Long-acting muscarinic antagonist regulates group 2 innate lymphoid cell-dependent airway eosinophilic inflammation

Short title: Effects of tiotropium on innate-type airway inflammation

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ABSTRACT

Background: Tiotropium bromide, a long-acting muscarinic antagonist, reduces the frequency of exacerbation in patients with moderate to severe asthma, but its underlying mechanism is not clear. Asthma exacerbations are associated with exposure to external stimuli, and group 2 innate lymphoid cells (ILC2s) are considered to be involved in the pathophysiology of asthma exacerbation. We investigated whether tiotropium modulates airway inflammation through ILC2 functions.

Methods: Mice were administered papain intranasally to induce innate-type airway inflammation with or without tiotropium pretreatment, and bronchoalveolar lavage fluids (BALF) and lung tissues were collected. Lung-derived ILC2s and bone-marrow-derived basophils were stimulated *in vitro* with IL-33 in the presence or absence of tiotropium. Muscarinic 3 receptor (M3R) expression on immune cells was assessed by RNA sequence.

Results: Papain induced airway eosinophilic inflammation, and tiotropium reduced the numbers of eosinophils in BALF. The concentrations of IL-4, IL-5 and IL-13, and the numbers of ILC2s in BALF were also reduced by tiotropium treatment. However, tiotropium did not affect IL-33-induced IL-5 and IL-13 production from ILC2s, suggesting that tiotropium regulates ILC2s indirectly. Gene-expression analysis showed that basophils predominantly expressed M3R mRNA among murine immune cells. Tiotropium reduced IL-4 production from basophils derived from mouse bone marrow and human basophils after stimulation with IL-33.

Conclusions: These findings suggest that tiotropium attenuates ILC2-dependent airway inflammation by suppressing IL-4 production from basophils and, subsequently, regulating ILC2 activation. The inhibitory effects of long-acting muscarinic antagonists on the innate response may contribute to reducing asthma exacerbation.

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Keywords:

Asthma, Basophils, Group 2 innate lymphoid cells, Tiotropium bromide

Abbreviations:

ACh: Acetylcholine

BALF: Bronchoalveolar lavage fluids

COPD: Chronic pulmonary obstructive disease

CysLT: Cysteinyl leukotriene

ICS: Inhaled corticosteroid

ILC2: Group 2 innate lymphoid cell

LABA: Long-acting β_2 agonist

LAMA: Long-acting muscarinic antagonist

Lin: Lineage

M3R: Muscarinic 3 receptor

OVA: Ovalbumin

PCR: Polymerase chain reaction

PBMC: Peripheral blood mononuclear cell

Th2 cell: T helper type 2 cell

TSLP: Thymic stromal lymphopoietin

INTRODUCTION

Bronchial asthma is a heterogeneous disease characterized by chronic airway inflammation, reversible airway obstruction, and airway hyperresponsiveness, and inhaled corticosteroids (ICSs) are the mainstay of therapy [1]. Although many patients with asthma are able to achieve good disease control, especially from ICS plus a long-acting β_2 agonist (LABA), a significant number of patients with asthma have uncontrolled, moderate-to-severe disease with recurrent exacerbations and persistent symptoms despite maximized standard-of-care controller therapy [2].

Acetylcholine (ACh) is a parasympathetic neurotransmitter that regulates various activities, including neurotransmission, smooth muscle contraction, and mucus secretion [3,4]. ACh is also synthesized and secreted by various types of cells, such as smooth muscle cells, fibroblasts, and inflammatory cells, and muscarinic receptors are expressed on inflammatory cells [5]. Five muscarinic receptor (M1–5) genes have been cloned, and M1–3 receptors are functionally recognized in the lungs and airways [3]. In asthma and chronic pulmonary obstructive disease (COPD), the bronchodilatory effect of a long-acting muscarinic antagonist (LAMA) is mainly attributed to Muscarinic 3 receptor (M3R) inhibition.

The addition of tiotropium bromide, LAMA, to a high-dose ICS plus LABA reduced the risk of asthma exacerbation [6,7]. Asthma exacerbations occur in response to exposure to external agents, such as virus infection, pollen, or air pollution. Viral infection and air pollution stimulate the production of epithelial-derived cytokines (IL-33, IL-25, thymic stromal lymphopoietin (TSLP)) [8]. Group 2 innate lymphoid cells (ILC2s)

release type-2 cytokines, IL-5 and IL-13, when stimulated by epithelial-derived cytokines [9,10]. ILC2s are considered to be involved in the pathogenesis of asthma exacerbation [1,11]. Previous studies demonstrated that tiotropium reduced airway eosinophilic inflammation by acting on $CD4^+$ T cells in an ovalbumin (OVA)-induced asthma model [12-14]. However, few studies have focused on the effect of tiotropium on ILC2-dependent airway inflammation.

Papain, a proteolytic enzyme, is known to cause occupational asthma [15,16] and triggers the release of IL-33, IL-25, and TSLP from airway epithelial cells, which subsequently activates ILC2s to induce airway eosinophilic inflammation through type-2 cytokine production [9,17,18]. This intranasal papain administration is considered to be a typical model of type-2 airway inflammation mediated by innate immunity because it does not require adaptive immunity [9,19]. We hypothesized that tiotropium could attenuate ILC2-dependent airway inflammation induced by papain. We showed that eosinophil accumulation and the production of type-2 cytokines induced by papain were reduced after tiotropium treatment. We next investigated the mechanisms by which tiotropium modulates ILC2-dependent airway inflammation.

METHODS

Mice

Male C57BL/6N mice 8–12 weeks old were maintained in a specific pathogen-free environment and had free access to food and water. All animal experiments and handling procedures were approved by the Animal Care and Use Committees of Kagoshima University and were performed in accordance with institutional guidelines.

Papain-induced airway inflammation

Mice were anesthetized with isoflurane, treated with 25 μg papain (Calbiochem, San Diego, CA, USA) in 40 μl saline intranasally for 3 consecutive days, and sacrificed 24 hours after the last papain administration. Bronchoalveolar lavage fluids (BALF) and lung tissues were collected. Total cell counts were determined by light microscopy, using a standard hemocytometer. BALF was centrifuged at 800 rpm for 5 min at 4°C. The cell pellet was resuspended in saline, and cytospins (Cytospin 3; Shandon, Pittsburgh, PA, USA) were prepared on glass slides and stained with Diff-Quik as previously described [20]. 200 cells were analyzed for differential cell counts by conventional morphological criteria [21]. For tiotropium treatment, 0.02 mg/kg of tiotropium (Cayman Chemical, Ann Arbor, MI, USA) in 40 μl PBS was administered intranasally 60 min prior to each papain administration as previously described [22].

Analyses of lung tissues by flow cytometry

Lung cell suspension was collected as previously described [23]. ILC2s and basophils were identified as lineage-negative (Lin^-) $Thy1.2^+$ $T1/ST2^+$ $KLRG1^+$ $CD45.2^+$ cells and $c\text{-}Kit^-$ $Fc\varepsilon RI^+$ $CD49b^+$ cells, respectively. For ILC2 sorting, naive ILC2s were

isolated as a fraction of Lin^- $Thy1.2^+$ $T1/ST2^+$ cells as previously described [23]. Briefly, ILC2s were a minor cell population in lung tissues, and to enrich the ILC2 population before sorting, we depleted lineage-positive cells by labeling them with a mixture of anti-mouse biotin-conjugated antibodies for lineage markers and using streptavidin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and the autoMACS Pro Separator (Miltenyi Biotec). These cells were analyzed and sorted using BD FACSCelesta (BD Biosciences, San Jose, CA, USA) and Cell Sorter SH800 (Sony Corporation, Tokyo, Japan). The purity of ILC2s was > 97%. All data were analyzed using FlowJo software (TreeStar, CA, USA). Antibodies used in this study are listed in Table S1.

In vitro stimulation of ILC2s and basophils

Purified ILC2s by cell sorting were seeded at a density of 5,000 cells per well in 96-well round bottom plates as previously described [23] and were stimulated with mouse IL-33 (BioLegend, San Diego, CA, USA) in the presence or absence of tiotropium at 37°C for 24 hours. Purified basophils were seeded at a density of 1×10^5 cells per well as previously described [24] and were stimulated with mouse IL-3 (Wako, Osaka, Japan) and mouse IL-33 in the presence or absence of tiotropium at 37°C for 24 hours. Further details are provided in the supporting information.

Isolation and stimulation of human basophils

For human basophils, peripheral blood mononuclear cells (PBMCs) were first isolated from the blood of healthy volunteers by diluting the blood 1:1 in PBS and using Ficoll-Paque PLUS (GE Healthcare, Chicago, IL, USA). Basophils were purified from

PBMCs by autoMACS Pro Separator using Basophil Isolation Kit II (Miltenyi Biotec). The purity of human basophils was confirmed to be >95% by Diff-Quik staining. Approval for all experiments with human cells was provided by the Human Ethics Committees of Kagoshima University, and written informed consent was obtained. Purified basophils were seeded at a density of 1 x 10^5 cells per well and were stimulated with human IL-3 (10 ng/ml) (R&D Systems, Minneapolis, MN, USA) and human IL-33 (10 ng/ml) (R&D Systems) in the presence or absence of tiotropium (10 to 50 μM) at 37°C for 24 hours.

Co-culture experiments

Both lung-derived ILC2s and bone-marrow-derived basophils were seeded at a density of 5 x 10^4 cells per well in 96-well round bottom plates. ILC2s co-cultured with basophils were stimulated with mouse IL-3 (2 ng/ml) and mouse IL-33 (1 ng/ml) in the presence or absence of tiotropium (10 to 50 μM) at 37°C for 24 hours. The levels of IL-4, IL-5 and IL-13 in the culture supernatant were measured by ELISA.

ELISA, flow cytometric analyses, quantitative real-time polymerase chain reaction (PCR), western blot analyses and RNA sequencing

Additional details regarding the method are provided in the supporting information.

Statistical analysis

Data are expressed as the mean ± SEM. Statistical differences among groups were analyzed using unpaired Student's t tests or an analysis of variance together with Dunnett or Tukey analysis. Data were analyzed with GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered significant.

RESULTS

Innate immunity-mediated type-2 airway inflammation is attenuated by tiotropium treatment

Intranasal administration of papain to mice *in viv*o for 3 consecutive days increased the numbers of eosinophils in BALF and lung tissues, and tiotropium treatment significantly suppressed papain-induced accumulation of eosinophils (Figure 1A, B and Figure S1A). Although there was an increase in the numbers of macrophages and neutrophils after papain, tiotropium did not affect these cell numbers. Papain also increased the concentrations of IL-5 and IL-13 in BALF, and tiotropium-treated mice exhibited decreased IL-5 and IL-13 levels after papain administration (Figure 1C). Cellular infiltration and mucus hyperproduction were suppressed by tiotropium treatment (Figure 1D, E). Glycopyrrolate, another LAMA, also suppressed papain-induced airway eosinophilia (Figure S2A–C).

It is reported that IL-33 and TSLP are constitutively expressed in the lungs and released from airway epithelial cells in response to papain [9,25]. M3R is expressed in almost all types of cells in the airways, including airway epithelial cells [5]. We confirmed M3R expression on airway epithelium and airway smooth muscle by immunohistochemistry, and there was no difference in M3R expression between naïve and papain-treated mice (Figure S3). We examined whether tiotropium regulates IL-33 and TSLP levels. Upregulation of IL-33 and TSLP mRNA expression in lungs was observed 3 hours after papain administration (Figure 1F). The concentrations of IL-33 and TSLP in BALF were also upregulated at 3 hours following papain exposure, and

then subsided at 24 hours; however, tiotropium did not affect the papain-induced upregulation of IL-33 and TSLP expression and production (Figure 1F, G). Next, intranasal administration of IL-33 for 3 consecutive days resulted in increased numbers of eosinophils in BALF and lung tissues and elevated levels of IL-5 and IL-13 in BALF (Figure 2A). Eosinophil accumulation and type-2 cytokine upregulations induced by IL-33 were reduced in tiotropium-treated mice (Figure 2B, C and Figure S1B). To assess whether tiotropium affects papain-induced IL-33 upregulation in airway epithelial cells, we conducted *in vitro* experiments using TGMBE-02-3 cell, a mouse tracheal epithelial cell line. Papain upregulated IL-33 mRNA expression in TGMBE-02-3 cells; however, tiotropium did not affect papain-induced upregulation of IL-33 (Figure S4). Taken together, these results indicate that tiotropium suppresses papain- and IL-33-induced airway inflammation without affecting IL-33 upregulation.

Tiotropium attenuates the proliferation and the type-2 cytokine production of ILC2s

We next sought to elucidate whether tiotropium regulates downstream cascades of IL-33. Tiotropium did not affect the numbers of lymphocytes in BALF (Figure 1B and 2B). ILC2s have been identified as a cell subset responsible for papain-induced lung inflammation [17], and IL-33 activates ILC2s [11,26]. Therefore, we focused on ILC2s, although ILC2s are non-T, non-B lymphocytes [11]. Intranasal administration of papain to mice increased the frequencies and numbers of ILC2s, which were enumerated using flow cytometry, in the lungs and in BALF (Figure S5A), and tiotropium significantly decreased ILC2 numbers in BALF (Figure 3A). The frequencies and numbers of ILC2s in the lungs had the same tendency to be reduced by tiotropium (Figure 3B). The

intracellular cytokine assay revealed that the production of IL-5 and IL-13 from lung ILC2s was reduced by tiotropium treatment (Figure 3C). In addition, the frequencies and numbers of ILC2s were enumerated in BALF and in the lungs in an IL-33-induced model (Figure 3D, E), and tiotropium significantly decreased ILC2 numbers in BALF (Figure 3D), similar to the papain-induced model.

We also evaluated whether tiotropium suppressed Th2 (T helper type 2) cells in a papain-induced model. Lin^+ $T1/ST2^+$ $CD4^+$ $CD45.2^+$ cell fractions are enriched for Th2 polarized cells [27,28]. Papain slightly increased the frequencies and numbers of these $CD4^+$ T cells in the lungs, but tiotropium failed to reduce $CD4^+$ T cell numbers and the production of type-2 cytokines from $CD4^+$ T cells (Figure S6A, B). ILC2s were more potent in producing IL-5 and IL-13 compared with $CD4^+$ T cells. These results suggest that tiotropium mainly inhibits the proliferation and the type-2 cytokine production of ILC2s in response to papain administration *in vivo*.

Tiotropium inhibits cytokine production from ILC2s indirectly

To study the effects of tiotropium on ILC2s, ILC2s were isolated from mouse lung using flow cytometry (Figure 4A and Figure S7A) and cultured with mouse IL-33 with or without tiotropium for 24 hours; the production of type-2 cytokines was then measured in the culture supernatant. Although the production of IL-5 and IL-13 from ILC2s increased with IL-33, tiotropium did not reduce the production of type-2 cytokines (Figure 4B). M1-3 receptors are functionally recognized in the airways. We also evaluated M3R expression in ILC2s. RNA sequence analysis and western blot analysis revealed that M3R expression was low in ILC2s (Figure 4C–E), confirming the inability

of tiotropium. These findings suggest that tiotropium indirectly suppresses type-2 cytokine production in ILC2s.

Tiotropium inhibits basophil-derived IL-4 production

Through RNA sequence analysis and western blot analysis, we noticed that basophils exhibited higher expression of M3R as compared with that of other immune or inflammatory cells (Figure 4C–E). Therefore, we first measured the numbers of basophils in the lungs of a papain-induced model by flow cytometry (Figure S5B); however, there was no difference in the frequencies and numbers of basophils between vehicle- and tiotropium-treated mice (Figure 5A). On the other hand, the concentrations of IL-4 in BALF were increased after papain, and tiotropium treatment significantly decreased IL-4 levels (Figure 5B). Intracellular cytokine assay also showed that tiotropium decreased IL-4-producing basophils in the lungs (Figure 5C).

Next, we stimulated bone-marrow-derived basophils with mouse IL-33 in the presence or absence of tiotropium *in vitro* using flow cytometry (Figure S7B). IL-33 enhanced IL-4 production from bone-marrow-derived basophils, and tiotropium suppressed IL-33-induced IL-4 production from basophils dose-dependently (Figure 5D). It has been reported that basophils also produced IL-13 and histamine in response to IL-33 [29,30]. We cultured basophils with mouse IL-33 and measured the levels of IL-13 and histamine in the culture supernatant. Although the levels of IL-13 and histamine increased with IL-33, tiotropium did not reduce the production of IL-13 and histamine (Figure S8A, B).

To determine whether tiotropium attenuates ILC2-dependent airway inflammation by suppressing basophil-derived IL-4, we co-cultured lung ILC2s with basophils in the presence or absence of tiotropium. Co-culture of basophils and ILC2s resulted in increasing IL-4, IL-5 and IL-13 levels in the culture supernatant after IL-33 stimulation, and tiotropium decreased IL-4, IL-5 and IL-13 levels (Figure 5E). ILC2s stimulated with IL-33 did not produce IL-4, and tiotropium did not reduce the production of IL-5 and IL-13 from ILC2s stimulated with IL-33 (Figure 5E). These results support the importance of IL-4 in our *in vivo* model and suggest that tiotropium decreases the production of type-2 cytokines from ILC2s via the basophil-IL-4-ILC2 axis.

IL-4 production from human basophils is reduced by tiotropium

Finally, we assessed the effects of tiotropium on human basophils. Peripheral blood basophils were isolated from healthy volunteers. Human basophils primed by human IL-3 also expressed M3R on their cell surfaces (Figure 6A). Isolated basophils were stimulated with human IL-33 in the presence or absence of tiotropium. As observed in murine cells, tiotropium reduced IL-33-induced IL-4 production of basophils (Figure 6B).

DISCUSSION

In the present study, for the first time, we demonstrated that tiotropium attenuated ILC2-dependent airway inflammation by reducing basophil-derived IL-4 production. Importantly, we showed that human basophil-derived IL-4 production was also reduced by tiotropium. So far, tiotropium has been used primarily as a bronchodilator. Our findings could provide a new anti-inflammatory mechanism of action for tiotropium on ILC2-dependent airway inflammation.

In an OVA-induced asthma model, it was reported that tiotropium inhibited the increase of eosinophils and goblet cell metaplasia and decreased the production of type-2 cytokines in the airways [12-14]. On the other hand, M3R-deficient mice did not exhibit decreased eosinophil accumulation and type-2 cytokine expression in an OVA-induced asthma model [31]. Type-2 cytokines in OVA-induced asthma model were predominantly produced by $CD4^+$ T cells, rather than ILC2s, and the anti-inflammatory effects of LAMAs on OVA-induced adaptive immune-mediated type-2 airway inflammation are equivocal.

In this study, we demonstrated that papain induced IL-5^+ and IL-13^+ $CD4^+$ T cells in the lungs, and that tiotropium did not affect $CD4^+$ T cell numbers and the production of type-2 cytokines from $CD4^+$ T cells. In the previous study, tiotropium suppressed type-2 cytokine production by $CD4^+$ T cells *in vitro* [13]. Although IL-4 derived from basophils promotes Th2 differentiation [32], there is a small number of $CD4^+$ T cells in the lungs and IL-4 has little impact on Th2 differentiation in a papain-induced model [9]. Papain-induced model is mainly dependent on cytokine production from ILC2s, where OVA-induced

model is depending on Th2 cells, and the discrepancy in the effects of tiotropium between our study and the previous report may be due to the difference in mouse models.

In the present study, tiotropium attenuated papain-induced type-2 airway inflammation. The papain model is considered to be a typical type-2 airway inflammation mediated by ILC2 and innate immunity because it does not require adaptive immunity in our studies [9,28]. It has been reported that ILC2s exacerbate allergic airway inflammation after viral infection [33]; however, ILC2s can also be protective because of their capacity to produce amphiregulin [34]. Recent findings support the conclusion that ILC2s and IL-33 contribute to virus-induced exacerbation [35,36].

All components of an ACh synthesis system, including high-affinity choline transporter 1, choline acetyltransferase, and vesicular acetylcholine transporter, have been demonstrated in various types of cells, such as epithelial cells and immune cells [37,38]. Muscarinic receptors are also detected in airway structural cells and immune cells [37,38]. This evidence suggests that non-neuronal cells can release ACh, which may participate in various biological reactions in an autocrine or paracrine manner [3]. Although it was not clear which type of cells are the major source of ACh in this study, basophils predominantly expressed M3R, and tiotropium decreased the production of IL-4 from basophils. Tiotropium indirectly suppressed type-2 cytokine production from ILC2s.

IL-4 is produced by Th2 cells, mast cells, basophils, and ILC2s [39]. IL-4 derived from basophils is a key cytokine for the differentiation of Th2 cells, Th2 cell-derived IL-4

induces isotype class switching of B cells to IgE synthesis and inflammatory cell recruitment by upregulating vascular cell adhesion molecule-1 expression [39,40]. IL-4 also play important roles in bioactivities of ILC2s. In mice, it has been reported that ILC2s constitutively express IL-4 receptors. IL-4 alone induces the production of IL-5, IL-9, IL-13 *in vitro* and enhances IL-33-mediated activation and proliferation on ILC2s [9]. These findings suggest that all IL-4-producing cells play an essential role in asthma pathogenesis. Basophil-deficient mice transferred with *Il4*$^{-/-}$ basophils showed a reduction of lung ILC2 numbers, and were failing to induce airway eosinophilic inflammation compared with control mice in papain-induced model [9]. Additionally, basophil-specific IL-4-deficient mice administered papain showed downregulation of IL-5 and IL-13 mRNA expression in lung ILC2s [9]. In the previous report, human ILC2s also express IL-4 receptors, and IL-4 acts in synergy with IL-33 to induce human ILC2 proliferation and the type-2 cytokine production [41]. In addition, ILC2s stimulated with LTD_4 are capable of producing IL-4, and IL-4 produced by ILC2s acts on ILC2s in an autocrine manner [42]. To address the contribution of ILC2-derived IL-4 to papain-induced model, we measured CysLT concentrations in BALF, and there was no difference of CysLT levels in BALF between vehicle- and tiotropium-treated mice (data not shown). Therefore, we consider that ILC2-derived IL-4 little affects ILC2 activation, and that basophil-derived IL-4 mainly contribute to the production of IL-5 and IL-13 from ILC2s in papain-induced model.

We demonstrated that tiotropium reduced IL-4 from basophils not affecting the frequencies and numbers of basophils in the lungs. However, underlying mechanism is not clear enough. Ca^{2+} influx is required for basophil activation, including IL-4

production after IL-3 stimulation [43]. On the other hand, IL-3-driven basophil proliferation depends on STAT5 signaling [44]. However, it is not clear that intracellular signal through M3R modulates STAT5 signaling. Therefore, tiotropium might decrease IL-4 production from basophils by inhibiting Ca^{2+} influx.

Airway epithelial cells express M3R [5,37,45]. The stimulation of M3R induces mucus secretion from airway epithelial cells, and LAMAs are known to inhibit mucus secretion [46]. Tiotropium is reported to inhibit IL-13-induced goblet cell metaplasia and ACh-induced LTB_4 release in human airway epithelial cells [47,48]. IL-33 and TSLP were constitutively expressed in the lungs, and papain upregulated their expression in agreement with previous observations [9,25]. Tiotropium did not affect the upregulation of IL-33 and TSLP. Therefore, it is considered that tiotropium acts on immune cells, but not on airway epithelial cells, to attenuate type-2 airway inflammation.

There are limitations to the interpretation of results obtained from human basophils isolated from peripheral blood rather than airway tissues. Basophils are a minor population (<1%) of leukocytes in the peripheral blood, and we consider that these experiments with human cells add value to our mouse model findings. We may need to assess the effects of tiotropium on airway inflammation in asthma patients, using an experimental viral infection model [49]. Human model and bronchial biopsy raise ethical concerns and are difficult; however, further investigation is required to clarify the anti-inflammatory action of tiotropium in asthma.

In conclusion, our data demonstrate that tiotropium attenuates ILC2-dependent airway inflammation by the basophil-IL-4-ILC2 axis, and we propose novel anti-

inflammatory effects of tiotropium on innate-type airway inflammation. The findings improve our understanding of the complex mechanisms of asthma exacerbation, which may lead to future therapeutic strategies targeting these mechanisms.

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FIGURE LEGENDS

Figure 1. Tiotropium bromide attenuates airway eosinophilic inflammation in a papain-induced model. A, Experiment protocol: Airway inflammation was induced by the administration of papain for 3 consecutive days. TIO or vehicle was administered intranasally 60 min before the administration of papain. B, C, The numbers of total cells, eosinophils (B), and the production of IL-5 and IL-13 (C) in BALF were reduced by the administration of TIO. D, The histology of the airway was examined by hematoxylin and eosin (x 200 or x 400), and Alcian blue/periodic acid-Schiff (PAS) staining (x 400). Scale bar: 100 μm. E, Semiquantitative analysis of peribronchial inflammation and the abundance of PAS-positive mucus-containing cells. Total lung inflammation and PAS-positive cells were defined as the average of the scores as described in the supporting information. F, G, TIO did not reduce the release and expression of epithelial-derived cytokines (IL-33, TSLP) in the lungs (F) and BALF (G). Lungs were collected 3 hours, and BALF was collected 3, 12 and 24 hours after the first papain administration. IL-33 and TSLP expression and production were measured by Real-time PCR (F) and ELISA (G). Mean ± SEM.: n = 4–7 for each group. *$p < 0.05$ measured by Dunnett analysis in (B), (C), (E) and (F), and Tukey analysis in (G). Data are representatives of three independent experiments. i.n. = intranasal, TIO = Tiotropium bromide.

Figure 2. Tiotropium bromide attenuates airway eosinophilic inflammation in the mouse IL-33-induced model. A, Experiment protocol: Airway inflammation was induced by the administration of mouse IL-33 for 3 consecutive days. TIO or vehicle

was administered intranasally 60 min before the administration of mouse IL-33. B, C, The numbers of total cells, eosinophils (B), and the production of IL-5 and IL-13 (C) in BALF were reduced by the administration of TIO. Mean ± SEM.: n = 4–6 for each group. *p < 0.05 measured by Dunnett analysis. Data are representatives of three independent experiments. i.n. = intranasal, TIO = Tiotropium bromide.

Figure 3. Tiotropium bromide reduces ILC2 numbers and type-2 cytokine production from ILC2s. The administration of TIO reduced the frequencies and numbers of ILC2s in BALF (A) and lungs (B), and the percentage of IL-5$^+$ and IL-13$^+$ lung ILC2 (C) in the papain-induced model (n = 4–6 for each group), and the frequencies and numbers of ILC2s in BALF (D) and lungs (E) in the mouse IL-33-induced model (n = 4–5 for each group) as measured by flow cytometry. Mean ± SEM.: n = 4–6 for each group. *p < 0.05 measured by Dunnett analysis. Data are representatives of three independent experiments. TIO = Tiotropium bromide.

Figure 4. Tiotropium bromide inhibits cytokine production from ILC2s indirectly. Isolated ILC2s were stimulated with mouse IL-33 (10 ng/ml) with or without TIO (1 to 50 μM) for 24 hours. A, The morphologies of purified ILC2s were examined by Diff-Quik staining (x 400). Scale bar: 25 μm. B, The production of IL-5 and IL-13 in the culture supernatant was not reduced by the administration of TIO as measured by ELISA. C, D, Basophils predominantly expressed M3R mRNA in a heat-map presentation (C) and in FPKM values (fragments per kilobase of transcript per million mapped reads) of

expressed muscarinic receptor genes (D). E, The expression of M3R was higher on basophils than on ILC2s as measured by western blot analysis. TGMBE-02-3 cell, a mouse tracheal epithelial cell line was used as positive control. Murine ILC2s were isolated from lungs in (A), (B) and (E), from mesentery in (C), (D). Mean ± SEM.: n = 4 for each group. *$p < 0.05$ measured by Dunnett analysis. Results in (A) and (B) are representatives of three independent experiments, and results in (C), (D) and (E) are representatives of two independent experiments. TIO = Tiotropium bromide, DC = Dendritic cells.

Figure 5. Tiotropium bromide reduces basophil-derived IL-4 production. A, There is no difference in the frequencies and numbers of basophils in the presence versus the absence of TIO. B, C, TIO reduced the IL-4 production in BALF as measured by ELISA (B) and the percentage of IL-4^+ lung basophils (C) as determined by flow cytometry. Isolated basophils were stimulated with mouse IL-33 (10 ng/ml) with or without TIO (10 to 50 μM) for 24 hours. D, The IL-4 production in the culture supernatant was reduced by the administration of TIO as measured by ELISA. The co-cultures of basophils and ILC2s were stimulated with mouse IL-33 (1 ng/ml) in the presence or absence of tiotropium (10 to 50 μM). E, In the co-cultures, the production of IL-4, IL-5 and IL-13 in the culture supernatant was reduced by the administration of TIO as measured by ELISA. Mean ± SEM.: n = 4–6 for each group. *$p < 0.05$ measured by Dunnett analysis in (A)–(D), and Tukey analysis in (E). Data are representatives of three independent experiments. TIO = Tiotropium bromide.

Figure 6. Tiotropium bromide reduces IL-4 production from human basophils. Basophils isolated from peripheral blood mononuclear cells were stimulated with human IL-33 (10 ng/ml) with or without TIO (10 to 50 μM) for 24 hours. A, Human basophils expressed M3R in flow cytometric analysis. B, TIO reduced the IL-4 production derived from human basophils as measured by ELISA. Mean ± SEM.: n = 7 for each group. *p < 0.05 measured by Dunnett analysis. Data are representatives of three independent experiments. TIO = Tiotropium bromide.

Figure 1

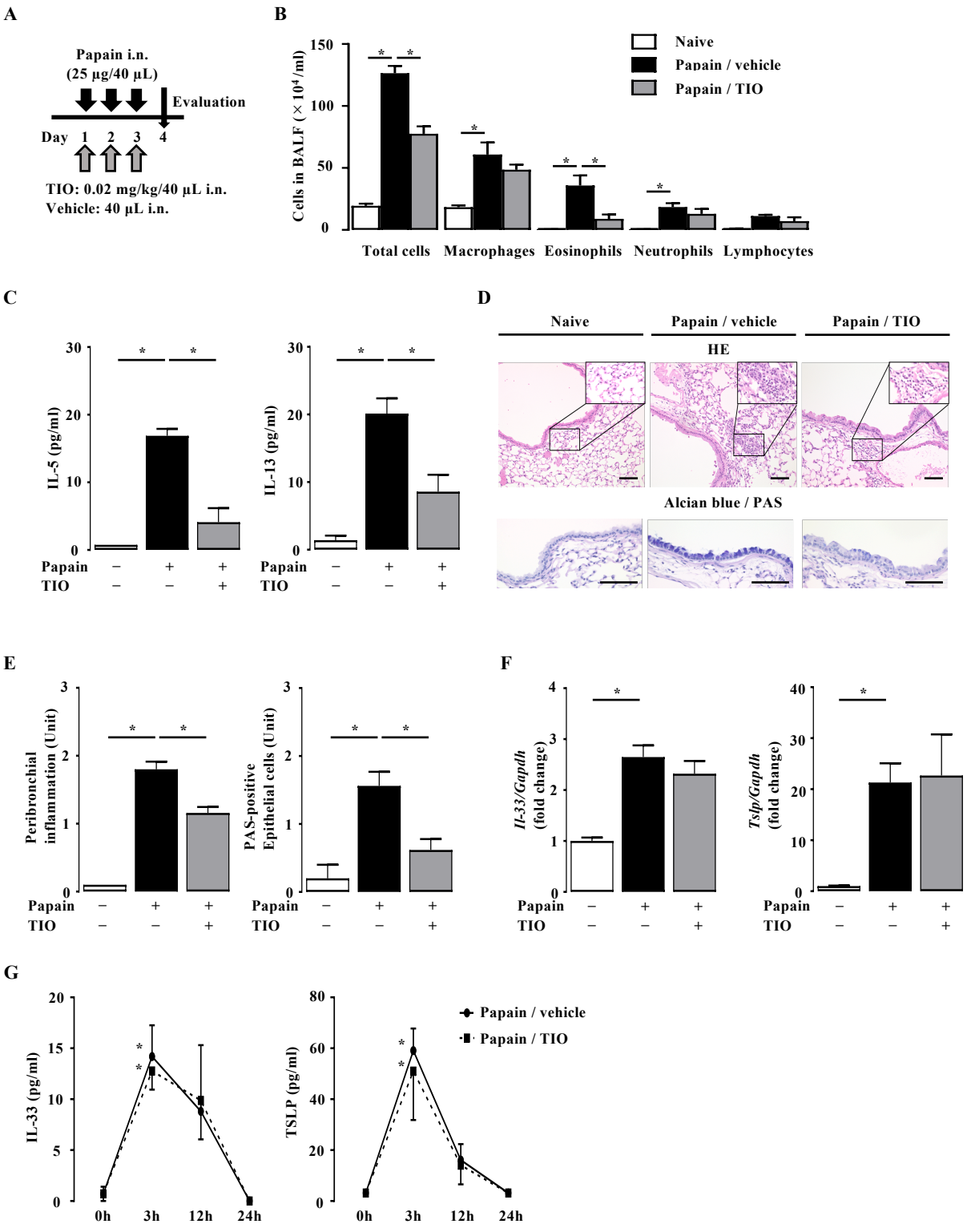

A
Papain i.n.
(25 μg/40 μL)
Evaluation
Day 1 2 3 4
TIO: 0.02 mg/kg/40 μL i.n.
Vehicle: 40 μL i.n.
B
Naive
Papain / vehicle
Papain / TIO
Cells in BALF (×10⁴/ml)
Total cells
Macrophages
Eosinophils
Neutrophils
Lymphocytes
C
IL-5 (pg/ml)
IL-13 (pg/ml)
Papain
TIO
D
Naive
Papain / vehicle
Papain / TIO
HE
Alcian blue / PAS
E
Peribronchial inflammation (Unit)
PAS-positive Epithelial cells (Unit)
F
Il-33/Gapdh (fold change)
Tslp/Gapdh (fold change)
G
IL-33 (pg/ml)
TSLP (pg/ml)
Papain / vehicle
Papain / TIO
0h
3h
12h
24h

Figure 2

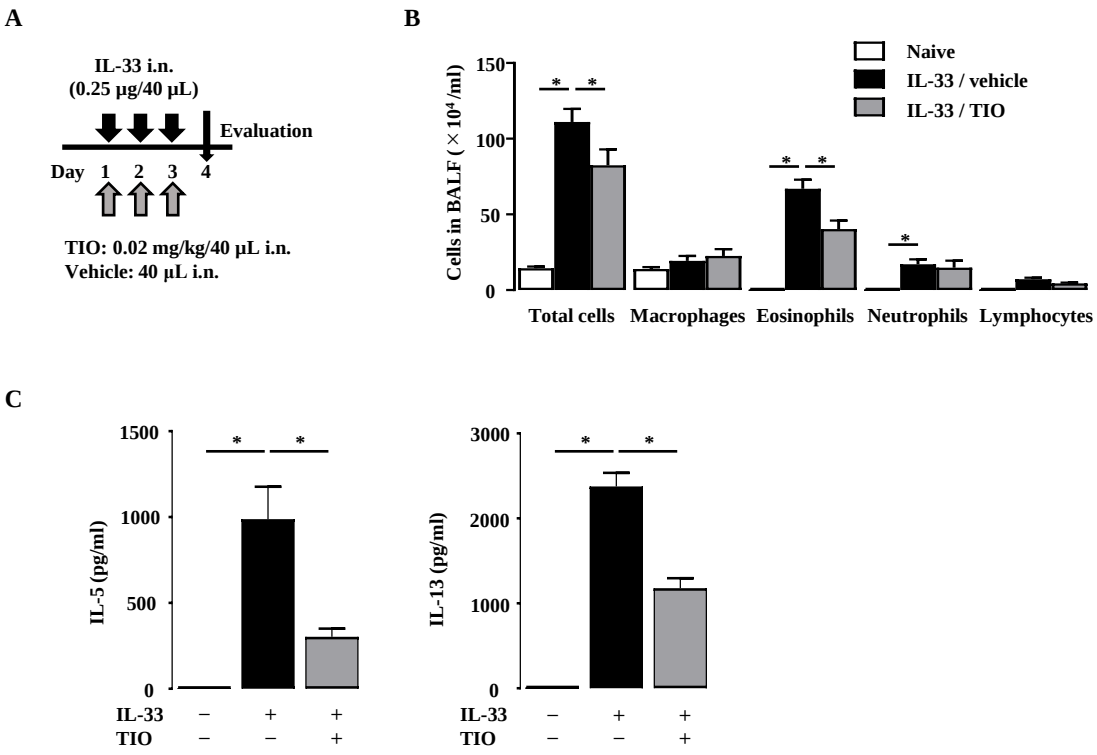

A
IL-33 i.n.
(0.25 μg/40 μL)
Evaluation
Day 1 2 3 4
TIO: 0.02 mg/kg/40 μL i.n.
Vehicle: 40 μL i.n.
B
Naive
IL-33 / vehicle
IL-33 / TIO
Cells in BALF (×10⁴/ml)
150
100
50
0
Total cells
Macrophages
Eosinophils
Neutrophils
Lymphocytes
C
IL-5 (pg/ml)
1500
1000
500
0
IL-13 (pg/ml)
3000
2000
1000
0
IL-33 − + +
TIO − − +

Figure 3

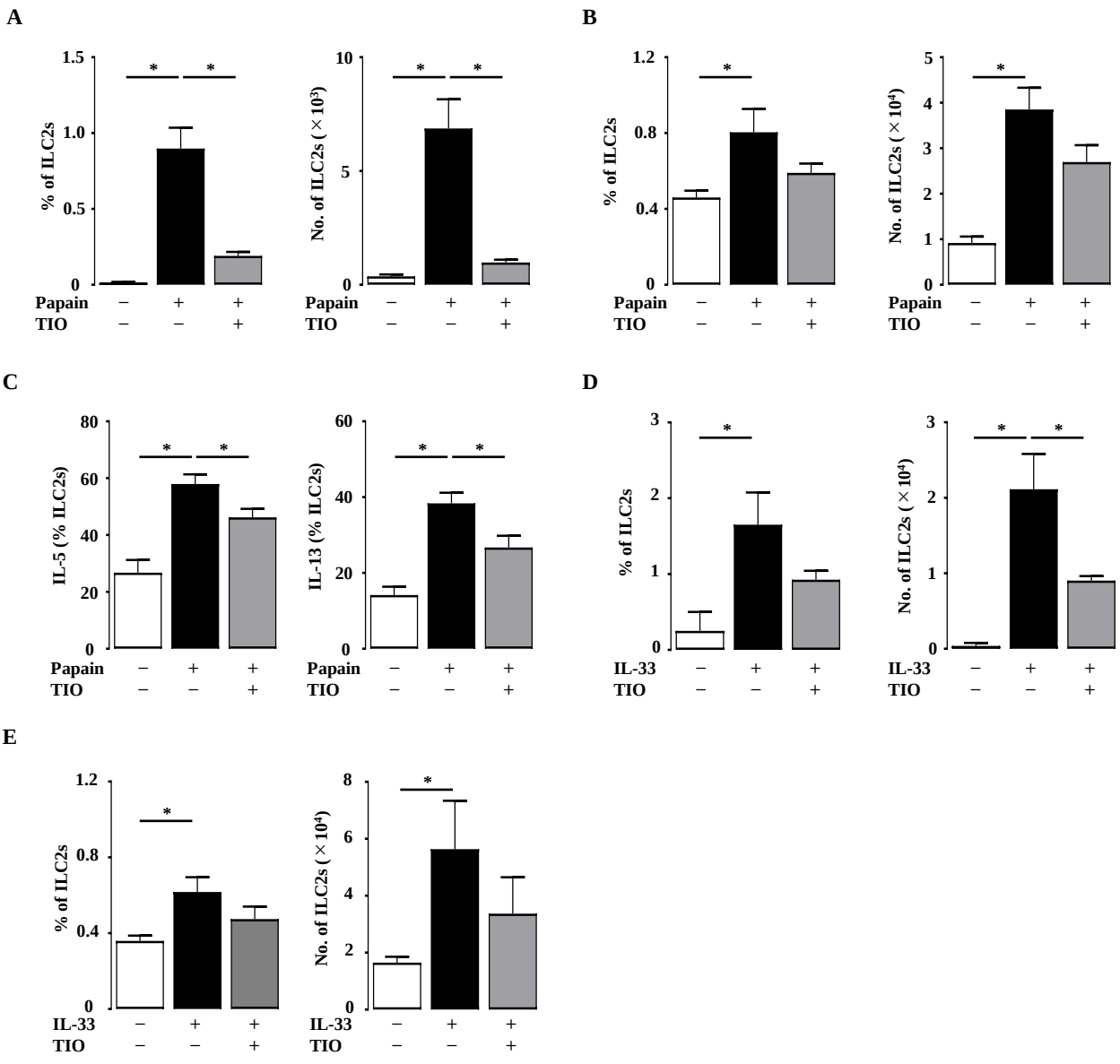

A
% of ILC2s
No. of ILC2s (×10³)
Papain
TIO
B
% of ILC2s
No. of ILC2s (×10⁴)
Papain
TIO
C
IL-5 (% ILC2s)
IL-13 (% ILC2s)
Papain
TIO
D
% of ILC2s
No. of ILC2s (×10⁴)
IL-33
TIO
E
% of ILC2s
No. of ILC2s (×10⁴)
IL-33
TIO

Figure 4

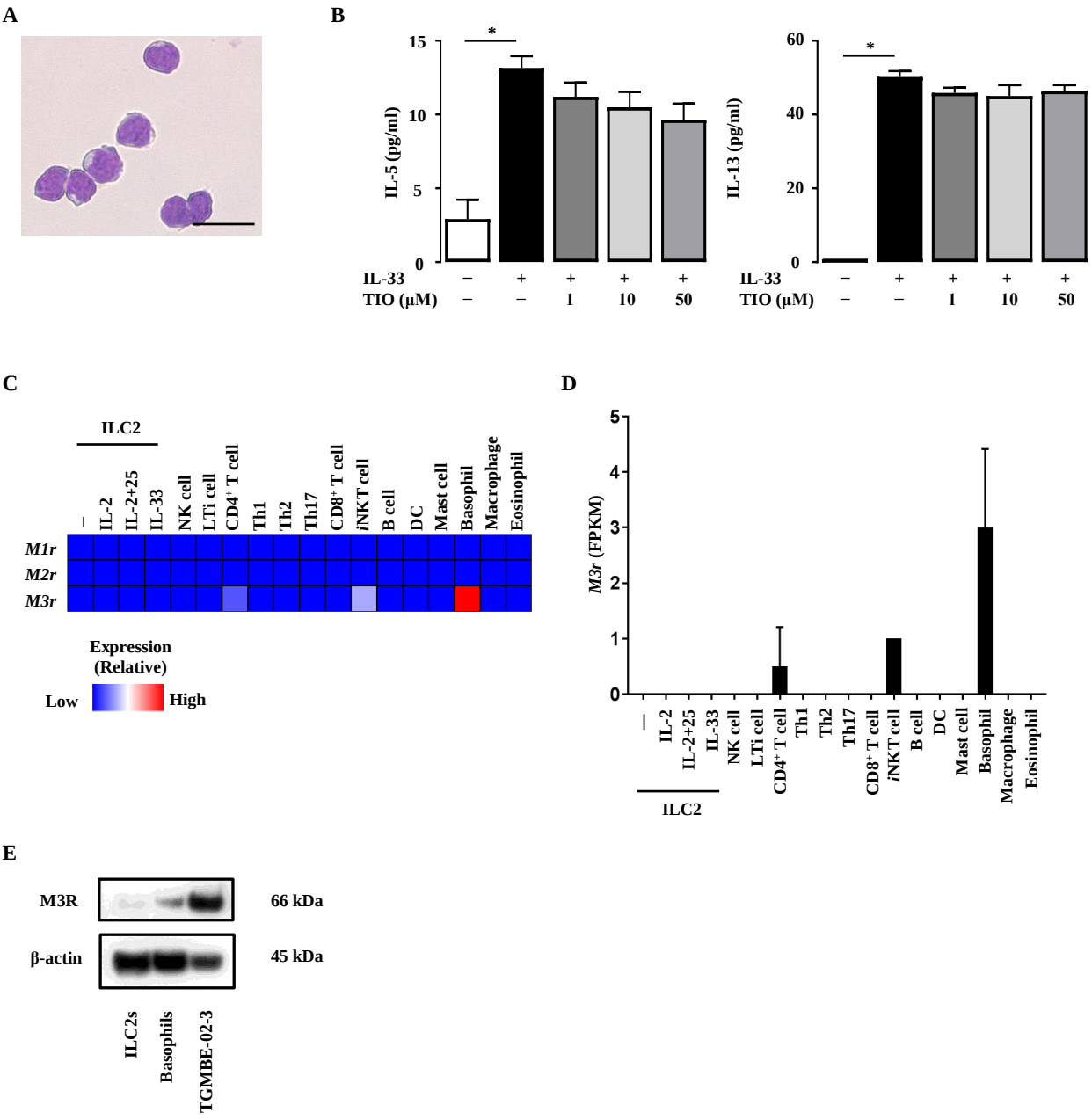

A
B
IL-5 (pg/ml)
IL-13 (pg/ml)
IL-33
TIO (μM)
C
ILC2
M1r
M2r
M3r
Expression (Relative)
Low
High
D
M3r (FPKM)
ILC2
E
M3R
β-actin
66 kDa
45 kDa
ILC2s
Basophils
TGMBE-02-3

Figure 5

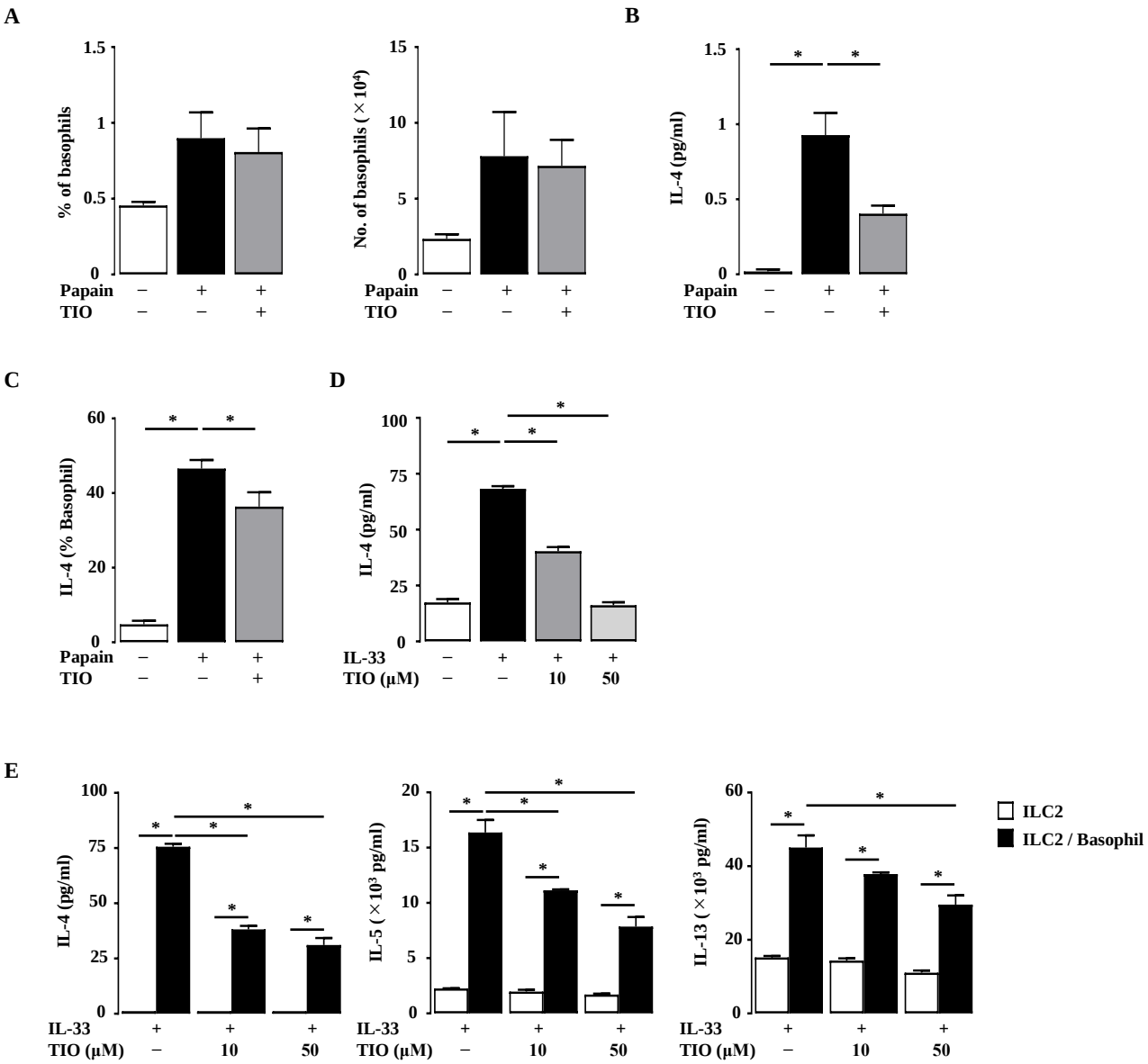
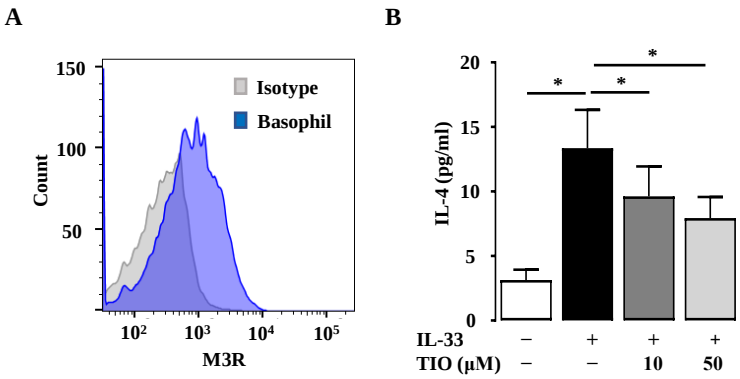

A
% of basophils
1.5
1
0.5
0
Papain – + +
TIO – – +
No. of basophils (×10⁴)
15
10
5
0
Papain – + +
TIO – – +
B
IL-4 (pg/ml)
1.5
1
0.5
0
*
*
Papain – + +
TIO – – +
C
IL-4 (% Basophil)
60
40
20
0
*
*
Papain – + +
TIO – – +
D
IL-4 (pg/ml)
100
75
50
25
0
*
*
*
IL-33 – + + +
TIO (μM) – – 10 50
E
IL-4 (pg/ml)
100
75
50
25
0
*
*
*
*
IL-33 + + +
TIO (μM) – 10 50
IL-5 (×10³ pg/ml)
20
15
10
5
0
*
*
*
*
IL-33 + + +
TIO (μM) – 10 50
IL-13 (×10³ pg/ml)
60
40
20
0
*
*
*
IL-33 + + +
TIO (μM) – 10 50
ILC2
ILC2 / Basophil

Figure 6


A
B
150
100
50
Count
Isotype
Basophil
10^2
10^3
10^4
10^5
M3R
20
15
10
5
0
IL-4 (pg/ml)
*
*
*
IL-33
TIO (μM)
–
+
+
+
–
–
10
50

Supporting information

Long-acting muscarinic antagonist regulates group 2 innate lymphoid cell-dependent airway eosinophilic inflammation

Takahiro Matsuyama, Kentaro Machida, Yasutaka Motomura, Koichi Takagi, Yoichi Doutake, Asako Tanoue-Hamu, Kiyotaka Kondo, Keiko Mizuno, Kazuyo Moro, Hiromasa Inoue

METHODS

Mouse IL-33-induced airway inflammation and glycopyrrolate treatment in papain-induced airway inflammation

For the administration of mouse IL-33, mice were treated with 0.5 μg mouse IL-33 (BioLegend, San Diego, CA, USA) in 40 μl PBS intranasally 60 min prior to papain treatment for 3 consecutive days and sacrificed 24 hours after the last administration. Bronchoalveolar lavage fluids (BALF) and lung tissues were collected and stained with Diff-Quik as previously described [1]. For glycopyrrolate treatment, 3 nmol/kg of glycopyrrolate (Cayman Chemical, Ann Arbor, MI, USA) in 40 μl PBS was administered intranasally 60 min prior to each papain administration.

Cell Culture, treatment

A mouse tracheal epithelial cell line, TGMBE-02-3, was cultured as previously described [2]. Briefly, the cells were cultured in a DMEM/F12 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% FBS (Gibco), 1% ITES (2 μg/ml insulin, 2 μg/ml transferrin, 122 ng/ml ethanolamine, and 9.14 ng/ml sodium selenite) (Wako, Osaka, Japan), and 10 ng/ml murine EGF (Wako). TGMBE-02-3 cells were incubated for 3 hours with papain (5 μM) in the presence or absence of tiotropium (0.1 to 10 μM) (Cayman Chemical). Cell lysates were collected.

Histological assessment

Lungs were fixed with 10% formalin, and tissue sections were stained with hematoxylin and eosin (HE) and Alcian blue/periodic acid-Schiff (PAS) to determine the presence of cellular infiltration and mucin glycoconjugates as previously described [1,3]. The severity of peribronchial inflammation was graded semiquantitatively for the following features: 0, normal; 1, few cells; 2, a ring of inflammatory cells one cell layer deep; 3, a

ring of inflammatory cells two to four cells deep; 4, a ring of inflammatory cells of more than four cells deep. The numerical scores for the abundance of PAS-positive mucus-containing cells in each airway were determined as follows: 0, >0.5% PAS-positive cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; 4, >75% [4]

Immunohistochemistry was carried out with a VECTASTAIN Universal Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. Lung sections were stained with Anti-muscarinic acetylcholine receptor M3/CHRM3 antibody (ab126168; Abcam, Cambridge, UK) or rabbit IgG, polyclonal-isotype control (ab37415; Abcam) as negative control.

Enzyme-linked immunosorbent assays (ELISA)

Cytokines in BALF and culture supernatant were measured with Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) or QuickPlex (Meso Scale Discovery, Rockville, MD, USA), and Histamine ELISA kit (Enzo Life Sciences, INC., Farmingdale, NY, USA) according to the manufacturer's protocol.

Antibodies

PerCP-Cy5.5 Mouse Lineage Antibody Cocktail with Isotype Control (CD3ε, CD11b, CD45RA/B220, Ly-76, Ly-6G/Ly-6C) (145-2C11, M1/70, RA3-6B2, TER-119, RB6-8C5), PE and BV421 anti-mouse T1/ST2 (U29-93), BV650 anti-mouse KLRG1 (2F1), FITC and BV786 anti-mouse Thy1.2 (30-H12), PE-Cy7 anti-mouse CD45.2 (104), biotin-conjugated anti-CD11c (HL3), BV421 anti-mouse IL-4 (11B11), BV421-Rat IgG1κ Isotype Control (R3-34), and Fixable Viability Stain 780 for removing dead cells were purchased from BD biosciences (San Diego, CA, USA). APC-conjugated anti-human/mouse IL-5 (TRFK5), PE and APC Rat IgG1κ Isotype Control (RTK2071), APC-conjugated anti-mouse c-Kit (2B8), Biotin and PE-conjugated anti-mouse FcεRI (MAR-1), FITC-conjugated anti-

mouse CD49b (DX5), FITC-conjugated anti-mouse CD3 (17A2), Alexa Fluor R700-conjugated anti-mouse CD4 (GK1.5), FITC Mouse Lineage Antibody Cocktail with Isotype Control (CD3ε, CD11b, CD45RA/B220, Ly-76, Ly-6G/Ly-6C) (145-2C11, M1/70, RA3-6B2, Ter-119, RB6-8C5), biotin-conjugated anti-CD4 (GK1.5), anti-CD5 (53-7.3), anti-CD8a (53-6.7), anti-CD19 (1D3), anti-F4/80 (BM8), anti-TER119 (TER-119), and propidium iodide (PI) for removing dead cells were from BioLegend. PE-conjugated anti-human/mouse IL-13 (eBio13A) was from eBioscience (La Jolla, CA, USA). Biotin-conjugated anti-CD3ε (145-2C11), anti-NK1.1 (PK136), and anti-Ly-6G (RB6-8C5) were from TONBO Biosciences (San Diego, CA, USA). Anti-muscarinic acetylcholine receptor M3/CHRM3 antibody (ab126168), rabbit IgG, polyclonal-isotype control (ab37415), and goat anti-rabbit IgG H&L (Allophycocyanin) preadsorbed (ab130805) were from Abcam. In this study, biotin-conjugated antibody mixtures for lineage markers (CD3ε, CD4, CD5, CD8α, CD11c, F4/80, CD19, NK1.1, Gr-1, TER119 and FcεRI) were used for cell sorting.

Intracellular flow cytometric analyses

For analysis of the intracellular cytokine expression of ILC2s, $CD4^+$ T cells, and basophils, isolated lung cells were stimulated at 37°C with Cell Activation Cocktail with Brefeldin A (BioLegend) for 3 to 4 hours. Cells were fixed and permeabilized with a BD Cytofix/Cytoperm Kit (BD Biosciences) and stained intracellularly with APC-conjugated anti-human/mouse IL-5, PE-conjugated anti-human/mouse IL-13, and BV421 anti-mouse IL-4. Dead cells were stained with Fixable Viability Stain 780 before fixation/permeabilization.

In vitro stimulation of ILC2s and basophils

Purified ILC2s by cell sorting were seeded at a density of 5,000 cells per well in 96-well round bottom plates in 200 μl RPMI complete medium (RPMI1640 medium (Sigma-

Aldrich, St Louis, MO, USA) containing 10% FCS (Gibco), 10 mM HEPES buffer (Sigma-Aldrich), 1 x MEM non-essential amino acids (Sigma-Aldrich), 100 U/ml penicillin-streptomycin (Wako), and 50 μM 2-mercaptoethanol (Gibco), and 1 mM sodium pyruvate (Gibco)) were stimulated with mouse IL-33 (10 ng/ml) in the presence or absence of tiotropium (1 to 50 μM) at 37°C for 24 hours. To isolate purified basophils, bone marrow cells were cultured in the presence of mouse IL-3 (2 ng/ml) (Wako) for 10 to 14 days, and then basophils were isolated as a fraction of c-Kit$^-$ FcεRI$^+$ cells by cell sorter. The purity of basophils was > 97%. Basophils were seeded at a density of 1 x 10^5 cells per well in 96-well round plates in 200 μl supplemented RPMI 1640 culture medium (RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin-streptomycin, 50 μM 2-mercaptoethanol, and 1 mM sodium pyruvate). Basophils were also stimulated with mouse IL-3 (2 ng/ml) and mouse IL-33 (10 ng/ml) in the presence or absence of tiotropium (10 to 50 μM) at 37°C for 24 hours.

Western blot analyses

Cytoplasmic extracts were prepared with Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA). Protein lysates (5 μg) were separated by SuperSep 7.5% gels (Wako), and incubated with Anti-muscarinic acetylcholine receptor M3/CHRM3 antibody (1:1000 dilution; ab126168; Abcam), and β-Actin Mouse mAb (1:5000 dilution; 8H10D10; Cell Signaling Technology) as primary antibodies at 4°C overnight. The membrane was washed and then incubated with secondary HRP-conjugated antibodies (GE Healthcare, Chicago, IL, USA). Immunoblotting was performed in accordance with the manufacturer's instructions. These assays were carried out as previously described [5].

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from lungs by using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), and cDNA was synthesized with High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). Quantitative PCR was performed with TaqMan PCR by using TaqMan Universal PCR Master Mix and StepOnePlus Real-Time PCR System (Life Technologies). The specific primers (Applied Biosystems, Foster City, CA, USA) used in this study are listed in Table S2.

RNA sequencing

ILC2s were sorted from mesentery of naïve mice. Cytokines (IL-2, IL-25 and IL-33) were added to the medium at a final concentration of 10 ng/ml for 48 hours. $CD4^+$ T cells, $CD8^+$ T cells, B cells, Th1 cells, Th2 cells, Th17 cells, NK cells, iNKT cells and eosinophils were sorted from spleen of naïve mice. LTi cells was sorted from fetal liver of naïve mice. Dendritic cells, mast cells, basophils, and macrophages were sorted from bone marrow of naïve mice [6]. Total RNA was isolated with Isogen (Nippon Gene, Toyama, Japan) or Trizol LS (Thermo Fisher Scientific, Waltham, MA, USA), and cDNA libraries were prepared with the TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA) according to the manufacturer's "low sample" protocol. A HiSeq 1500 system (Illumina) was used for 50 single-end-base sequencing. Sequenced reads were trimmed for adaptor sequences and were masked for low-complexity or low-quality sequences, and were then mapped to the reference genome (mm9 assembly of the mouse genome) with Bowtie2 software, version 2.1.0, and TopHat2 software, version 2.0.8. The abundance of transcripts was estimated as FPKM values (fragments per kilobase of exon million fragments mapped) with Cufflinks software, version 2.1.1. Heat maps were produced by the microarray data-analysis tool MeV (http://mev.tm4.org/).

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Supplemental Table S1

Table S1. A list of antibody sources

Antigen	Clone	Label	Source
Lineage cocktail (CD3ε, CD11b, CD45RA/B220, Ly-76, Ly-6G/Ly-6C)	145-2C11, M1/70, RA3-6B2, TER-119, RB6-8C5	PerCP-Cy5.5 FITC	BD bioscience Biolegend
T1/ST2	U29-93	BV421 PE	BD bioscience
KLRG1	2F1	BV650	BD bioscience
Thy1.2	30-H12	BV786 FITC	BD bioscience BioLegend
CD45.2	104	PE-Cy7	BD bioscience
FcεRI	MAR-1	PE Biotin	BioLegend
CD49b	DX5	FITC	BioLegend
c-Kit	2B8	APC	BioLegend
NK1.1	PK136	Biotin	TONBO Biosciences
Ly-6G/Ly-6C	RB6-8C5	Biotin	TONBO Biosciences
TER-119	TER-119	Biotin	BioLegend
F4/80	BM8	Biotin	BioLegend
CD3ε	145-2C11	Biotin	TONBO Biosciences
CD4	GK1.5	Alexa Fluor R700 Biotin	BioLegend
CD5	53-7.3	Biotin	BioLegend
CD8α	53-6.7	Biotin	BioLegend
CD11c	HL3	Biotin	BD bioscience
CD19	1D3	Biotin	BioLegend
IL-4	11B11	BV421	BD bioscience
IL-5	TRFK5	APC	BioLegend
IL-13	eBio13A	PE	eBioscience
streptavidin	-	APC	BD bioscience
Streptavidin MicroBeads	-	-	Miltenyi Biotec
M3R	-	unconjugated	Abcam

Supplemental Table S2

Table S2. A list of primers used in quantitative RT-PCR

Primer	Assay ID; Applied Biosystems
Mouse *Gapdh*	Mn99999915_g1
Mouse *Il-33*	Mn00505403_m1
Mouse *Tslp*	Mn01157588_m1

SUPPLEMENTARY FIGURES

Supplemental Figure S1. Tiotropium bromide attenuates lung eosinophilic inflammation in papain- and mouse IL-33-induced model. The numbers of eosinophils in lung cells were reduced by the administration of tiotropium bromide in papain-induced (A) and mouse IL-33-induced model (B). Mean ± SEM.: n = 5 for each group. *$p < 0.05$ measured by Dunnett analysis. Data are representatives of two independent experiments. i.n. = intranasal, TIO = Tiotropium bromide.

Supplemental Figure S2. Glycopyrrolate attenuates airway eosinophilic inflammation in the papain-induced model. A, Experiment protocol: Airway inflammation was induced by the administration of papain for 3 consecutive days. Glyco or vehicle was administered intranasally 60 min before the administration of papain. B, C, The numbers of total cells, eosinophils (B), and the production of IL-5 and IL-13 (C) in BALF were reduced by treatment with Glyco as measured by ELISA. Mean ± SEM.: n = 5–6 for each group. *$p < 0.05$ measured by Dunnett analysis. Data are representatives of three independent experiments. i.n. = intranasal. Glyco = Glycopyrrolate.

Supplemental Figure S3. Immunohistochemical analysis of M3R expression in lungs in papain-induced model. Representative images of M3R immunostaining were shown in airway epithelium and airway smooth muscle (x 400). No staining is detected in samples stained with isotype control as negative control. Scale bar: 100 μm.

Supplemental Figure S4. Tiotropium bromide has no effect on the upregulation of IL-33 expression in airway epithelial cells exposed to papain. TGMBE-02-3 cells were cultured with papain (5 μM) in the presence or absence of TIO (0.1 to 10 μM) for 3 hours. IL-33 mRNA expression was measured by Real-time PCR. Mean ± SEM.: n = 4

for each group. *p < 0.05 measured by Dunnett analysis. Data are representatives of two independent experiments. TIO = Tiotropium bromide.

Supplemental Figure S5. Gating strategy for (A) ILC2s and (B) basophils in flow cytometry. Mouse lung cells were stained with indicated antibodies and examined by flow cytometry. FSC^{low} SSC^{low} gates were used for the detection of ILC2s and basophils. A. ILC2s were identified as Lin^- $Thy1.2^+$ $T1/ST2^+$ $KLRG1^+$ $CD45.2^+$ cells. B. Basophils were identified as $c\text{-}Kit^-$ $Fc\varepsilon RI^+$ $CD49b^+$ cells. For intracellular cytokine staining, positive staining was determined using isotype controls.

Supplemental Figure S6. Tiotropium bromide does not attenuate $CD4^+$ T cell numbers and the production of type-2 cytokines in the papain-induced model. There was no difference in the frequencies and numbers of $CD4^+$ T cells (A) and the type-2 cytokine-producing ability of $CD4^+$ T cells (B) between the presence and absence of TIO. Mean ± SEM.: n = 4–5 for each group. *p < 0.05 measured by Dunnett analysis. Data are representatives of three independent experiments. TIO = Tiotropium bromide.

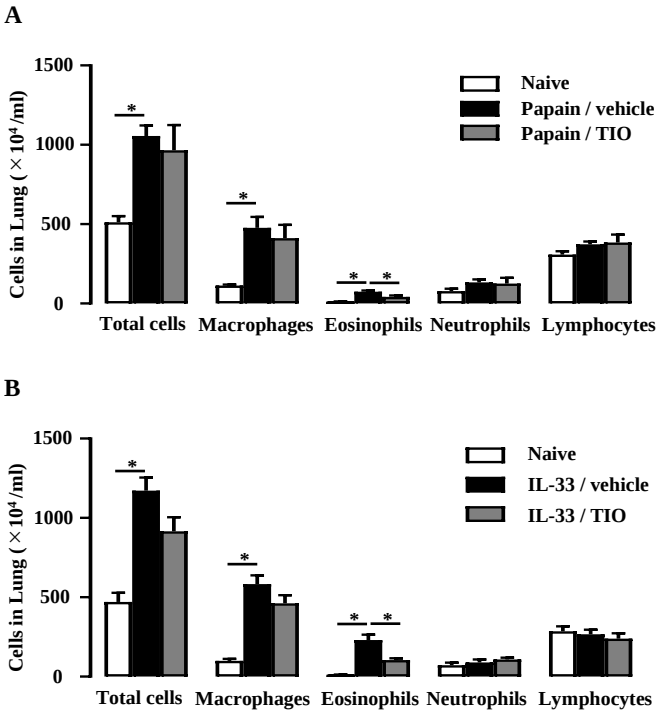
Supplemental Figure S7. Flow cytometric gating strategies for isolation and sorting of lung-derived ILC2s and bone-marrow-derived basophils. A, C, Representative gating strategy for sorting lung ILC2s (A) and bone-marrow-derived basophils (C). B, D, Purity of post-sorted lung ILC2s (B), and bone-marrow-derived basophils (D). FSC^{low} SSC^{low} gates were used for the detection of ILC2s and basophils. A, B, ILC2s were identified as Lin^- $Thy1.2^+$ $T1/ST2^+$ cells. C,D, Basophils were identified as $c\text{-}Kit^-$ $Fc\varepsilon RI^+$ cells.

Supplemental Figure S8. Tiotropium bromide does not reduce the production of IL-13 and histamine from basophils. The production of IL-13 (A) and histamine (B) in the culture supernatant was measured by ELISA. Mean ± SEM.: n = 3–4 for each group. *p < 0.05 measured by Dunnett analysis. Results are representatives of three independent experiments. TIO = Tiotropium bromide.

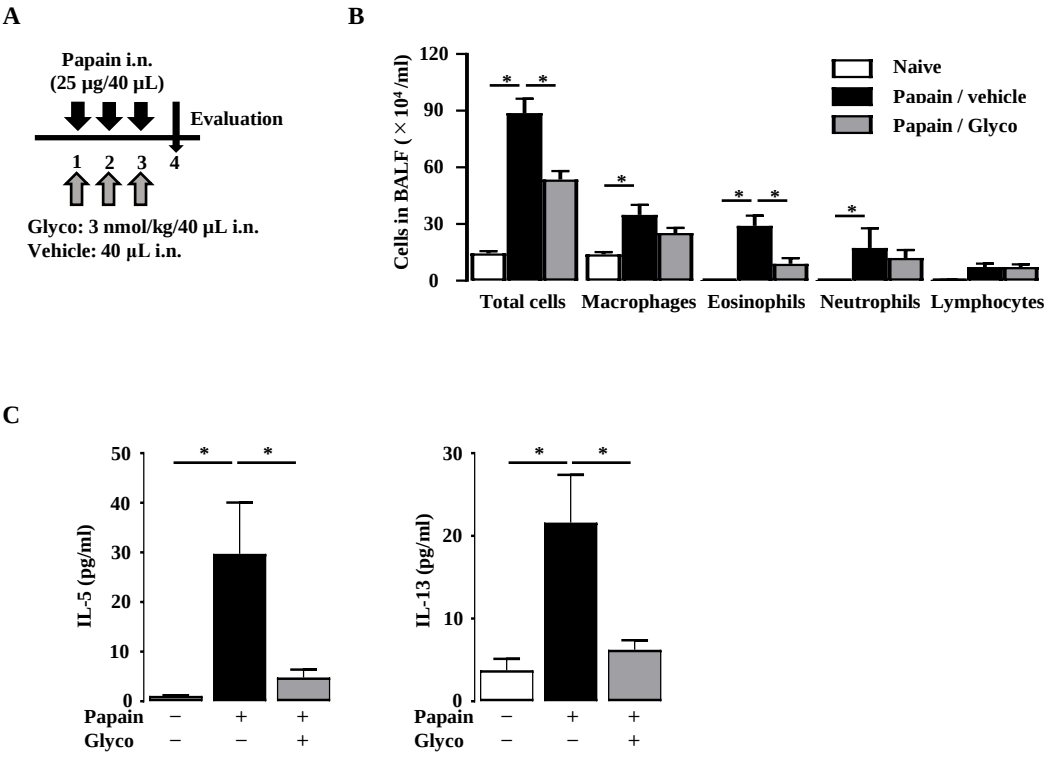
Supplemental Table S1: A list of antibody sources

Supplemental Table S2: A list of primers used in quantitative RT-PCR

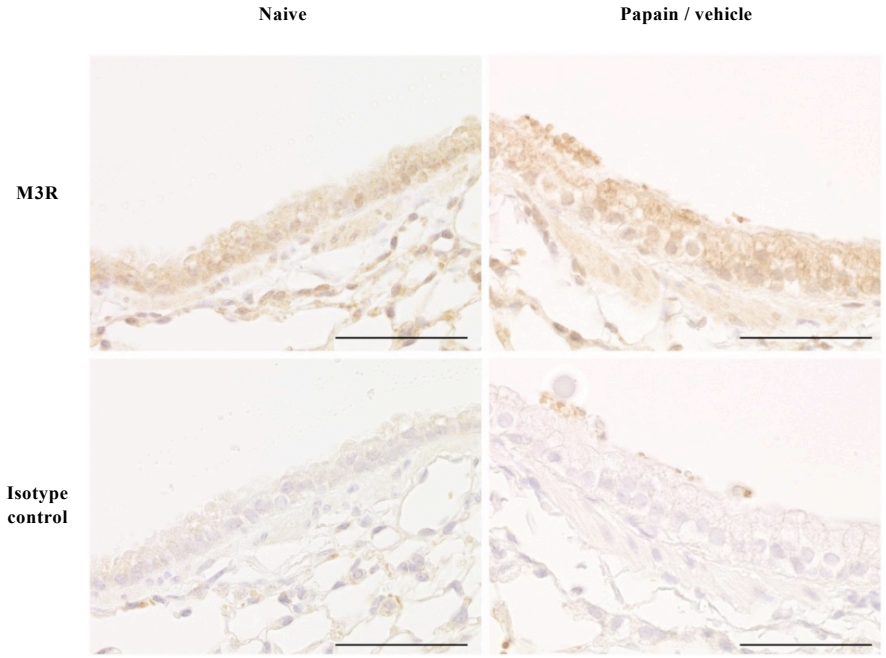
Supplemental Figure S1



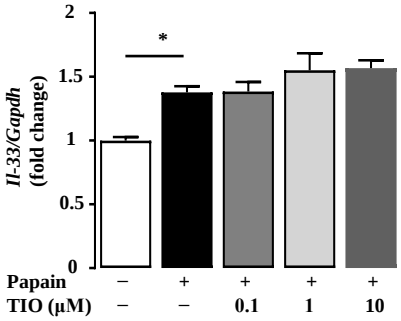
Supplemental Figure S2



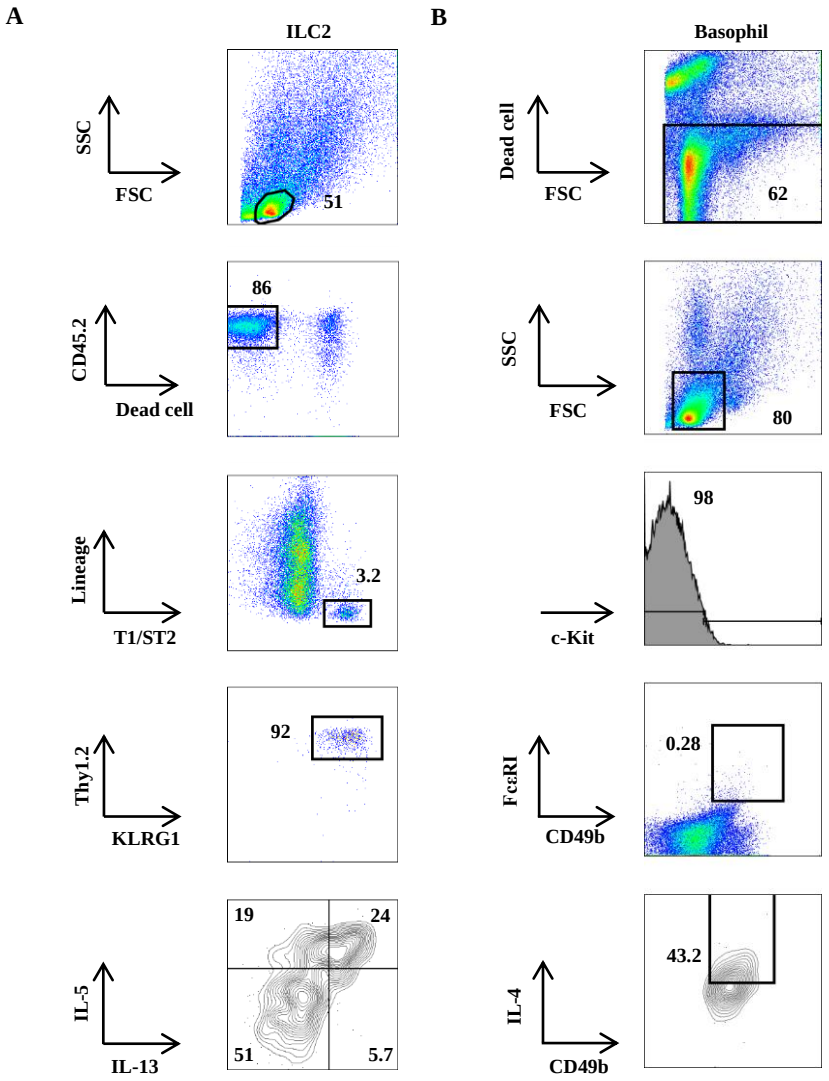
Supplemental Figure S3



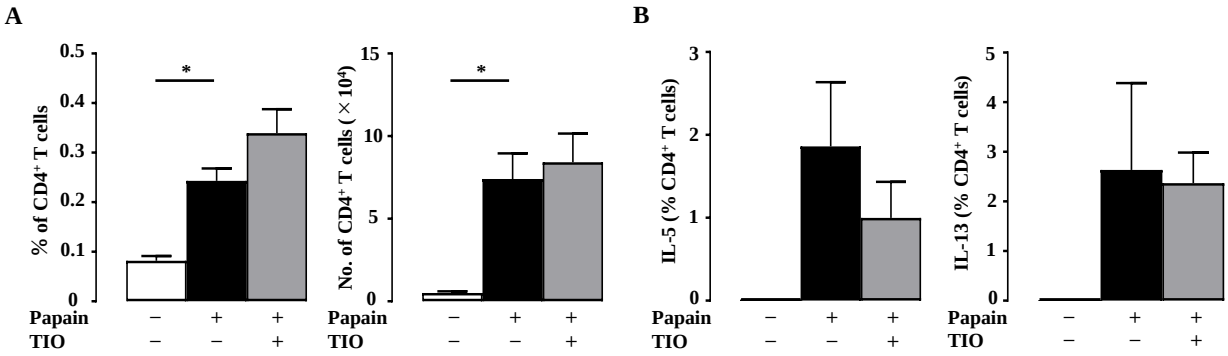
Supplemental Figure S4



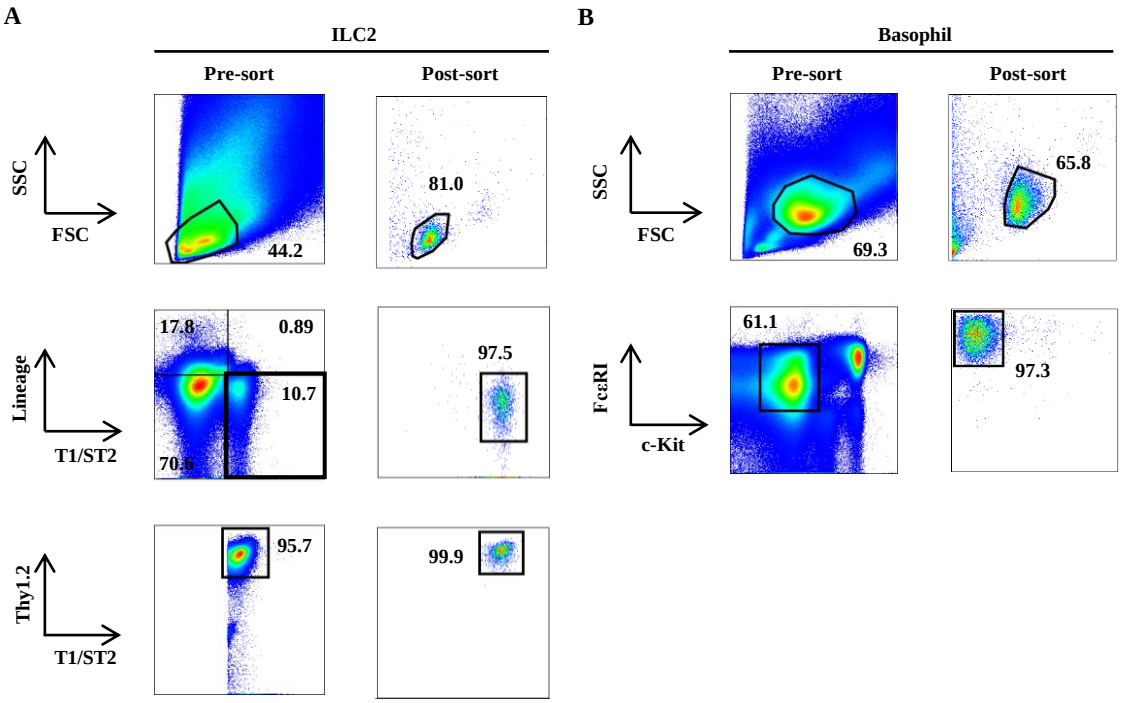
Supplemental Figure S5



Supplemental Figure S6



Supplemental Figure S7



Supplemental Figure S8