E), papain addition to OVA administration did not enhance OVA-specific IgG1 production (Fig. 1F) and OVA-induced nasal T_H2 cell activation (Fig. 1B). OVA-plus-papain administration

activated both T_H2 cells and ILC2s in noses (Fig. 1B and D). Therefore, the nasal administration of OVA or papain preferentially activated T_H2 cells or ILC2s, respectively.

Activation of type 2 immunity in noses induced nasal mucosal hyperplasia and eosinophilia that resulted in nasal congestion in AR and polyp generation in CRS (3,5). We examined the effect of the activation of T_H2 cells and/or ILC2s on nasal epithelial thickness in the posterior lateral meatus and on nasal eosinophilia. Both OVA- and papain-alone administration induced nasal epithelial thickening (Fig. 2A and B) and eosinophilia (Fig. 2C), and coadministration of OVA together with papain induced significantly stronger nasal type 2 inflammation compared with OVA- or papain-alone. In addition, another ILC2-activator *Alternaria alternata* extract, an environmental fungus that may cause CRS (38), also exacerbated OVA-induced nasal epithelial thickening (Fig. 2D). These results suggest that the simultaneous activation of T_H2 cells and ILC2s in noses additively exacerbates nasal type 2 inflammation.

T_H2 cells and ILC2s contribute in parallel to OVA-plus-papain-induced nasal type 2 inflammation.

Papain did not enhance OVA-induced nasal CD4⁺ T cell activation or OVA-specific IgG1 production. However, because papain administration induced papain-specific IgG1, papain-induced T_H2 cells, rather than papain-induced ILC2s, might contribute to the exacerbation of nasal symptoms. To directly examine whether ILC2s contribute to papain-induced exacerbation of nasal type 2 inflammation, we used T- and B-cell-deficient mice (*Rag2*^{-/-} mice), and ILC2-deficient mice (*Rora*^{sg/sg} → WT BMT mice). In *Rag2*^{-/-} mice, nasal epithelial thickening and eosinophilia induced by OVA-alone administration were completely abrogated (Fig. 3A and B). OVA-plus-papain-administration-induced nasal epithelial thickening and eosinophilia were also largely attenuated in *Rag2*^{-/-} mice (Fig. 3A and B), indicating the essential role of T_H2 cells in this model. *Rag2*^{-/-} mice, however, showed a slight increase in nasal epithelial thickness and number of eosinophils by OVA-plus-papain-administration indicating that activation of ILC2 alone can induce nasal symptoms even though relatively weakly. The *Rora*^{sg/sg} → WT BMT system has been well defined as ILC2 deficient with normal numbers of T_H2 cells. Papain-induced ILC2s were completely depleted in the nasal mucosa of *Rora*^{sg/sg} → WT BMT mice (Fig. 3C). Although OVA-alone-induced nasal epithelial thickening and eosinophilia were comparable between WT → WT and *Rora*^{sg/sg} → WT BMT mice, the papain-induced exacerbation of nasal thickening and eosinophilia were suppressed to levels comparable with OVA-alone in *Rora*^{sg/sg} → WT BMT mice (Fig. 3D and E). Because ILC2s can

affect T_H2 cell priming or activation in the lungs (14-18), we investigated whether depletion of ILC2s influenced T_H2 cell responses. mRNA levels of *Il4*, *Il5* and *Il13* in nasal CD4⁺ T cells in OVA-plus-papain-administered *Rora*^{sg/sg} → WT BMT mice were not decreased, but rather increased, compared with those in OVA-plus-papain-administered WT → WT BMT mice (Fig. 3F). These results suggested that the papain-induced exacerbation of nasal type 2 inflammation was mediated by the direct additive effect of ILC2 activation, but not through the augmentation of T_H2 cell responses. Therefore, papain-induced ILC2s and OVA-induced T_H2 cells cooperate in parallel to induce nasal allergic symptoms.

IL-33/ST2-signaling contributes to the ILC2-mediated exacerbation of nasal type 2 inflammation.

The IL-33/ST2-signaling pathway is essential for papain-induced ILC2 activation (13,31). First, to examine whether papain promotes IL-33 secretion from nasal epithelial cells, we performed immunohistochemistry for IL-33 in nasal mucosa. Mice were administrated PBS, OVA, or OVA plus papain as in Fig. 1A, and IL-33 expression in the nasal mucosa was evaluated before and after the final nasal challenge. Before the final challenge, nasal IL-33 levels were higher in OVA-plus-papain-administered mice compared with PBS- and OVA-administrated mice. Nasal

IL-33 levels were decreased at 1 hour, and partially recovered by 24 hours after OVA-plus-papain-administration (Fig. 4A). However, PBS- or OVA-alone-administration did not affect nasal IL-33 levels. Of note, IL-33 was not detected in OVA-plus-papain-administered *Il33*^{-/-} mice. These results suggested that papain induced temporal IL-33 secretion from nasal epithelial cells, and chronic nasal exposure to papain enhanced tissue IL-33 level. Next, to investigate the contribution of IL-33/ST2-signaling in OVA-plus-papain-induced nasal type 2 inflammation, we used *Il33*^{-/-} and *Il1rl1*^{-/-} (ST2 deficient) mice. OVA-plus-papain-induced nasal epithelial thickening and eosinophilia were significantly abrogated in *Il33*^{-/-} mice and the levels were comparable to OVA-alone-administered mice, while OVA-alone-induced epithelial thickening and eosinophilia were comparable between wild-type and *Il33*^{-/-} mice (Fig. 4B and C). Similarly, nasal epithelial thickening and eosinophilia were not exacerbated by papain in *Il1rl1*^{-/-} mice, and were comparable between OVA-plus-papain- and OVA-alone-administered *Il1rl1*^{-/-} mice (Fig. 4D and E). To investigate whether IL-33/ST2-signaling is important for the activation of nasal T_H2 cells and/or ILC2s, we examined the expression of *Il4*, *Il5*, and *Il13* mRNA levels in CD4⁺ T cells and the number of IL-5/IL-13 producing ILC2s in the nose. *Il4*, *Il5*, and *Il13* levels in CD4⁺ T cells were comparable between OVA- or OVA-plus-papain-administrated wild-type and *Il33*^{-/-} mice (Fig. 4F), while the number of IL-5/IL-13 producing ILC2s in OVA-plus-papain-administrated *Il33*^{-/-} mice were significantly lower than that in wild-type mice

(Fig. 4G). These results demonstrate that IL-33/ST2-signaling contributes to the ILC2-mediated exacerbation of nasal type 2 inflammation. However, IL-33/ST2-signaling has little contribution to T_H2 cell activation in this OVA and papain induced nasal allergy model.

Nasal eosinophilia is dispensable for nasal epithelial thickening.

Because eosinophils might be involved in epithelial thickening and tissue remodeling in allergic pulmonary inflammation by producing proteases (39-42), we depleted eosinophils in wild-type mice by the i.p. administration of anti-IL-5 antibody. OVA-alone- and OVA-plus-papain-induced nasal epithelial thickening were not suppressed by anti-IL-5 antibody, although numbers of nasal eosinophils were markedly reduced (Fig. 5A and B). In addition, nasal epithelial thickening was not ameliorated in OVA-alone and OVA-plus-papain treated eosinophil-deficient Δ dblGATA mice (Fig. 5C). Therefore, even though T_H2 cells and ILC2s induce strong nasal eosinophilia by producing IL-5, eosinophils are not major contributor to OVA- and papain-induced nasal epithelial thickening.

IL-13 is responsible for ILC2-mediated exacerbation of nasal type 2 inflammation.

IL-13 induces eosinophil accumulation, epithelial thickening, and mucous cell hyperplasia in lungs (43). To examine the effect of IL-13 in OVA- and papain-induced nasal type 2 inflammation, *Il13*^{-/-} mice were i.n. administered OVA-alone or OVA-plus-papain. Papain-induced exacerbation of nasal type 2 inflammation was abrogated in *Il13*^{-/-} mice as OVA-alone and OVA-plus-papain administration induced comparable levels of nasal epithelial thickening and eosinophilia (Fig. 6A and B). However, OVA-alone-induced epithelial thickening and eosinophilia were not ameliorated in *Il13*^{-/-} mice (Fig. 6A and B). Therefore, IL-13 derived from ILC2s, but not from T_H2 cells, contributes to papain-induced exacerbation of nasal epithelial thickening and eosinophilia.

Exacerbated nasal epithelial thickening by papain is refractory to corticosteroid treatment.

ILC2s and IL-33 are assumed to mediate corticosteroid-treatment resistance in nasal (44) and lung allergic responses (45). To investigate whether papain-induced ILC2 activation confers corticosteroid resistance in nasal allergy, OVA- or OVA-plus-papain-administered mice were i.p. injected with dexamethasone (2.5 mg/kg or 5 mg/kg) at 24 and 2 hours before each nasal challenge. OVA-induced nasal epithelial thickening was suppressed by dexamethasone in a dose-dependent manner and high-dose dexamethasone administration almost completely

ameliorated the symptoms (Fig. 7A). Even though low-dose dexamethasone suppressed nasal epithelial thickening in OVA-plus-papain-administered mice with a similar efficacy to that in OVA-alone-administered mice, high-dose dexamethasone did not have a greater suppressive effect and the symptoms remained in the OVA-plus-papain administered group (Fig. 7A). Because the IL-33/ST2-pathway mediates papain-induced ILC2 activation (Fig. 4), the nasal epithelial thickness in OVA-plus-papain-administered *Il1rl1*^{-/-} mice treated with high-dose dexamethasone was comparable to that of the PBS-administered groups (Fig. 7B). Nasal eosinophilia induced by OVA-alone or OVA-plus-papain was similarly suppressed by dexamethasone and was dose-dependent (Fig. 7C). Thus, although nasal eosinophilia was well controlled by corticosteroids regardless of the initial cause, papain-exacerbated nasal epithelial thickening was corticosteroid resistant. High-dose dexamethasone completely suppressed OVA-plus-papain-induced expression of *Il4*, *Il5*, and *Il13* in CD4⁺ T cells (Fig. 7D). Although dexamethasone decreased the number of IL-5/IL-13 producing ILC2s even in PBS-administered mice (Fig. 7E), the percentage of IL-5/IL-13 producing ILC2s in OVA-plus-papain-administered mice was not decreased by dexamethasone (Fig. 7F). Taken together, these results suggest that nasal ILC2 activation via the IL-33/ST2-pathway induces corticosteroid resistant nasal type 2 inflammation.

Discussion

In this study, we demonstrated that nasal ILC2s additively exacerbated T_H2 cell-induced nasal type 2 inflammation in mice. Interestingly, IL-13 induced by ILC2s, but not T_H2 cells, was critical for the exacerbation of nasal allergic symptoms. Furthermore, ILC2s activated by the IL-33/ST2-pathway conferred corticosteroid resistance.

Although T_H2 cells have a central role in allergic diseases by secreting type-2 cytokines, ILC2s may also be an important innate source of cytokines (1,7). To investigate the cooperation between T_H2 cells and ILC2s in nasal allergy, we generated a mouse model allowing the simultaneous activation of T_H2 cells and ILC2s in the nasal mucosa. We successfully induced the selective activation of nasal T_H2 cells and ILC2s by using OVA and low dose papain, respectively. Using this mouse model, we demonstrated that the activation of ILC2s additively enhanced T_H2 cell-induced nasal type 2 inflammation during the early chronic stage of disease. In the lungs, ILC2s induce type-2 immunity by inducing T_H2 cell priming and/or activation (14-18). In the nose, however, ILC2s and T_H2 cells are independently activated by different stimuli and contribute in parallel to nasal type 2 inflammation.

IL-13 can be produced from several cell types other than Th2 cells and ILC2s including mast cells, basophils, eosinophils, and NKT cells. However, the data from *Rag2*^{-/-} mice, which

lack NKT cells, and Δ dblGATA mice, which lack eosinophils, exclude the involvement of NKT cells and eosinophils in papain-induced IL-13 essential for augmented nasal symptoms. In addition, papain did not enhance OVA-induced upregulation of mast-cell- and basophil-specific genes in the nose (data not shown). Therefore, ILC2s may be the major cell population for IL-13 production in response to papain in our model.

Currently, it is believed that ILC2s are associated with the development of nasal polyps in CRSwNP because the presence of ILC2s in nasal polyps in CRSwNP patients was reported by several groups (22-26). However, this is controversial for cases of AR. A study reported a comparable number of ILC2s in PBMCs from AR patients and healthy controls (46), whereas circulating ILC2s were increased in patients with seasonal AR during the allergen season (19,20) and in patients with cat allergy immediately after nasal provocation (21). In the present study, we showed that the i.n. administration of IL-33 inducers such as papain and *Alternaria alternata* extract increased the number of ILC2s in the nasal mucosa, but that the administration of OVA, a T-cell activating antigen, did not. Thus, we hypothesized that ILC2s activated in nasal allergic disorders are associated with the affected allergens or microbes. When allergens that induce IL-33 production by nasal epithelial cells are inhaled, ILC2s are activated in the nasal mucosa and the symptoms of AR may be exacerbated or polyp

generation might be induced in CRS. Indeed, a study demonstrated that the number of circulating ILC2s in AR patients was different depending on the specific sensitizing allergens (19-21,46,47).

The development of anti-type-2 cytokine therapies for the treatment of allergic disorders is currently being investigated (48,49). Anti-IL-5 antibody (mepolizumab) significantly reduced nasal polyp size and improved computational tomography scores in CRSwNP, while some symptoms of CRS such as nasal congestion and rhinorrhea were not improved by the treatment (50). Our current study also demonstrated that anti-IL-5 antibody treatment did not suppress OVA-alone- and OVA-plus-papain-induced nasal epithelial thickening, despite a significant reduction in nasal eosinophilia. In contrast, both nasal epithelial thickening and nasal eosinophilia induced by OVA-plus-papain administration were significantly suppressed in *IL13^{-/-}* mice. Therefore, IL-13 might be a better therapeutic target for the treatment of nasal allergic diseases. However, OVA-alone-induced nasal epithelial thickening was not suppressed in *IL13^{-/-}* mice, suggesting that T_H2 cells produce other factor(s) that mediate nasal type 2 inflammation. IL-4 might be such a factor, because it signals through both IL-4 and IL-13 receptors and contributes to epithelial thickening (51). Human monoclonal antibody against the IL-4 receptor α subunit (dupilumab), which inhibits the effects of both IL-4 and IL-13,

significantly improved nasal polyp scores and nasal symptoms, including nasal congestion and rhinorrhea, in patients with CRS and nasal polyposis refractory to intranasal corticosteroids (52). Thus, it will be interesting to investigate whether the T_H2-cell-derived factor that mediated OVA-induced nasal epithelial thickening in the current study was IL-4.

Corticosteroid-treatment resistance is a significant clinical problem in CRSwNP and asthma. IL-33 administration conferred ILC2-dependent corticosteroid resistance in an OVA-induced allergic pulmonary inflammation model (45). In addition, increased levels of nasal IL-33 levels were associated with corticosteroid-resistant CRSwNP (44). The present study indicated that exacerbated nasal epithelial thickening induced by papain administration was dexamethasone treatment resistant. Because papain induced the IL-33/ST2 pathway-dependent activation of ILC2s, and OVA-plus-papain-induced nasal epithelial thickening was controlled in dexamethasone treated ST2-deficient mice, ILC2 activation may confer corticosteroid resistance during nasal type 2 inflammation. Thus, the regulation of ILC2 activation or IL-33 production might control refractory and recurrent nasal polyps in CRSwNP.

In summary, we identified a relationship between T_H2 cells and ILC2s in nasal type 2 inflammation, whereby ILC2s additively exacerbate T_H2-dependent nasal inflammation. ILC2-derived IL-13 might play a potent role in the exacerbation of nasal allergic symptoms.

Furthermore, ILC2s contributed to corticosteroid-treatment resistance in nasal type 2 inflammation. Therefore, ILC2s and the ILC2 activator, IL-33, might be promising therapeutic targets for the treatment of refractory nasal allergic disorders. Further studies specifically focusing on nasal ILC2s and T_H2 cells are required to fully determine the underlying pathological mechanisms in refractory nasal allergic disorders.

Abbreviations:

AR: allergic rhinitis

BMT: bone marrow transplantation

CRS: chronic rhinosinusitis

CRSwNP: chronic rhinosinusitis with nasal polyps

ILC2s: Group 2 innate lymphoid cells

OVA: ovalbumin

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Author contributions

T.M., A.F., and K.M. designed the study; T.M., and A.F. performed experiments; T.M., A.F. and

K.M. analyzed the data; S.A., N.I., and K.Y. prepared experimental materials; T.M., A.F., and

K.M. wrote the manuscript; S.F. and T.Y. supervised the study.

Conflict of Interest

The authors have no financial conflicts of interest.

Figure Legends

Fig. 1. Nasal administration of OVA or papain preferentially activated T_H2 cells or ILC2s, respectively.

(A) Experimental schema. Mice were intranasally (i.n.) administered PBS, OVA, papain, or OVA plus papain. Mice were analyzed 24 hours after the final challenge. (B) Expression of mRNAs (*Il4*, *Il5*, and *Il13*) in nasal CD4⁺ T cells was evaluated by RT-PCR. Data are mean $\pm$ SEM. (C and D) The gating strategy (C) and the number of nasal ILC2s (Lin⁻CD45⁺ST2⁺Thy1.2⁺) (D) analyzed by flow cytometry. FSC, Forward scatter; SSC, side scatter. Wild-type mice were treated as in Fig. 1A. (E and F) Mouse sera were collected at days 0 and 14. Serum papain- (E) and OVA-specific IgG1 (F) were measured by ELISA. Data are mean $\pm$ SEM. n=5 (B and D), n=4 (E and F). **P* < 0.05, ***P* < 0.01. N.S., Not significant.

Fig. 2. OVA and papain cooperatively promote nasal type 2 inflammation.

(A) Representative images of nasal mucosa. An image of posterior nasal cavity (left bigger panel) and magnified images of nasal mucosa (boxed area from left panel) from each group (4 right panels) are shown. Scale bars indicate 1 mm (left panel) and 20 μ m (4 right panels). (B)

Nasal epithelial thicknesses were measured at 6 different points in each mouse. Each dot represents an individual mouse and the bars indicate mean $\pm$ SEM of each group. (C) Eosinophils in nasal mucosa were counted. (D) Mice were intranasally administered PBS, OVA, *Alternaria alternate*, or OVA plus *Alternaria alternate*. Mice were analyzed 24 hours after the final challenge. Nasal epithelial thicknesses were measured as in Fig. 2B. Each dot represents an individual mouse and the bars indicate mean $\pm$ SEM of each group. , n=10–11 (B), n=6 (C), n=5 (D). * $P < 0.05$, ** $P < 0.01$.

Fig. 3. ILC2s and T_H2 cells additively exacerbate nasal type 2 inflammation.

Wild-type (WT) and *Rag2*^{-/-} mice (A and B) or WT and *Rora*^{sg/sg} bone-marrow transferred mice (C to F) were intranasally administered PBS, OVA, or OVA plus papain. Mice were analyzed 24 hours after the final challenge. (A and D) Nasal epithelial thicknesses were measured at 6 different points in each mouse. Each dot represents an individual mouse and the bars indicate mean $\pm$ SEM of each group. (B and E) Eosinophils in nasal mucosa were counted. (C) Nasal ILC2s from WT and *Rora*^{sg/sg} bone marrow transferred mice serially administered papain nasally for 4 days were analyzed by flow cytometry. (F) WT and *Rora*^{sg/sg} BMT mice were administered PBS, OVA, or OVA plus papain. Expression of mRNAs (*Il4*, *Il5*, and *Il13*) in nasal CD4⁺ T cells was

evaluated by RT-PCR. Data are mean $\pm$ SEM. n=6 (A), n=3 (B), n=5 (C, D and E), n=2–4 (F). * P < 0.05, ** P < 0.01.

Fig. 4. IL-33/ST2-signaling contributes to nasal type 2 inflammation.

(A) Mice were treated as in Fig. 1A. IL-33 expression in nasal tissues was examined before (pre) and 1 or 24 hours after the final administration of PBS, OVA or OVA plus papain. Green, IL-33; blue, DAPI. (B-E) Wild-type (WT) and *Il33*^{-/-} mice (B and C) or WT and *Il1rl1*^{-/-} mice (D and E) were intranasally administered PBS, OVA, or OVA plus papain. Mice were analyzed 24 hours after the final challenge. (B and D) Nasal epithelial thicknesses were measured at 6 different points in each mouse. Each dot represents an individual mouse and the bars indicate mean $\pm$ SEM of each group. (C and E) Eosinophils in nasal mucosa were counted. (F) Expression of mRNAs (*Il4*, *Il5*, and *Il13*) in nasal CD4⁺ T cells in WT and *Il33*^{-/-} mice was evaluated by RT-PCR. (G) Whole nasal cells were stimulated with PMA and ionomycin for 4 hours in the presence of Golgi-Stop. The number of IL-5/IL-13 producing ILC2s was evaluated by flowcytometry. Data are mean $\pm$ SEM. n=3 (A), n=5–8 (B), n=3–4 (C), n=6–7 (D), n=5–6 (E), n=4–5 (F and G). * P < 0.05, ** P < 0.01. N.S., Not significant.

Fig. 5. Nasal eosinophilia is dispensable for nasal epithelial thickening.

(A and B) Wild-type (WT) mice intraperitoneally injected with PBS or anti-IL-5 monoclonal antibody (α IL-5 mAb) were intranasally administered PBS, OVA, or OVA plus papain. Mice were analyzed 24 hours after the final challenge. (A) Nasal epithelial thicknesses were measured at 6 different points in each mouse. Each dot represents an individual mouse and the bars indicate mean $\pm$ SEM of each group. (B) Eosinophils in nasal mucosa were counted. Data are mean $\pm$ SEM. (C) WT and Δ dblGATA mice were intranasally administered PBS, OVA, or OVA plus papain. Mice were analyzed 24 hours after the final challenge. Nasal epithelial thicknesses were measured at 6 different points in each mouse. Each dot represents an individual mouse and the bars indicate mean $\pm$ SEM of each group. n=4 (A and B), n=6–7 (C). * P < 0.05, ** P < 0.01. N.S., Not significant.

Fig. 6. ILC2-derived IL-13 is involved in nasal type 2 inflammation.

Wild-type (WT) and $Il13^{-/-}$ mice were intranasally administered PBS, OVA, or OVA plus papain. Mice were analyzed 24 hours after the final challenge. (A) Nasal epithelial thicknesses were measured at 6 different points in each mouse. Each dot represents an individual mouse and

the bars indicate mean $\pm$ SEM of each group. (B) Eosinophils in nasal mucosa were counted.

Data are mean $\pm$ SEM. n=5–7 (A), n=3–4 (B). ** $P < 0.01$.

Fig. 7. Papain-induced ILC2 activation contributes to the corticosteroid-resistance of nasal epithelial thickening.

Wild-type (WT) mice and *Il1rl1*^{-/-} mice were intranasally administered PBS, OVA, or OVA plus papain. The mice were treated intraperitoneally with PBS or dexamethasone (Dex; 2.5 mg/kg or 5 mg/kg) at 24 and 2 hours before each intranasal challenge. Mice were analyzed 24 hours after the final challenge. (A and B) Nasal epithelial thicknesses in WT mice (A) and *Il1rl1*^{-/-} mice (B) were measured at 6 different points in each mouse. Each dot represents an individual mouse and the bars indicate mean $\pm$ SEM of each group. (C) Eosinophils in nasal mucosa were counted. (D) Expression of mRNAs (*Il4*, *Il5*, and *Il13*) in nasal CD4⁺ T cells in WT mice was evaluated by RT-PCR. (E and F) Whole nasal cells were stimulated with PMA and ionomycin for 4 hours in the presence of Golgi-Stop. The number (E) and percentage (F) of IL-5/IL-13 producing ILC2s were evaluated by flowcytometry. Data are mean $\pm$ SEM. n=3–5 (A and C), n=2–4 (B), n=5 (D, E and F). ** $P < 0.01$. N.S., Not significant.

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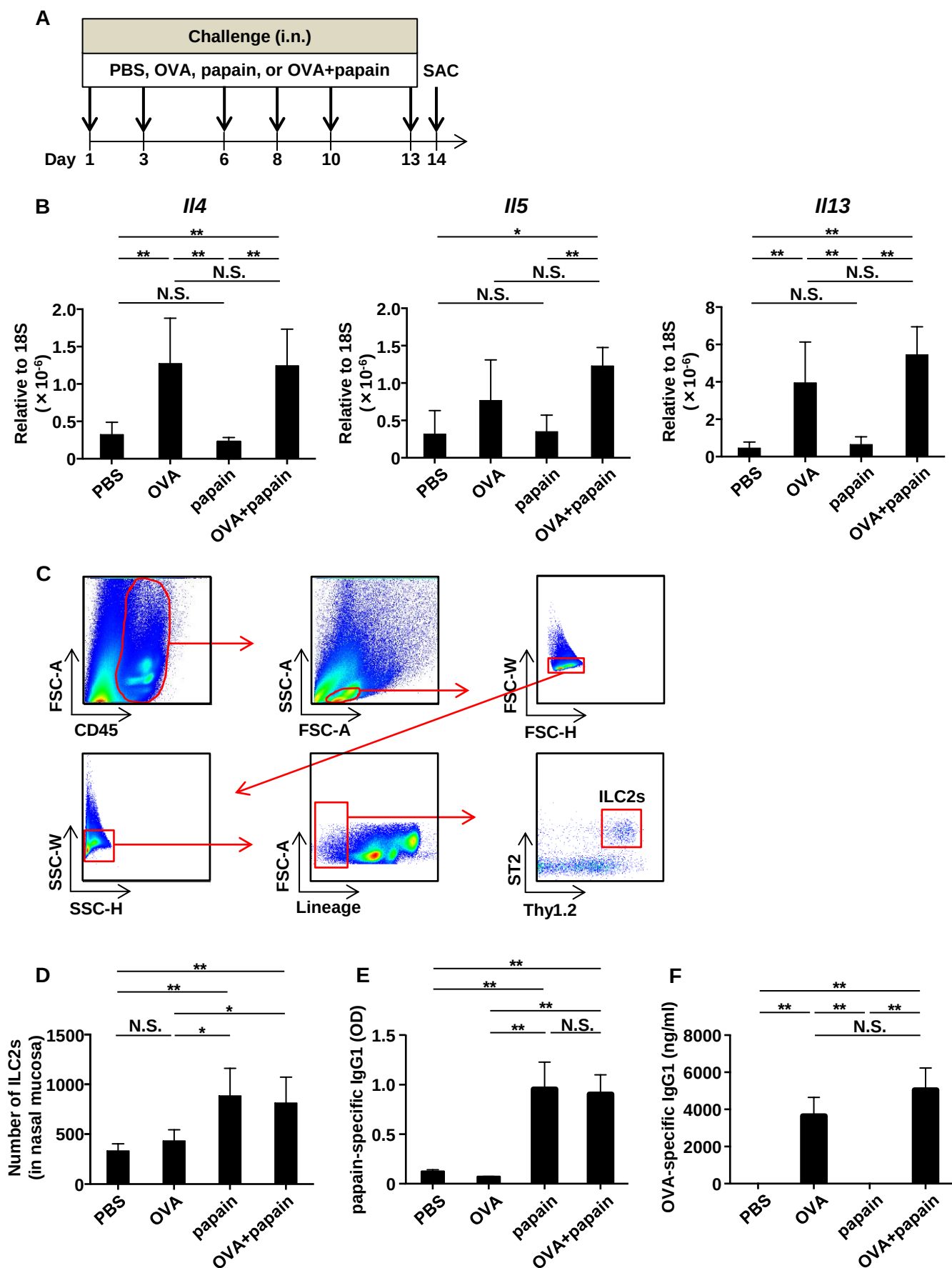
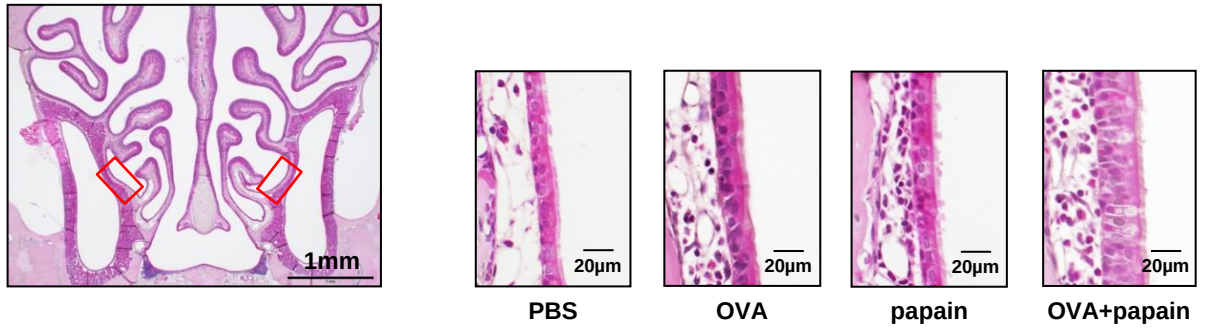
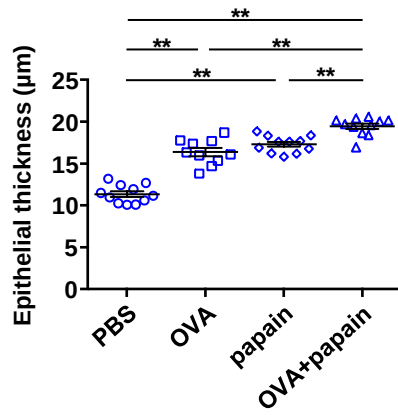


Figure 1

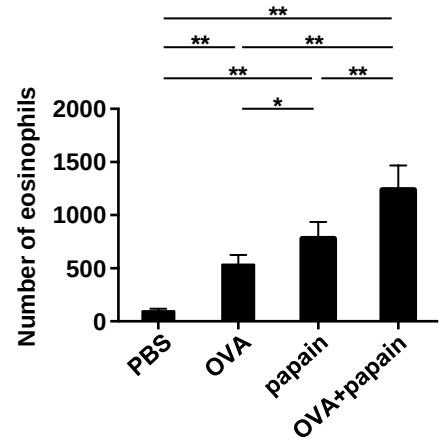
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B



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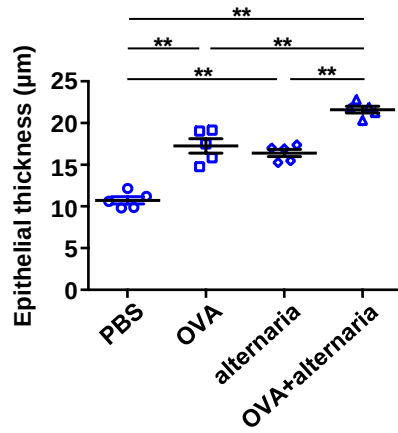


Figure 2

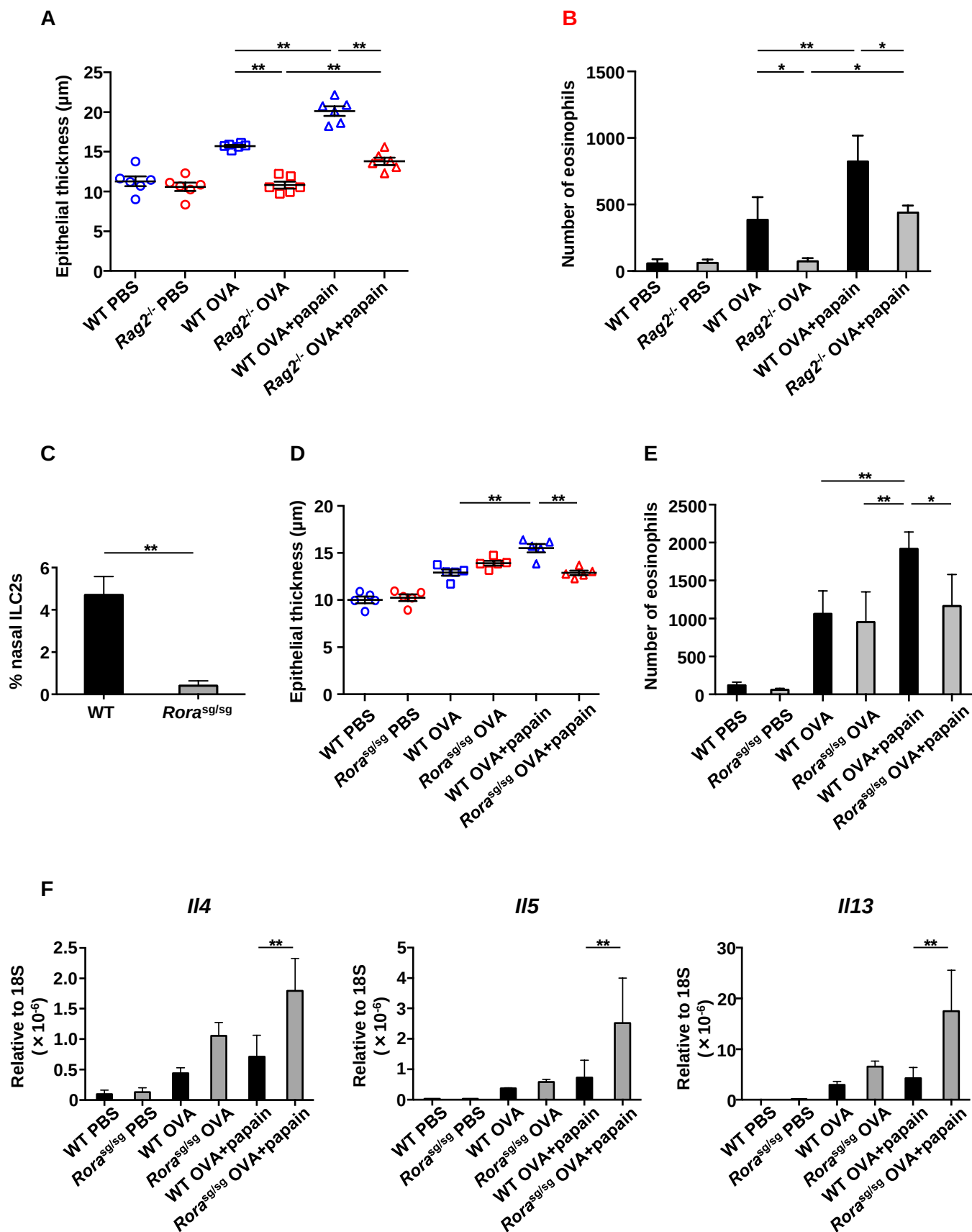


Figure 3

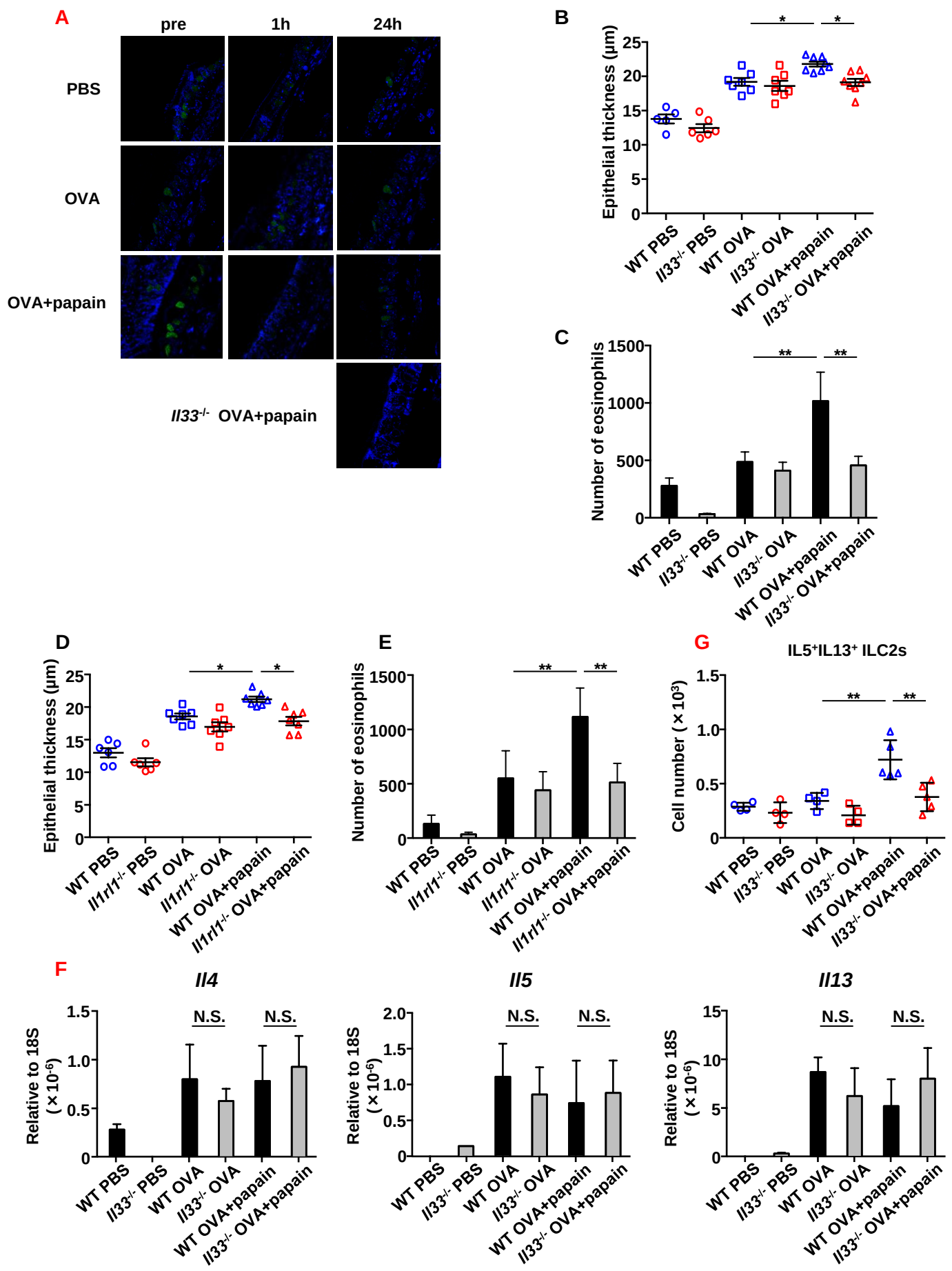
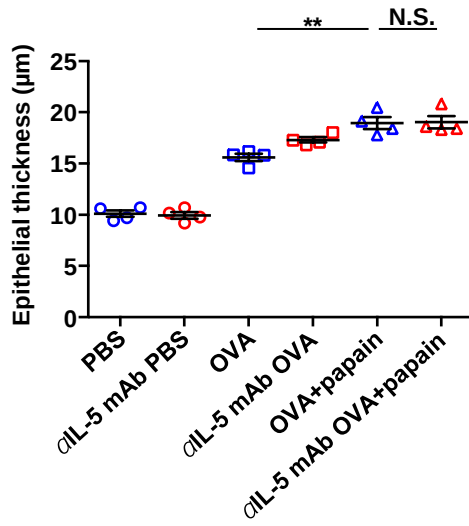
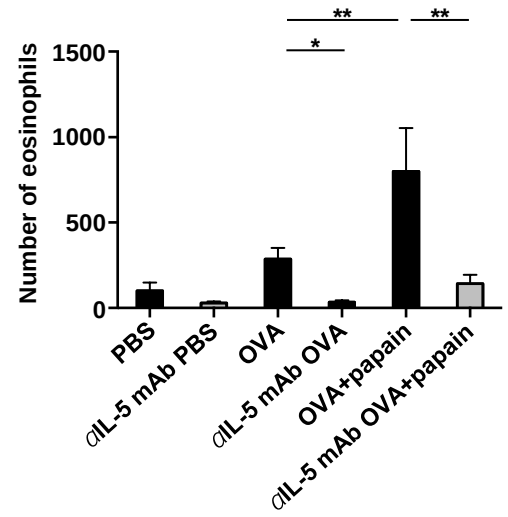


Figure 4

A



B



C

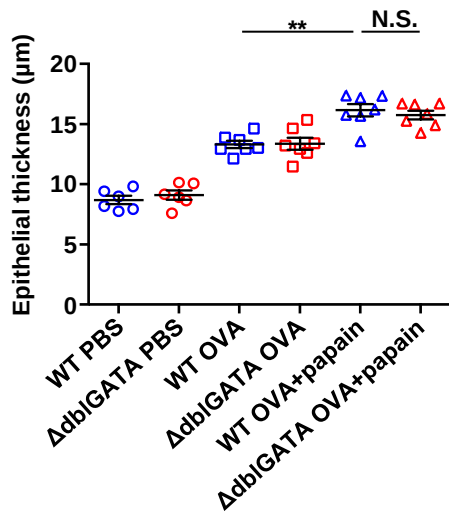


Figure 5

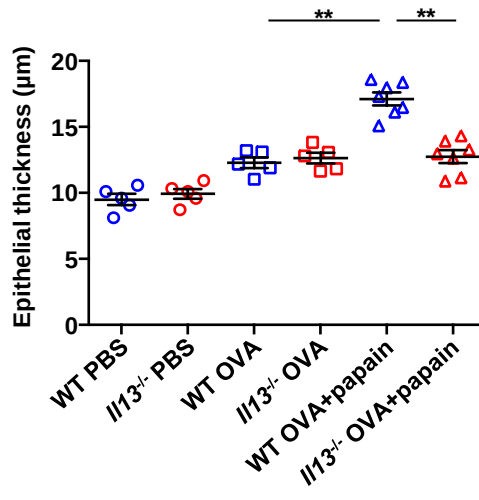
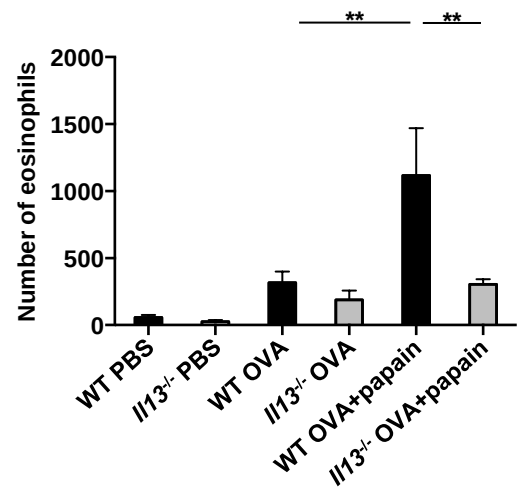
A**B**

Figure 6

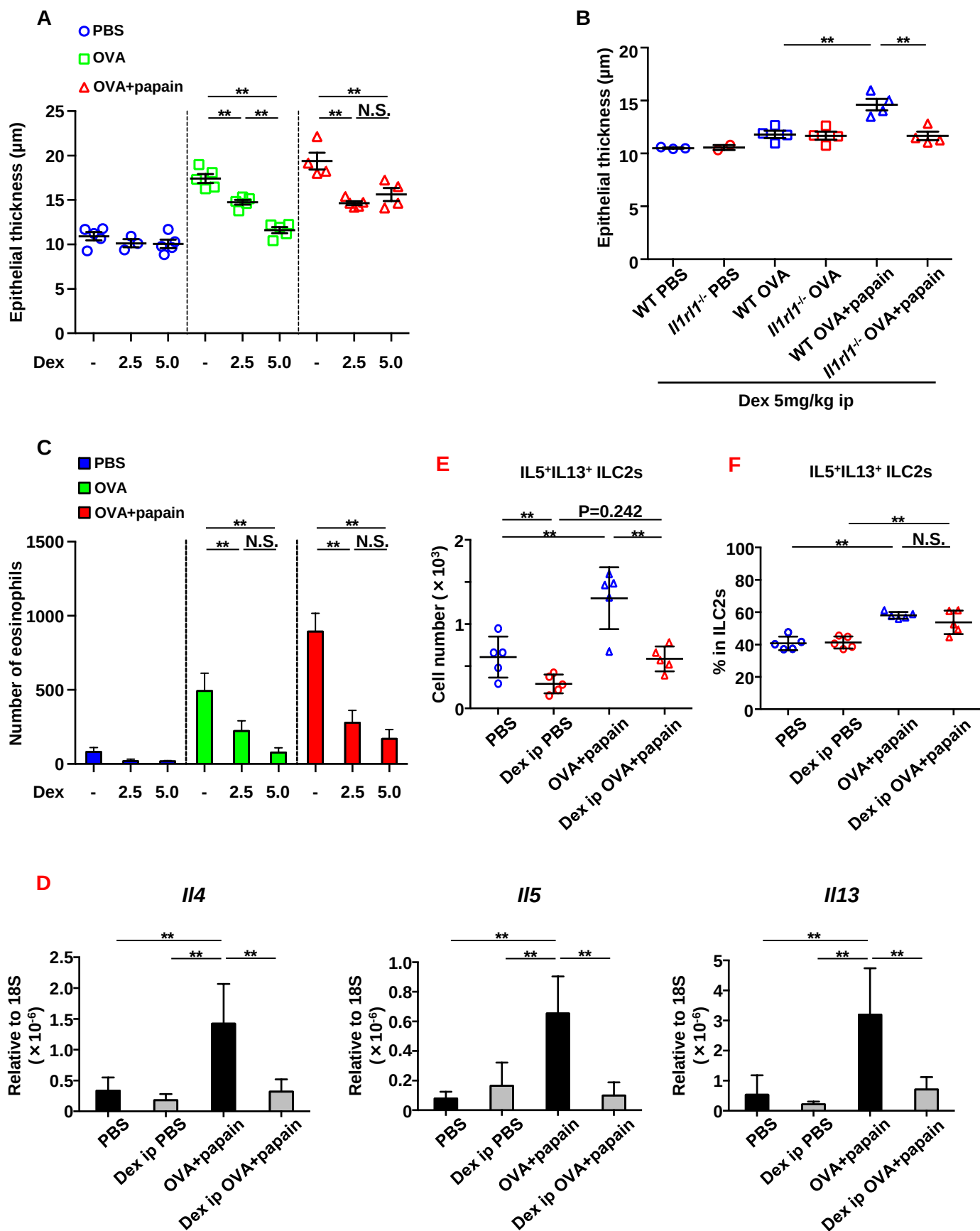


Figure 7