

Research paper

Cyclosporin A indirectly attenuates activation of group 2 innate lymphoid cells in papain-induced lung inflammation

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ARTICLE INFO

Keywords:

Cyclosporin A
ILC2
Th2 cytokine
Asthma

ABSTRACT

Cyclosporin A (CsA) is a well-known immunosuppressant that is used against steroid-resistant asthma. Group 2 innate lymphoid cells (ILC2s) and type 2 helper T (Th2) cells produce Th2 cytokines including IL-5 and play important roles in asthma pathogenesis. Here, we studied the effects of CsA in allergen-induced lung inflammation in mice and found that CsA decreased the number of lung ILC2s and attenuated papain-induced activation of ILC2s accompanied with IL-5 expression. The ILC2 suppression mediated by CsA was not observed in culture or in lymphocyte-deficient *Rag2*^{-/-} mice. Thus, we propose a new suppressive effect of CsA, i.e., administration of CsA indirectly suppresses maintenance and activation of lung ILC2s in addition to direct suppression of T-cell activation and cytokine production.

1. Introduction

Cyclosporin A (CsA) is a potent immunosuppressive drug that is used to prevent allograft rejection and to treat autoimmune diseases such as rheumatoid arthritis and psoriasis [1–3]. CsA primarily inhibits the activity of calcineurin and the calcineurin-dependent transcription factors, nuclear factor of activated T cells (NFATs) [4]. Through that inhibition, CsA blocks T cells' production of NFAT-dependent cytokines such as interleukin-2 (IL-2) and it suppresses the activation and functions of effector T cells including helper T (Th) cells [4,5].

Allergic asthma is characterized by airway hyperreactivity (AHR), pulmonary eosinophilia, and excessive mucus production. This response is induced by cytokines such as IL-4, IL-5, IL-9 and IL-13 produced by type 2 Th (Th2) cells and also by group 2 innate lymphoid cells (ILC2s) [6–8]. Administration of CsA to patients with severe steroid-resistant asthma has provided some improvement in lung function [9–11]. In addition to the suppression of T cells, recent studies have confirmed that calcineurin/NFAT inhibitors have a much broader effect and have shown that CsA targets innate immunity and the regulation of homeostasis of innate cells [5]. For example, CsA inhibits degranulation of eosinophils as well as IL-4 production by mast cells [12,13].

ILC2s produce a large amount of Th2 cytokines when stimulated with IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which is

derived from damaged epithelial cells and other cells [14]. In combination with those epithelial cell-derived cytokines, IL-2 derived from activated T cells is also important for full activation and expansion of ILC2s [15,16]. Recent progress has revealed critical roles of ILC2s for progression of asthma pathogenesis [6,17]; however, the effects of CsA on ILC2 function have not been addressed in detail and are not fully understood.

In addition to cytokines described above, the activation and maintenance of ILC2s are influenced by cell-to-cell signaling through surface receptors, such as inducible T cell costimulator (ICOS) [6]. ILC2s express both ICOS and ICOS-ligand (ICOSL), and the ICOS-ICOSL interaction augments survival and function of ILC2s [18,19]. Interaction of ICOSL on ILC2s with ICOS on Treg cells suppresses ILC2s together with IL-10 and TGF- β produced by Treg cells [20]. In contrast, ILC2s promote Treg cell accumulation via ICOS-ICOSL interaction in adipose tissues [21].

In this study, we employed mice in which IL-5 expression on live cells was monitored by Venus expression [22], and we found that activation and maintenance of ILC2s were suppressed by CsA in papain-induced lung inflammation. The suppression was not a direct effect of CsA on ILC2s, and it was not observed in lymphocyte-deficient *Rag2*^{-/-} mice. In spite of the decrease of Treg cells in the lungs of CsA-treated mice, IL-10 levels in BAL (bronchoalveolar lavage) fluids of CsA-treated mice were increased, suggesting that IL-10-producing cell other

Abbreviations: BAL, bronchoalveolar lavage; CsA, cyclosporin A; ICOS, inducible T cell costimulator; ICOSL, inducible T cell costimulator ligand; ILC2s, group 2 innate lymphoid cells; NFAT, nuclear factor of activated T-cells; Th2, type 2 helper T; TSLP, thymic stromal lymphopoietin

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<http://dx.doi.org/10.1016/j.cellimm.2017.10.010>

Received 24 April 2017; Received in revised form 17 September 2017; Accepted 26 October 2017
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than Treg cells might be involved in the machinery for ILC2 suppression. Our results may add a previously unrecognized mechanism by which CsA attenuates asthmatic inflammation. Thus, we suggest that CsA suppresses activation and cytokine expression not only from T cells but also simultaneously from ILC2s.

2. Materials and methods

2.1. Mice and reagents

IL5-Venus knock-in (*IL5^{+/Venus}*) mice [22] were crossed with *Rag2^{-/-}* mice to generate *IL5^{+/Venus} Rag2^{-/-}* mice on the C57BL/6 background. They were maintained under specific pathogen-free conditions, and mice at 9–17 weeks of age were used for all experiments. Mice were handled in accordance with the Guidelines for Animal Experiments of the Research Institute, National Center for Global Health and Medicine. Cyclosporin A (CsA) and papain were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. In vivo stimulation and lung cell preparation

Mice were injected subcutaneously with CsA dissolved in olive oil or vehicle alone at a dose of 20 mg/kg, 6 times a week for 2 weeks and on days 14, 15, and 16. Mice were anesthetized intraperitoneally with an anesthetic mixture (medetomidine 0.3 mg/kg, midazolam 4 mg/kg, and butorphanol 5 mg/kg), followed by intranasal injection of papain (50 µg) in 20 µL PBS on days 14, 15, and 16, and were analyzed on day 17. Lungs were minced and digested in HBSS containing type I collagenase (1 mg/mL; Sigma-Aldrich, St. Louis, MO) and DNase I (200 U/mL; Roche Applied Science, Mannheim, Germany) for 1 h at 37 °C. Tissue samples were next mashed through a 70 µm cell strainer and washed with HBSS supplemented with 10% FBS. Red blood cells were removed by incubation for 2 min at room temperature (RT) in ammonium chloride (ACK) lysis buffer.

2.3. BAL fluid

Lungs were lavaged twice with 0.5 mL PBS and the BAL fluid was centrifuged to pellet cells and supernatants were collected. Total protein was measured by Bradford's method with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

2.4. Cytokine measurements

The levels of IL-10 in BAL fluids were determined with a Bio-Plex Pro™ Mouse Cytokine 23-plex Assay (Bio-Rad Laboratories) in a Bio-Plex 3D System (Bio-Rad Laboratories) according to the manufacturer's instructions. Briefly, undiluted culture supernatant and standards were added to 96-well microplates containing washed beads and incubated at room temperature (RT) for 1 h with shaking. After washing, biotin-labeled antibody was added to the wells. After 30 min of incubation at RT with shaking, streptavidin-phycoerythrin was added followed by 10 min of incubation with shaking. Data were collected with xPONENT for FLEXMAP 3D software, version 4.2 (Luminex Corporation, Austin, TX, USA) and the data were analyzed with Bio-Plex Manager 6.1 software (Luminex Corporation). The levels of IL-33 in BAL fluids were measured using mouse IL-33 ELISA Ready-SET-Go!® (eBioscience, San Diego, CA) according to the manufacturer's instructions.

2.5. Antibodies and flow cytometry

Single cell suspensions were preincubated with anti-CD16/32 (2.4G2, BD Biosciences) to block Fc receptors. Cells were stained with combinations of the following monoclonal antibodies conjugated with phycoerythrin: anti-CD25 (PC61; BioLegend, San Diego, CA), anti-

CD127 (SB/199; eBioscience, San Diego, CA), anti-CD275 (ICOSL) (HK5.3; BioLegend), anti-ST2 (DIH9; BioLegend), anti-Siglec-F (E50-2440; BD Biosciences, San Jose, CA), anti-TCRβ (H57-597; BioLegend), and anti-Gr-1 (RB6-8C5; eBioscience). The following allophycocyanin-conjugated antibodies were used: anti-CD25 (PC61; BioLegend), anti-CD127 (A7R34; BioLegend) and anti-CD278 (ICOS) (C398.4A; BioLegend). Phycoerythrin-Cy7-conjugated antibodies included anti-Thy1.2 (30-H12; BioLegend) and anti-CD11c (HL3; BD Biosciences). We also used allophycocyanin-Cy7-conjugated anti-CD11b (M1/70; BioLegend) as well as biotin-conjugated anti-CD3e (145-2C11; BioLegend), anti-CD4 (GK1.5; BioLegend), anti-CD8 (53-6.7; BioLegend), anti-CD11b (M1/70; BioLegend), anti-CD11c (N418; BioLegend), anti-CD19 (6D5; BioLegend), anti-B220 (RA3-6B2; BioLegend), anti-NK1.1 (PK136; BioLegend), anti-TCRγδ (GL3; BD Biosciences) and anti-TER-119 (TER-119; Tonbo Biosciences, San Diego, CA) followed by allophycocyanin-Cy7-conjugated streptavidin. Dead cells were excluded by staining with 2 µg/mL 7-aminoactinomycin D (7AAD) (Sigma-Aldrich, St. Louis, MO). Identification of ILC2s was based on the expression of Thy1.2 and CD25, and the absence of lineage markers (Lin⁻) defined below. Eosinophils were identified as CD45⁺, Siglec-F⁺, and CD11c⁻ cells. ILC2 sorting was performed with a FACSAria III (BD Biosciences), and phenotypic analysis was performed using a BD FACSCanto II (BD Biosciences). Data were analyzed using FlowJo (Tree Star, Inc. San Carlos, CA).

2.6. ILC2 culture

ILC2s were sorted from lung cell suspensions and cultured for 19 h in the presence or absence of CsA (0.2 µg/mL) in complete RPMI media supplemented with 10% FBS and 50 µM β-mercaptoethanol at a density of 1.6×10^3 in 100 µL medium. Lung ILC2s were stimulated with IL-33 (10 ng/mL; R & D Systems, Minneapolis, MN) and IL-7 (10 ng/mL; PeproTech, Rocky Hill, NJ). After incubation, cells were stained with allophycocyanin-conjugated anti-CD25. Cells were washed, resuspended in 7AAD and analyzed using a BD FACSCanto II.

2.7. Quantitative RT-PCR analysis

Total lung cells were isolated and total RNA was prepared using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. cDNA was synthesized using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using TaqMan Fast Universal PCR Master Mix and primer/probe sets for the genes of interest in a StepOne Real-Time PCR System (Applied Biosystems). The primers were as follows: *IL33* (Mm00505403_m1) and *Gapdh* (Mm99999915_g1) (all Applied Biosystems). Target gene quantification was normalized to *Gapdh* expression.

2.8. Lung histology

Lungs obtained from mice were fixed by 20% (vol/vol) formalin and embedded in paraffin. Lung sections were stained with hematoxylin and eosin for histological analysis [23].

2.9. T cell adoptive transfer

Splenocytes from *IL5^{+/Venus}* mice were treated with biotin-labeled anti-B220 and anti-CD11b antibodies. Then, B220⁺ and CD11b⁺ cells were depleted to enrich T cells using anti-biotin conjugated beads and the MACS system (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's recommendations. Splenic T cells (5×10^6 cells/mouse) were adoptively transferred to *IL5^{Venus/Venus} Rag2^{-/-}* mice.

2.10. Statistical analysis

Data were analyzed using Prism v.6 software (GraphPad Software, San Diego, CA). Unpaired two-tailed Student's *t* tests were used to determine significance. *P* values < .05 were considered statistically significant.

3. Results

3.1. CsA reduced IL-5 expression and the number of ILC2s in papain-induced lung inflammation

CsA blocks T cells' production of IL-2 and other NFAT-dependent cytokines. It is used to treat severe steroid-resistant asthma. ILC2s also produce large amount of Th2 cytokines [14,15], including IL-5, which is involved in eosinophil development and accumulation in various tissues [24]. It also has important roles in the pathogenesis of asthmatic inflammation. However, the effects of CsA on ILC2 function have not been examined. Therefore, we studied the issue using a mouse model of papain-induced lung inflammation. Papain, an allergen with cysteine protease activity induces airway inflammation by damaging airway tissues and releasing IL-33 in the lung [17]. We utilized *Il5*^{+/-Venus} knock-in mice in which *Il5* expression can be monitored by the expression of Venus, a modified GFP [22]. Mice were treated with DMSO or CsA for 2 weeks. Then, papain was intranasally administered to the mice for 3 consecutive days. Cells in the lungs were harvested, stained and ILC2s were identified as CD45⁺ Thy1.2⁺ CD25^{high} cells that were lineage negative, i.e., no expression of CD3e, CD4, CD8, CD19, B220, CD11b, CD11c, NK1.1, TCRγδ, or TER119 (Fig. S1). CD25^{high} ILC2s were activated by papain and exhibited Venus (IL-5) expression (27% in Lin⁻ Thy1.2⁺ cells, 52 ± 9.7% of CD25^{high} ILC2s) in DMSO-treated mice (Fig. 1A–C). In mice treated with CsA and papain, The frequency of Venus⁺ cells (9% in Lin⁻ Thy1.2⁺ cells) and ILC2 numbers in lung were significantly decreased (Fig. 1A, B). The percentage of Venus (IL-5)⁺ cells in ILC2s was decreased (33 ± 9.1% of CD25^{high} ILC2s) (Fig. 1C) and the expression levels of Venus (IL-5) were also reduced in lungs from CsA-treated mice (Fig. 1D). CsA treatment also decreased the number of total lung cells and eosinophils (Fig. 1E, F). The percentages of CD45⁺ cells in total cells were comparable (DMSO, 82 ± 4.9% and CsA, 81 ± 2.6%) (data not shown). Meanwhile, there was no difference in the levels of *Il33* mRNA in the lung or protein in BAL fluids (Fig. 1G). Lung histological analysis showed that CsA treatment suppressed inflammatory cell infiltration (Fig. 1H). The severity of peribronchial inflammation semiquantitatively graded by the accumulation of inflammatory cells was reduced in CsA-treated mice compared to DMSO-treated *Il5*^{+/-Venus} mice (Fig. S2).

3.2. Lung ILC2s were reduced by CsA treatment of naïve mice

We assessed whether lung ILC2s were influenced by injection of CsA before papain treatment. *Il5*^{+/-Venus} mice were treated with CsA for 2 weeks, and then lung ILC2s were identified (Fig. 1A). The number of ILC2s was reduced by CsA treatment (Fig. 1B), but the percentages of Venus (IL-5)⁺ ILC2s and the expression levels of Venus (IL-5) in ILC2s were comparable between DMSO and CsA-treated mice (Fig. 2C, D). The numbers of eosinophils (Fig. 2E) and total lung cells (data not shown) were not significantly affected by CsA treatment.

3.3. CsA did not directly suppress activation of purified ILC2 in culture

To examine whether CsA directly regulated the expression of IL-5 in ILC2s, we purified ILC2s from lungs of naïve mice and stimulated them with IL-33 and IL-7 in the presence or absence of CsA. The percentages of Venus (IL-5)⁺ cells in ILC2s cultured with CsA were comparable to DMSO-treated ILC2s (Fig. 3A, B). The survival of cultured ILC2s was comparable with or without CsA (data not shown), and total cell

numbers were not affected between DMSO-treated and CsA-treated groups (DMSO-treated ILC2s; 815 ± 73, CsA-treated ILC2s; 742 ± 21). There was no difference in the expression levels of Venus (IL-5) indicated by the MFI between CsA-treated and DMSO-treated ILC2s (Fig. 3C). These results indicated that CsA did not directly suppress cytokine expression or survival of ILC2s, and suggested that the suppressive effects of CsA on ILC2 were mediated by other target cell components *in vivo*.

3.4. Suppressive effects of CsA on ILC2s were not obvious in *Rag2*^{-/-} mice

IL-33 is critical for the activation of ILC2s, but IL-2 or IL-7 is required for efficient IL-5 production by ILC2s *in vitro* [15]. Since CsA inhibits IL-2 production by activated T cells [25], we examined whether CsA induced suppression of ILC2s in *RAG2*-deficient mice that have ILC2s but no T, B or NKT cells. *Il5*^{+/-Venus} *Rag2*^{-/-} mice were treated with DMSO or CsA for 2 weeks, followed by stimulation with papain. In contrast to CsA-treated wild-type mice (Fig. 1), CsA-treated *Il5*^{+/-Venus} *Rag2*^{-/-} mice did not exhibit decreased numbers of ILC2s (Fig. 4A, B). Neither the percentage of Venus (IL-5)⁺ cells in ILC2s (Fig. 4C) nor the expression levels of Venus (IL-5) were influenced by CsA treatment (Fig. 4D). The total number of lung cells in CsA-treated *Il5*^{+/-Venus} *Rag2*^{-/-} mice was comparable to DMSO-treated mice (Fig. 4E). Consistent with the direct inhibitory effect of CsA [12], eosinophils were significantly reduced by CsA treatment (Fig. 4F). Whereas lung ILC2s were reduced in CsA-treated naïve wild-type mice before papain administration (Fig. 2), CsA-treated naïve *Il5*^{+/-Venus} *Rag2*^{-/-} mice did not exhibit reduction of ILC2s (data not shown). We adoptively transferred splenic T cells into *Il5*^{Venus/Venus} *Rag2*^{-/-} mice and examined the effect of CsA treatment on ILC2 activation and expansion. B220⁺ and CD11b⁺ cell-depleted splenocytes were adoptively transferred to *Il5*^{Venus/Venus} *Rag2*^{-/-} mice. These mice were subcutaneously injected with DMSO or CsA for 2 weeks, followed by intranasal administration of papain for 3 days. We observed that expansion and activation of ILC2s were suppressed by CsA in T cell-transferred *Rag2*^{-/-} mice (Fig. S3). These results further support that the suppressive effects of CsA on maintenance and activation of ILC2s are induced by a lymphocyte-mediated mechanism.

3.5. CD25⁺ T cells decreased but IL-10 levels increased in lungs treated by CsA

ILC2s express both ICOS and ICOSL, and ICOS-ICOSL interaction promotes cytokine production and survival of ILC2s [18,19]. To investigate CsA-induced inhibitory mechanisms of IL-5 production in ILC2s, we analyzed ICOS and ICOSL expression by ILC2s after CsA treatment for 2 weeks. ICOS expression on ILC2s was upregulated by CsA treatment (Fig. S4A), but ICOSL expression by CsA-treated mice was comparable with DMSO-treated mice (Fig. S4B). The binding of ICOSL on ILC2s and ICOS on Treg cells induces ILC2 suppression [20]. We next analyzed ICOS expression on CD25⁺ T cells, which include Treg cells. Expression levels in DMSO-treated and CsA-treated mice were comparable (Fig. S4C).

We noticed that expression levels of CD25 (IL-2 receptor) on CD25⁺ T cells (including Treg cells) were significantly downregulated after CsA treatment (Fig. S4D), and the number of CD25⁺ T cells was decreased (Fig. 5A). In addition, our measurement of cytokines in BAL fluids collected from papain and CsA-treated mice revealed that the levels of IL-10 were increased in *Il5*^{+/-Venus} mice compared to DMSO control mice (Fig. 5B). The levels of IL-10 were elevated similarly in BALs obtained from DMSO- and CsA-treated *Il5*^{+/-Venus} *Rag2*^{-/-} mice (Fig. 5C) over the levels observed in CsA-treated lymphocyte sufficient *Il5*^{+/-Venus} mice (compare Fig. 5B and 5C). These results suggest the involvement of IL-10 derived from non-lymphoid cell fractions in the suppression of ILC2 by CsA.

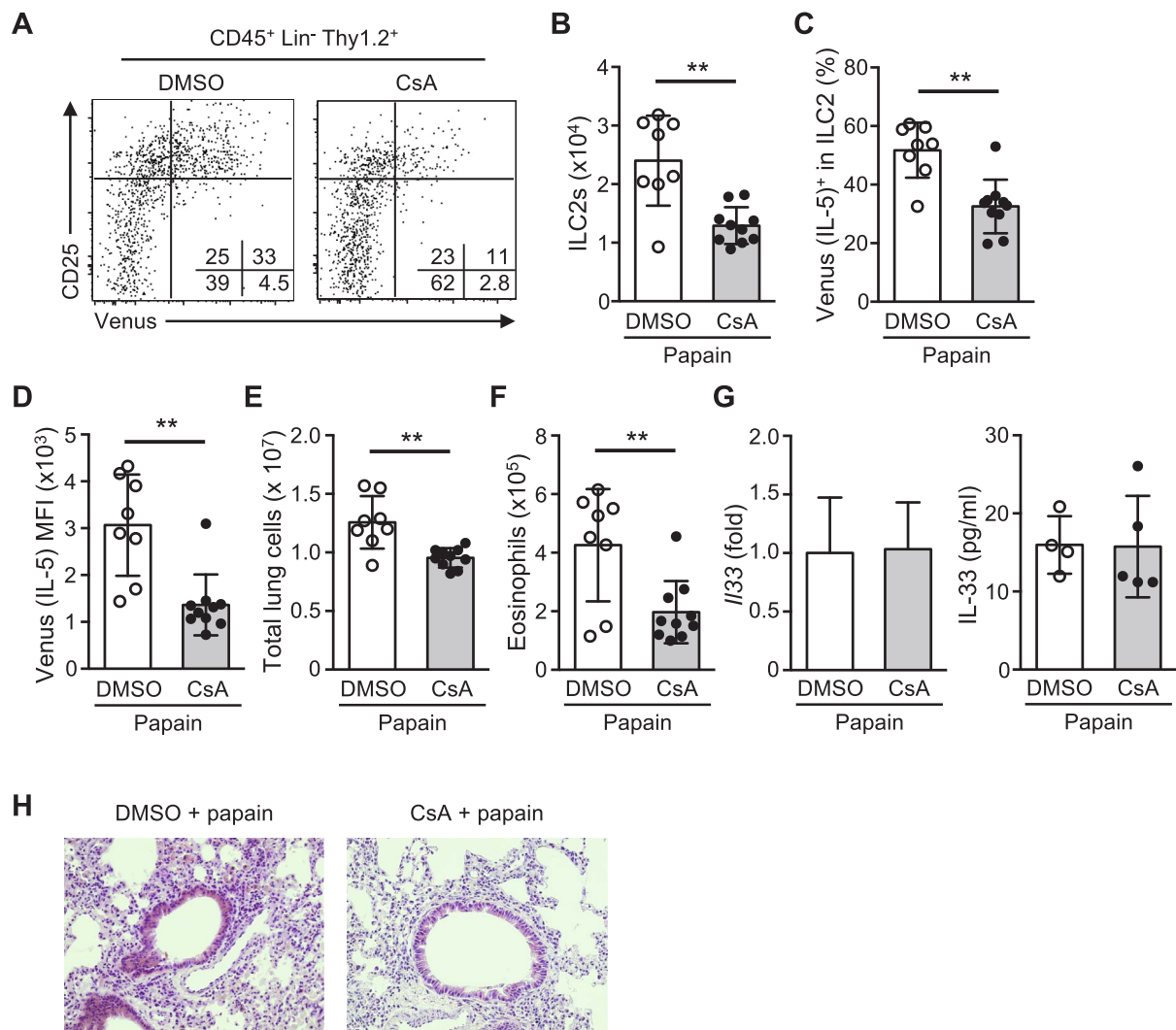


Fig. 1. CsA suppressed papain-induced ILC2 activation in the lung. *IL5⁺/Venus* mice were subcutaneously injected with DMSO or CsA for 2 weeks, followed by intranasal administration of papain for 3 days, in addition to DMSO or CsA treatment. The mice were sacrificed 19 h after final injection. (A) Flow cytometric analysis of leukocytes in the lung obtained from papain-stimulated DMSO-injected (left dot plots) or CsA-injected (right dot plots) *IL5⁺/Venus* mice. Representative dot plots show Venus and CD25 expression, which were gated on CD45⁺ Lineage (Lin)⁻ Thy1.2⁺ cells. ILC2s are identified as Lin⁻ Thy1.2⁺ and CD25^{high} cells. (B–D) Absolute number of ILC2s in the lung (B). Percentage of Venus (IL-5)⁺ cells in ILC2s (C) and mean fluorescence intensity (MFI) of Venus (IL-5) (D) in total ILC2s. (E and F) Number of total lung cells (E) and eosinophils (F) in the lung. (G) Expression of *Il33* mRNA (left) in the lung and concentrations of IL-33 in BAL fluids (right). The mRNA levels were normalized and presented as the percent of maximal expression observed in lung cells from DMSO-treated mice. Data shown are combined results obtained from 5 independent experiments. Each symbol represents individual mice (n = 8–10), and bars represent the mean of each group of mice. Error bars show SD. *, *P* < .05; **, *P* < .01 (two-tailed Student's *t* test). (H) Representative sections were obtained from DMSO-treated or CsA-treated *IL5⁺/Venus* mice. Sections were stained with hematoxylin and eosin. Tissue was examined by light microscopy (original magnification 200X).

4. Discussion

CsA is a well-known immunosuppressive agent that acts by inhibiting T cell activation [3,4,10,26,27]. Moreover, recent studies have shown that calcineurin/NFAT inhibitors, such as CsA and tacrolimus (FK506), also contribute to innate immune regulation [5,9–11,28,29]. In this study, we used a papain-induced asthma model [15]. We found that CsA decreased the number of ILC2s in the lung and reduced IL-5 expression by activated ILC2s. In naïve mice, CsA decreased the number of ILC2s but did not suppress basal production of IL-5. CsA suppresses expansion or maintenance of ILC2s in lung and their IL-5 production upon activation, however, it has little effect on basal or spontaneous production of IL-5 by ILC2s in steady state. The inhibition by CsA was not a direct effect on ILC2s, but was mediated by T cells or B cells. Our results suggest that suppression of asthmatic inflammation by CsA might involve inhibition of ILC2 maintenance and cytokine production in addition to the suppression of Th cells and other innate cell functions.

We previously reported that IL-5 and IL-13 production in ILC2s was

suppressed by IFN- γ , and that the suppression was directly mediated through the IFN- γ receptor expressed on ILC2 [30]. Our *in vitro* data in the current study revealed that CsA did not directly suppress IL-5 expression by ILC2s. ILC2s are activated and produce type 2 cytokines by stimulating IL-33, which is released by damaged epithelial cells and other cells [31]. We ruled out the possibility that IL-33 levels released into BAL fluids might be downregulated by CsA treatment. The expression levels of *Il33* mRNA in lung and IL-33 protein in BAL were comparable between control and CsA-treated mice.

ILC2s express cysteinyl leukotriene receptor 1 (CysLT1R), which stimulates production of IL-4, IL-5, and IL-13 and promotes calcium influx [32–34]. Recently, von Moltke et al. showed that leukotriene (LT) signaling activated NFAT in ILC2s and synergized with IL-33 to activate ILC2s [35]. Moreover, CsA inhibited cytokine production from ILC2s by stimulation with LT but not with IL-33 [35]. Our observation in which ILC2 activation was partially suppressed by CsA administration *in vivo* might be associated in part with ILC2 activation by LTs produced by papain exposure. On the other hand, LTs are largely

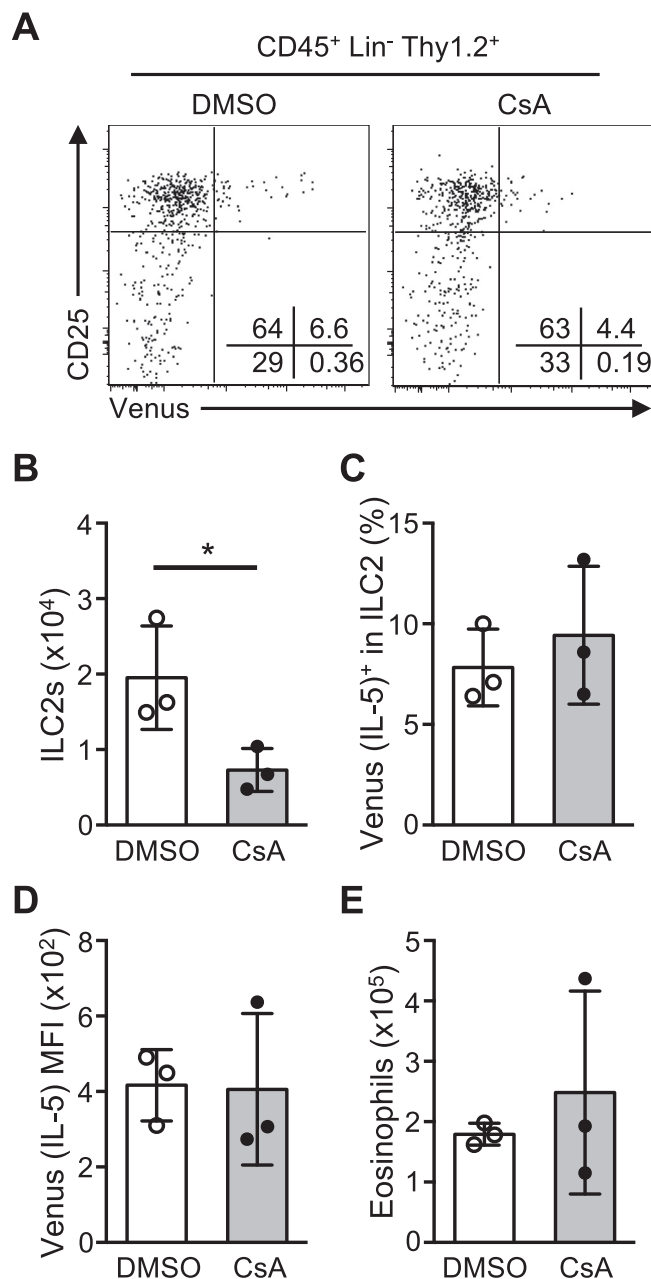


Fig. 2. CsA decreased the number of ILC2s in lung. *IL5*^{+/Venus} mice were subcutaneously injected with DMSO or CsA for 2 weeks. (A) Representative dot plots of CD45⁺ Lineage (Lin)⁻ Thy1.2⁺ cells obtained from DMSO-treated *IL5*^{+/Venus} mice (left) or from CsA-treated *IL5*^{+/Venus} mice (right). (B-D) Absolute numbers of ILC2s in the lung from DMSO-treated mice or CsA-treated mice (B). Percentage of Venus (IL-5)⁺ cells in ILC2s (C) and MFI of Venus (IL-5) (D) in total ILC2s. (E) Number of eosinophils (Siglec-F⁺ CD11c⁺) in the lung. Data are expressed as the mean $\pm$ SD (n = 3). Each symbol represents an individual mouse, and bars represent the mean of each group of mice. *, $P < .05$ (two-tailed Student's *t* test).

dispensable for ILC2 homeostasis [35]. The reduction of ILC2 by CsA treatment *in vivo* suggested the presence of a regulatory mechanism for ILC2 maintenance other than LTs that was affected by CsA and lymphocytes.

The inhibitory effects on ILC2s were not observed in *IL5*^{+/Venus} *Rag2*^{-/-} mice after papain treatment. These results suggest that the suppressive effects of CsA on maintenance and activation of ILC2s were induced by a lymphocyte-mediated mechanism. ILC2 activation is enhanced by adding IL-2 to ILC2 culture with IL-33 [15]. Although IL-2 in BAL fluids from papain-stimulated mice was undetectable (data not shown), CD25⁺ T cell numbers decreased and the expression levels of

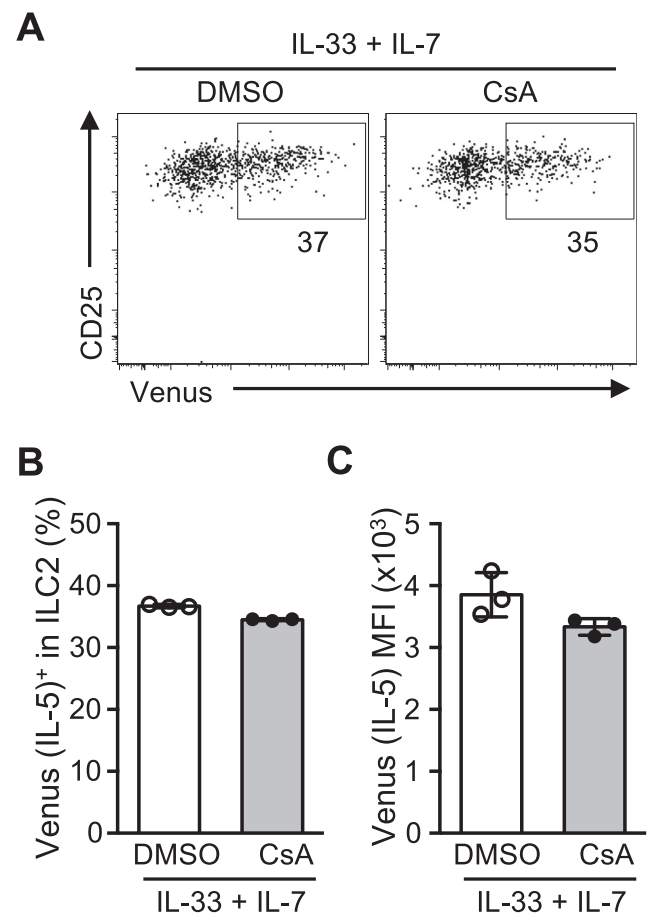


Fig. 3. CsA did not directly suppress IL-5 expression by activated ILC2s. Purified ILC2s from naïve *IL5*^{+/Venus} mice were cultured in the presence of IL-33 and IL-7 with DMSO or CsA for 19 h. (A) Representative dot plots show Venus and CD25 expression on cultured ILC2s. (B and C) The percentage of Venus (IL-5)⁺ cells (B) and MFI of Venus (IL-5) expression (C). Data are expressed as the mean $\pm$ SD (n = 3).

CD25 on CD25⁺ T cells were downregulated in CsA treatment. These data might indicate that IL-2 levels in lung were limited. In this regard, CD25⁺ T cell expansion is suppressed by CsA [36], while IL-2 enhances Treg expansion [37]. These observations were consistent with the depletion of IL-2 in CsA treated mice, and the IL-2 exhaustion might be one reason for ILC2 reduction [38].

ILC2s express both ICOS and ICOSL, and their interaction is involved in ILC2 function and homeostasis [18,19]. ICOS expression on ILC2s was elevated in CsA-treated mice, suggesting that the machinery to maintain ILC2 numbers at normal levels may operate through up-regulation of ICOS on ILC2s. However, given ICOSL is downregulated upon binding to ICOS [18], the maintained ICOSL expression on ILC2 in CsA-treated mice may indicate little or impaired ICOS/ICOSL interaction on or between ILC2s. Alternatively, ICOS upregulation on ILC2s may indicate a certain compensatory mechanism for Treg reduction by IL-2 exhaustion. Treg cell accumulation has been shown to be partially dependent on interaction between ICOS on Treg cells and ICOSL on ILC2s in adipose tissue [21]. Conversely, ILC2s are suppressed by the interaction of ICOSL on ILC2s with ICOS on Treg cells [20]. The ICOS-ICOSL interaction in this case was presumably not a major reason for ILC2 reduction by CsA, since CD25⁺ T cells also significantly decreased in CsA treated mice.

IL-10, an anti-inflammatory cytokine, has a crucial role in preventing inflammatory and autoimmune pathologies [39]. ILC2 activation is also suppressed by Treg cell-derived IL-10 [20,40]. We found that IL-10 in BAL fluids was increased in CsA-treated mice, and that higher levels of IL-10 were observed in *Rag2*^{-/-} mice than in

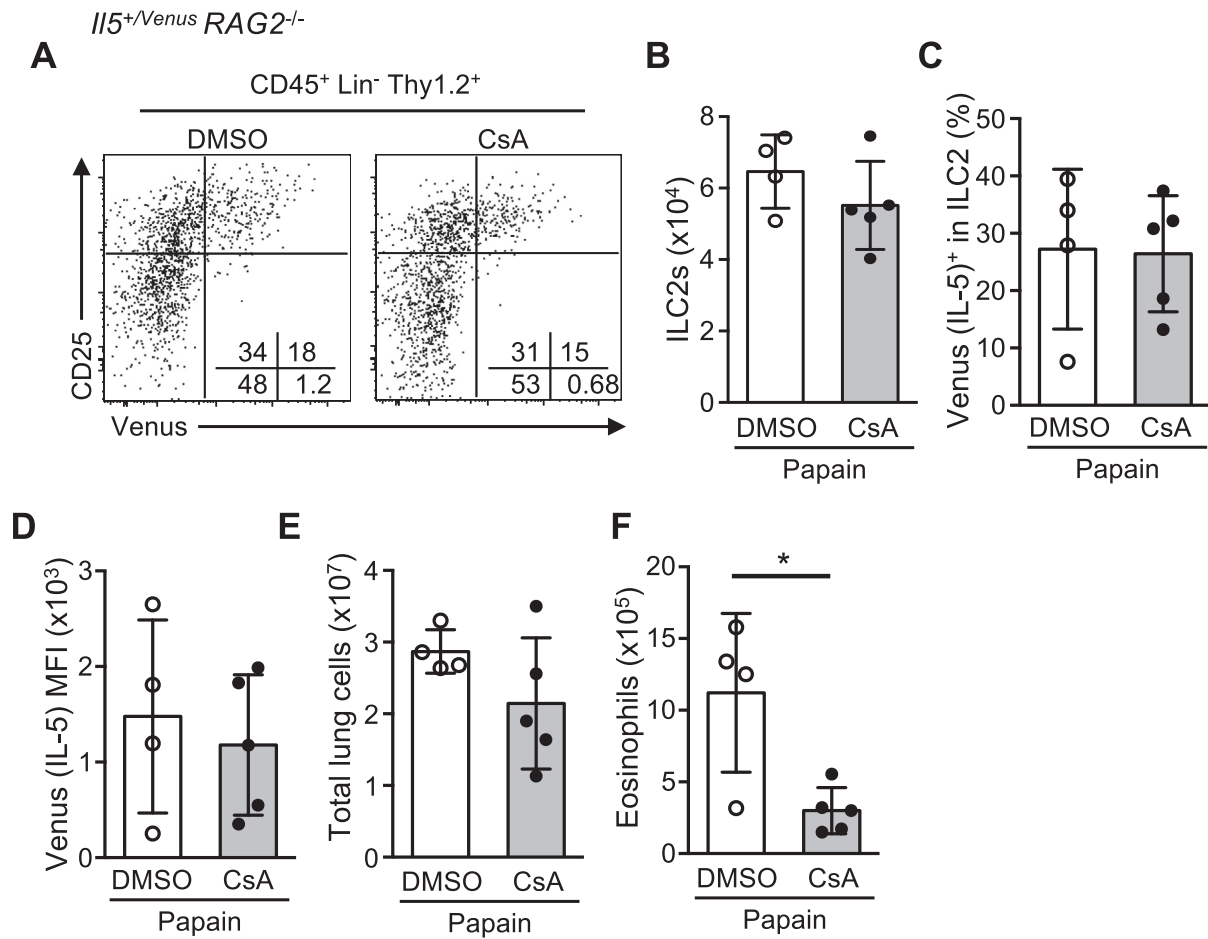


Fig. 4. RAG2-deficient mice failed to exhibit inhibition of ILC2 activation. *Il5⁺/Venus RAG2^{-/-}* mice were subcutaneously injected with DMSO or CsA for 2 weeks, followed by intranasal administration of papain for 3 days, in addition to DMSO or CsA treatment. Mice were sacrificed 19 h after the final injection. (A) Representative dot plots of CD45⁺ Lin⁻ Thy1.2⁺ cells obtained from DMSO-treated *Il5⁺/Venus* mice (left) or from CsA-treated *Il5⁺/Venus RAG2^{-/-}* mice (right). (B) Absolute number of ILC2s in the lung. (C) Percentage of Venus (IL-5)⁺ cells in ILC2s (C) and MFI of Venus (IL-5) (D) in total ILC2s. (E and F) Number of total lung cells (E) and eosinophils (F) in the lung. Data shown are combined results obtained from 2 independent experiments and expressed as the mean $\pm$ SD (n = 4–5). Each symbol represents an individual mouse, and bars represent the mean of each group of mice. *, $P < .05$ (two-tailed Student's *t* test).

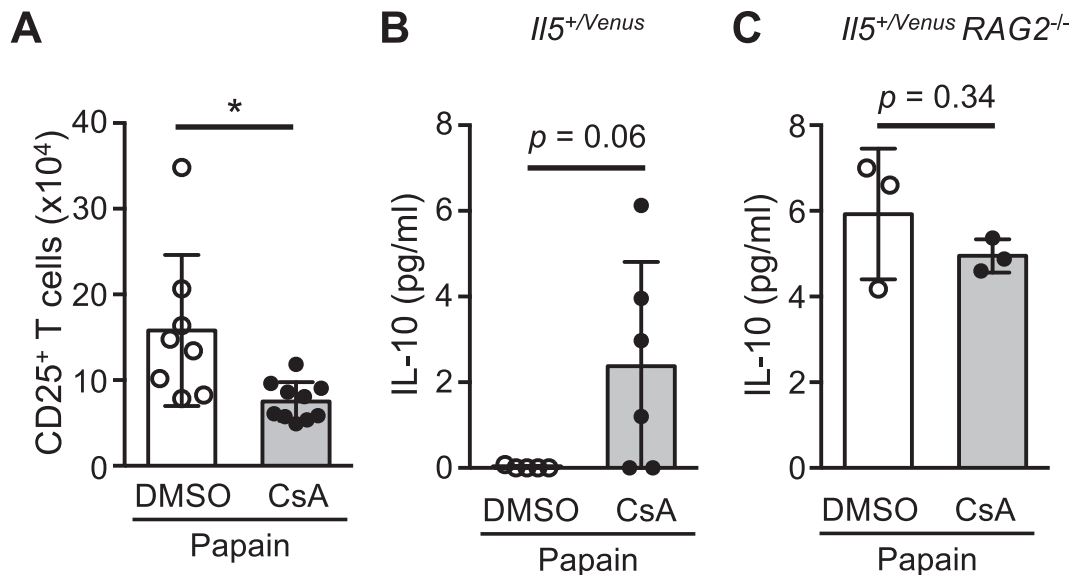


Fig. 5. Treg cells were reduced and IL-10 levels were upregulated by CsA. (A) The absolute number of CD25⁺ T cells (Treg cells) in the lung obtained from papain-stimulated DMSO-injected or CsA-injected *Il5⁺/Venus* mice. (B and C) Concentrations of IL-10 in BAL fluids in papain-stimulated DMSO-injected or CsA-injected *Il5⁺/Venus* mice (B) and *Il5⁺/Venus RAG2^{-/-}* mice (C). Each symbol represents an individual mouse. Data are expressed as the mean $\pm$ SD (n = 3–10). *, $P < .05$ (two-tailed Student's *t* test).

lymphocyte-sufficient mice. These data suggest that IL-10 is produced by non-lymphoid cells. Thus, IL-10 levels were well correlated with the suppression of ILC2 activation in our papain-induced lung inflammation model. In the lung, IL-10 is constitutively produced by interstitial macrophages (IMs) [41], and IL-10-producing IMs reduce neutrophil infiltration and mucus production in house dust mite-induced asthma [42]. We evaluated IL-10 expression in CD11b⁺ CD11c^{mid} MHCII⁺ F4/80⁺ IMs by real-time PCR, however, it was not enhanced by CsA treatment (data not shown). Thus far, we could not determine the source of IL-10 produced more in CsA treated animals compared to DMSO treated control animals, and in Rag-deficient mice compared to normal mice. Further studies are required to confirm whether IL-10 is critical for ILC2 suppression by CsA, what is the source of IL-10 and also how T cells regulate the suppression.

In conclusion, our studies show that CsA inhibits ILC2 expansion and activation in papain-stimulated lung and also ILC2 maintenance. The ILC2 suppression is indirectly induced by CsA and may be associated with IL-10 and is lymphocyte-dependent. This study provides insight into the therapeutic effects of CsA on asthma via regulation of ILC2s.

5. Author contributions

FK and ST designed the experiments and wrote the manuscript. FK performed experiments and analyzed data with cooperation and discussion with MI (Ikutani) and MI (Iseki).

Disclosure statement

All authors declare that no competing financial interests exist.

Acknowledgments

We thank our colleagues for helpful discussions and for technical assistance. This work was supported by JSPS KAKENHI Grant Numbers 16K19111 (F.K.), 15K09036, 25293097 (S.T.), and The Grants for National Center for Global Health and Medicine (25-107, 26-110) (S.T.). We thank Dr. H. Nakajima and Dr. T. Ito for help with histological analysis. We also thank N. Yamanaka for technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cellimm.2017.10.010>.

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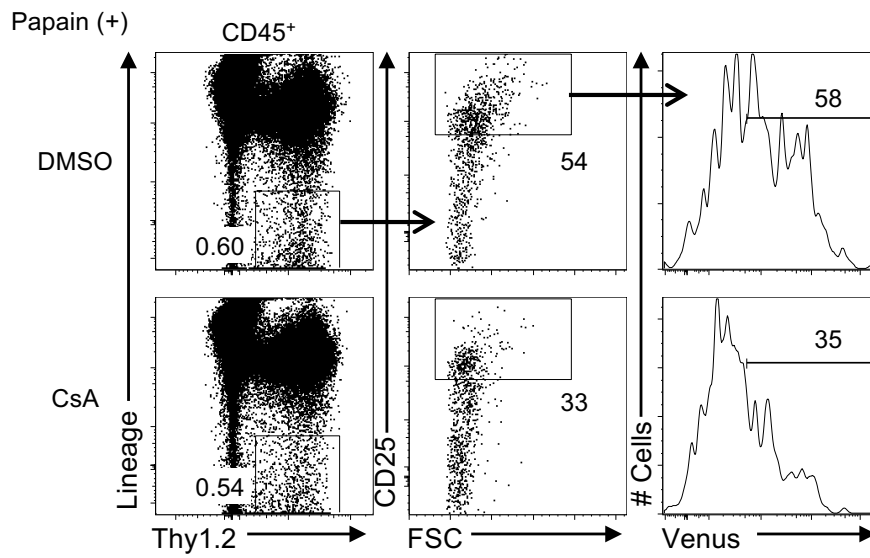


Fig. S1. Surface markers and IL-5 expression on ILC2s. Flow cytometric analysis of CD45⁺ cells in the lung obtained from papain-stimulated DMSO-injected (top dot plots) or CsA-injected (bottom dot plots) *Il5*^{+/Venus} mice. ILC2s were identified as CD45⁺ Lin⁻ Thy1.2⁺ CD25^{high} cells, and IL-5-expressing ILC2s were positive for Venus expression.

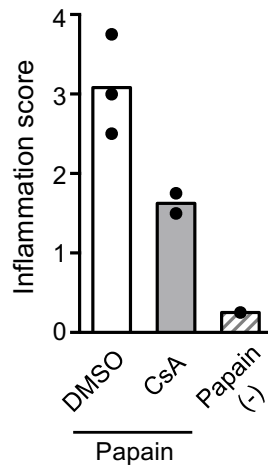


Fig. S2. CsA suppressed papain-induced lung inflammation . Histological inflammation scores of lung sections from DMSO-treated, CsA-treated or untreated *Il5^{+/Venus}* mice. The sections were stained with hematoxylin and eosin. The severity of peribronchial inflammation was graded semiquantitatively 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 2–4 cells deep; 4, a ring of inflammatory cells of >4 cells deep. Each symbol represents mean score of an individual mouse, and bars represent the mean of each group of mice (n=1-3).

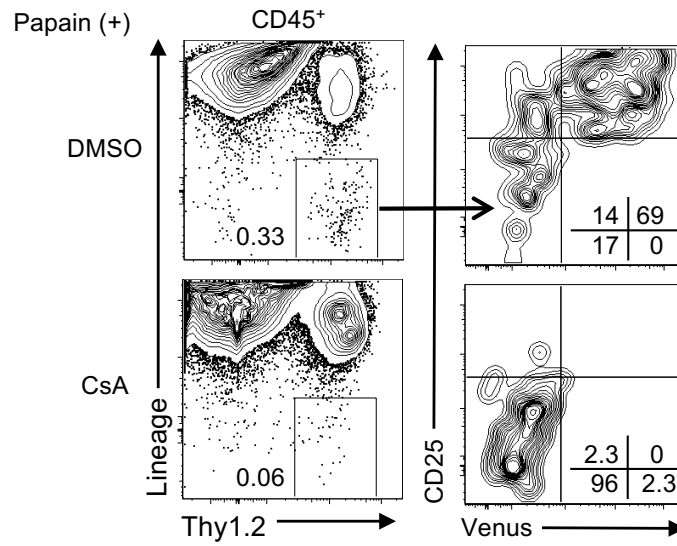


Fig. S3. Expansion and activation of ILC2s were suppressed by CsA in T cell-transferred *Rag2*^{-/-} mice. Splenic T cells were adoptively transferred into *Il5*^{Venus/Venus} *Rag2*^{-/-} mice. These mice were subcutaneously injected with DMSO or CsA for 2 weeks, followed by intranasal administration of papain for 3 days, in addition to DMSO or CsA treatment. Mice were sacrificed 19 h after the final injection. Representative dot plots of CD45⁺ cells obtained from DMSO-treated *Il5*^{Venus/Venus} *Rag2*^{-/-} mice (top dot plots) or from CsA-treated *Il5*^{Venus/Venus} *Rag2*^{-/-} mice (bottom dot plots) (n=2).

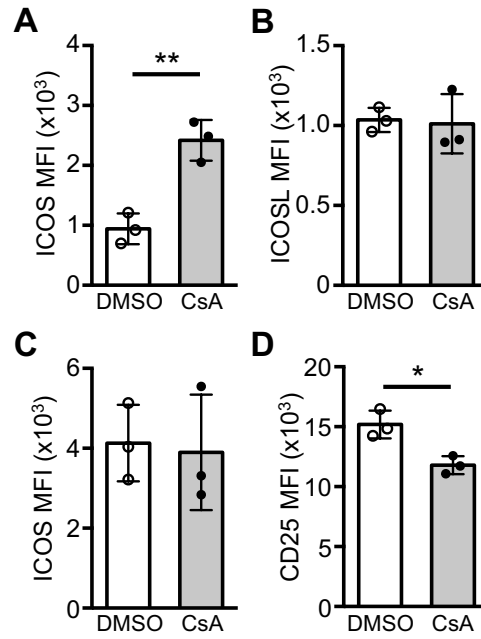


Fig. S4. ICOS expression was upregulated on ILC2s in the lung of CsA-treated mice. (A, B) Expression of ICOS (A) and ICOSL (B) on ILC2s from DMSO-treated and CsA-treated *Il5^{+/Venus}* mice. (C, D) Expression of ICOS (C) and CD25 (D) on CD25⁺ Thy1.2⁺ T cells from DMSO-treated and CsA-treated *Il5^{+/Venus}* mice. Data are expressed as the mean $\pm$ SD (n=3). Each symbol represents an individual mouse, and bars represent the mean of each group of mice. *, $P < 0.05$; **, $P < 0.01$ (two-tailed Student's *t* test).