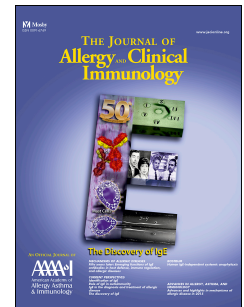


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IL-10, TGF- β and glucocorticoid prevent the production of type 2 cytokines in human group 2 innate lymphoid cells

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Capsule summary:

Induction of IL-10 and TGF- β and treatment with glucocorticoids may have a beneficial role in type 2 inflammatory diseases by suppressing production of type 2 cytokines in ILC2s.

Key words

Glucocorticoid; IL-10; ILC2; Nasal polyps; TGF- β ; Type 2 inflammation.

Abbreviations

GR; Glucocorticoid receptor

CRS; Chronic rhinosinusitis

CRSwNP; CRS with nasal polyps

ILC; Innate lymphoid cell

ILC2; Group 2 innate lymphoid cell

NP; Nasal polyp

TSLP; Thymic stromal lymphopoietin

Treg; Regulatory T cell

To the Editor:

Allergic diseases are characterized by accumulation and activation of inflammatory cells including Th2 cells and mast cells. Recent studies indicate that group 2 innate lymphoid cells (ILC2s) also produce a large quantity of type 2 cytokines, and have pathogenic roles in allergic and type 2 inflammatory diseases including allergic asthma and chronic rhinosinusitis with nasal polyps (CRSwNP).¹⁻³ Although the mechanisms of suppression of Th2 cells and mast cells have been extensively investigated, there is only minimal data on corresponding mechanisms of suppression for ILC2s in type 2 inflammation. Studies in mice indicate that immunosuppressive cytokines, IL-10 and TGF- β , and glucocorticoids suppress ILC2-mediated type 2 inflammation.⁴⁻⁶ However, the role of IL-10 and TGF- β in human ILC2s is still unclear and only mRNA expression of the IL-10 receptor has been reported.⁴ Although Walford *et al.* reported that the frequency of ILC2 in nasal polyps (NPs) was reduced by treatment with systemic glucocorticoid,⁶ two other groups showed that ILC2s were resistant to glucocorticoids especially in patients with asthma.^{7, 8} However, both studies examined the role of glucocorticoids in ILC2s using PBMC, which are a mixture of cells, and identified the presence of intracellular cytokine⁺ ILC2s by flow cytometry after activation.^{7, 8} Therefore the direct effect of glucocorticoid on human ILC2s is also still unclear.

To clarify the role of IL-10 and TGF- β in human ILC2, we identified ILC2s by flow cytometry (see Fig. E1 in this article's Online Repository at www.jacionline.org),³ and investigated the presence of receptors for IL-10 and TGF- β on human ILC2s in peripheral blood, tonsil (a model of lymphoid tissue) and NP (a model of type 2 inflammatory disease). Detailed methods for experiments, our study population (see Table E1) and supplemental figures E1-E6 are given in this article's Online Repository at www.jacionline.org. We found that IL-10 receptor

subunits, IL-10RA and IL-10RB, and a TGF- β type II receptor (TGFB2) were expressed on ILC2s from blood, tonsil and NP (Fig. 1AB, see Fig. E2 in this article's Online Repository at www.jacionline.org and not shown). Levels of IL-10RA and TGFB2 on ILC2 were similar to Th2 cells which are well known to react with IL-10 and TGF- β (see Fig. E3 in this article's Online Repository at www.jacionline.org). Although we detected the IL-10 receptor complex, the level of protein expression on blood ILC2s was not high based on flow cytometry (Fig. 1A and see Fig. E2B). We next investigated whether IL-10 was able to induce phosphorylation of STAT3 in blood ILC2s since IL-10 is a well known activator of STAT3. We found that IL-10 dose-dependently and significantly induced the phosphorylation of STAT3 in blood ILC2s (Fig. 1C). This result suggests that functional IL-10 receptor complex is present on human ILC2s.

To investigate the functional role of IL-10 and TGF- β in ILC2s, we examined whether IL-10 and TGF- β suppressed activation of ILC2s sorted from human blood. IL-33 is known to induce morphological changes in ILC2s and induces the production of type 2 cytokines.^{1, 2} We therefore incubated the sorted blood ILC2s with IL-33 and TSLP in the presence or absence of IL-10 or TGF- β 1 for 4 days and examined the morphology of ILC2s by cytospin and production of type 2 cytokines from ILC2s by Luminex. We found that the morphological changes to ILC2s induced by IL-33+TSLP were almost completely blocked by IL-10 but not by TGF- β (Fig. 2A). We found that IL-10 significantly suppressed IL-33- and IL-33+TSLP-mediated production of IL-4, IL-5, IL-9 and IL-13 in blood ILC2s (Fig. 2B and see Fig. E4A in this article's Online Repository at www.jacionline.org). We also found that TGF- β 1 significantly suppressed IL-33- and IL-33+TSLP-mediated production of IL-4, IL-5 and IL-13 but significantly enhanced production of IL-9 in human blood ILC2s (Fig. 2C and see Fig. E4B). These results suggest that

IL-10 and TGF- β suppress the activation of ILC2s in humans, although the effect of TGF- β may be weaker than IL-10.

To examine the role of glucocorticoids, we incubated sorted human blood ILC2s with IL-33 and TSLP in the presence or absence of 0.01% DMSO (vehicle control) or 100 nM dexamethasone for 4 days. We found that dexamethasone suppressed the morphological changes to ILC2s and the production of IL-4, IL-5, IL-9 and IL-13 induced by IL-33+TSLP in blood ILC2s (Fig. 2AD). To confirm whether the dexamethasone mediated inhibition was via glucocorticoid receptors (GRs), we added a GR antagonist, RU-486 (100 nM), together with dexamethasone. We found that RU-486 significantly inhibited dexamethasone-mediated suppression of type 2 cytokine production in ILC2s (see Fig E5 in this article's Online Repository at www.jacionline.org). In contrast to our result (Fig. 2D), Liu *et al.* reported that glucocorticoid could not suppress IL-33-mediated activation of human ILC2s in the presence of TSLP using PBMC.⁸ This discrepancy suggests that TSLP and/or IL-33 may also act directly and/or indirectly on other immune cells present in PBMCs and factors from these other immune cells may cancel the glucocorticoid-mediated suppressive effect on ILC2s when cultured together. Future studies will be required to identify these factors.

Although we found that IL-10, TGF- β 1 and dexamethasone inhibited the function of human ILC2s, the mechanism and degree of inhibition by these factors might be different. We found that only dexamethasone induced apoptosis and cell death in human blood ILC2s (not shown). We also found that the inhibitory effect of TGF- β may be weaker than IL-10 and glucocorticoids, and that TGF- β 1 enhanced IL-33-mediated production of IL-9 in human ILC2. These results suggest that production of IL-9 is differentially regulated in ILC2s compared to the

classical Th2 cytokines IL-4, IL-5 and IL-13. Future study will be required to identify the mechanisms of suppression for each inhibitor.

Recent studies showed that peripheral blood ILC2s were increased during pollen season and that this seasonal increase was abrogated in seasonal allergic rhinitis patients who received grass pollen immunotherapy.⁹ Successful immunotherapy was associated with the induction of Treg cells that produce IL-10 and TGF- β .⁹ Our current study showed that IL-10 and TGF- β 1 potently suppressed the activation of ILC2s (Fig. 2). This suggests that immunotherapy may not only suppress Th2 cell-mediated reactions but also inhibit ILC2-mediated inflammation via induction of Treg-mediated cytokines.

Since allergic diseases are characterized by chronic inflammation, it is therefore important to ask whether inhibitory factors can also suppress previously activated ILC2. We recently found that NP ILC2s were already activated in NP *in vivo*, and sorted NP ILC2s but not blood ILC2s spontaneously released type 2 cytokines without additional stimuli.³ Interestingly, IL-10, TGF- β 1 and dexamethasone suppressed this spontaneous production of IL-5 and IL-13 in NP ILC2s within a small cohort (see Fig. E6 in this article's Online Repository at www.jacionline.org and not shown). This result suggests that these inhibitors may reduce the ongoing ILC2-mediated inflammation that is found in chronic type 2 inflammatory diseases. However, it will require a larger study to confirm the current findings in NP and other diseases.

In conclusion, we report here that functional receptors for IL-10, TGF- β and glucocorticoids are expressed on human ILC2s and IL-10, TGF- β and glucocorticoids strongly suppress the activation of human ILC2s. Our data suggests that induction of allergen-specific Tregs, IL-10 and TGF- β and treatment with glucocorticoids would have strong benefits in

allergic and type 2 inflammatory diseases by virtue of suppressing local T cells, mast cells and ILC2s.

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Competing interests: The authors declare no conflict of interest as to the interpretation and presentation of this manuscript.

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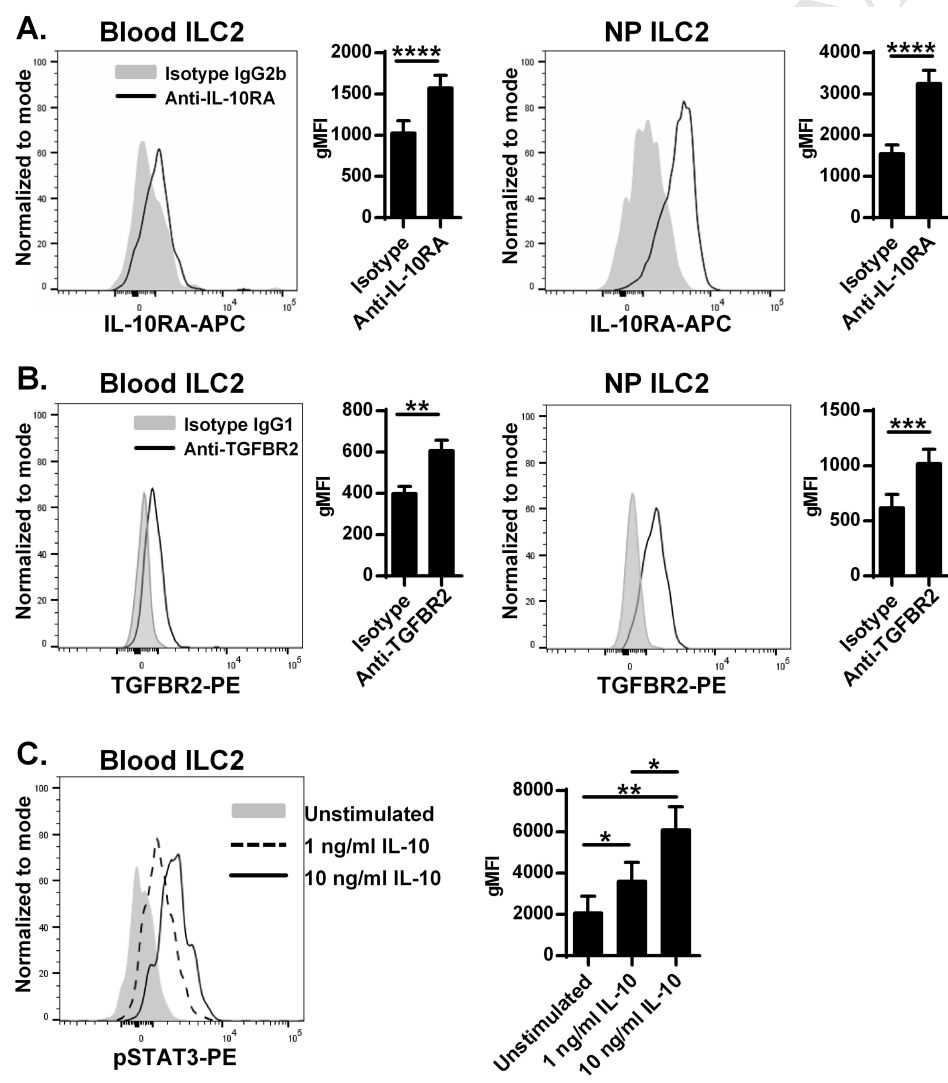
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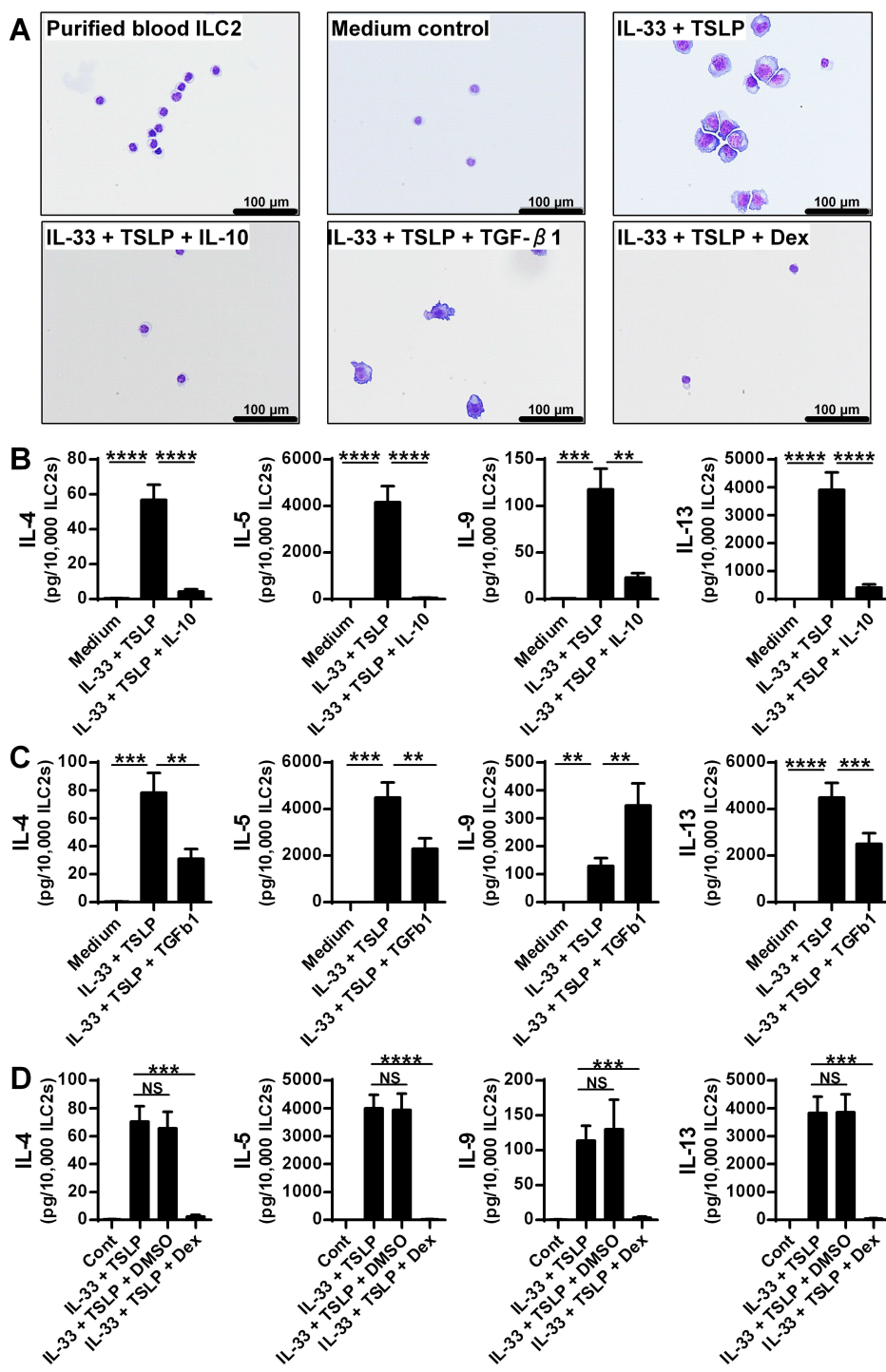
Figure 1. Presence of receptors for IL-10 and TGF- β on human ILC2.

Representative histograms of flow cytometric plots for IL-10RA (A) and TGFBR2 (B) in ILC2s from a blood sample and a NP are shown. Levels of cell surface expression of receptors on ILC2s from blood (n=10) and NPs (n=10) are shown by geometric mean fluorescence intensity (gMFI). PBMC were stimulated with medium control (filled), 1 (dashed line) or 10 (solid) ng/ml IL-10 for 15 minutes. The level of intracellular phospho-STAT3 in blood ILC2s was detected by flow cytometry (C, n=6). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, by the Paired t test (A and B) and one-way ANOVA (C).

Figure 2. IL-10, TGF- β and dexamethasone inhibit activation of human ILC2.

Sorted blood ILC2s were cultured with 10 ng/ml IL-33 and 10 ng/ml TSLP in the presence or absence of 10 ng/ml IL-10 (B, n=8), 20 ng/ml TGF- β 1 (C, n=6), 0.01% DMSO (vehicle control) and 100 nM dexamethasone (Dex) (D, n=6) for 4 days. The morphology of ILC2s was examined by cytopsin with Diff-Quik staining (A). The concentrations of IL-4, IL-5, IL-9 and IL-13 were measured by using Luminex (B-D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, by one-way ANOVA.





Online Repository

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Methods

Patients and tissue collection

Patients with CRSwNP were recruited from the Otolaryngology clinic and the Northwestern Sinus Center of Northwestern Medicine. All patients met the criteria for CRSwNP as defined by the European Position Paper on Rhinosinusitis and Nasal Polyps 2012. Patients with an established immunodeficiency, pregnancy, coagulation disorder or diagnosis of Churg-Strauss syndrome or cystic fibrosis were excluded from the study. NP tissue was collected during endoscopic sinus surgery. Tonsil tissue was obtained when patients underwent tonsillectomy for chronic tonsillitis, recurrent acute tonsillitis or obstructive sleep apnea as collected by faculty of the Otolaryngology Department of Northwestern Medicine. Several patients were taking a variety of medications, including corticosteroids. Details of patients' characteristics are included in Table E1. All subjects signed informed consent and the study was approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine (IRB Project Number: STU00080917). Human peripheral blood leukopaks (STEMCELL Technologies, Vancouver, BC, Canada) were obtained from healthy subjects for isolation of peripheral blood mononuclear cells (PBMC).

Cell isolation and flow cytometric analysis

Cells were isolated from tonsil and NP tissues using a previously described method. Tissue samples were weighed, washed by dPBS, fragmented and then incubated with 30 μ g/ml DNase I and 1 mg/ml type I collagenase containing media at 4°C overnight. Following this, tissues were minced using a gentleMACS dissociator (Miltenyi Biotec, Auburn, CA) and the cells were

46 filtered through 70 μ m nylon mesh (BD Biosciences, San Jose, CA). Cells were then treated with
47 red blood cell lysis solution (Miltenyi Biotec) and washed with dPBS before counting and
48 staining for flow cytometric analysis.

49 Cells were first treated with Aqua LIVE/DEAD fixable dead cell staining reagent
50 (Invitrogen, Carlsbad, CA) as a live/dead discriminator. Cells were then incubated with an Fc
51 Block reagent (Miltenyi Biotec) for 10 minutes at 4°C in the dark. All antibodies were obtained
52 from BioLegend (San Diego, CA), unless otherwise stated. The following antibodies and
53 dilutions were used to stain the surface of the cells: FITC anti-human Lineage Cocktail (CD3,
54 CD14, CD16, CD19, CD20, CD56, 1:20), 2 μ g/ml FITC anti-Fc ϵ R1a (AER-37), 4 μ g/ml FITC
55 anti-CD11c (Bu15), 4 μ g/ml FITC anti-CD303 (201A), 5 μ g/ml PE/Cy7 anti-CD3 (HIT3a), 2.5
56 μ g/ml PE/Cy7 anti-CD4 (RPA-T4), 1 μ g/ml Brilliant Violet 421 anti-CD127 (A019D5), 7.5
57 μ g/ml PE/Dazzle 594 anti-CRTH2 (Bim16), 1.25 μ g/ml PerCP/Cy5.5 anti-CD45 (HI30), 5
58 μ g/ml APC/Cy7 anti-CD161 (HP-3G10), 10 μ g/ml Alexa Fluor 647 anti-IL-10RA (714212,
59 Novus Biologicals, Southpark Way, CO), 10 μ g/ml Alexa Fluor 647 control mouse IgG2b
60 (MPC-11, Novus Biologicals), 10 μ g/ml PE anti-IL-10RB (90220, R&D systems, Minneapolis,
61 MN), 5 μ g/ml PE anti-TGFBR2 (25508, R&D systems) and 10 μ g/ml PE control mouse IgG1
62 (11711, R&D systems). Cells were stained for 30 minutes at 4°C in the dark, and washed with
63 MACS buffer (Miltenyi Biotec). Cells were fixed with a BD Cytofix/Cytoperm Kit,
64 resuspended in MACS buffer and stored at 4°C in the dark before analysis on a CytoFLEX flow
65 cytometer (Beckman Coulter, Indianapolis, IN). All analysis was performed with FlowJo
66 software, version 10.1 (TreeStar, Ashland, OR), and each experiment contained the proper
67 single-stained control beads (BD Biosciences and eBiosciences) and fluorescence minus one
68 (FMO) negative controls.

ILC2 sorting

Human PBMC were isolated from a human peripheral blood leukopak by centrifugation on a Ficoll-Paque PREMIUM density gradient (GE Healthcare, Piscataway, NJ). After lysis of red blood cells, PBMC were incubated with Fc Block reagent and FITC anti-human Lineage Cocktail for 15 minutes at 4°C in the dark. FITC⁺ (Lin⁺) cells were depleted by human FITC positive selection kit (STEMCELL Technologies) using Big EasySep Magnets (STEMCELL Technologies). After depletion of Lin⁺ cells, cells were counted and further stained with FITC anti-FcεRIa, FITC anti-CD11c, FITC anti-CD303, PE anti-CD4, Brilliant 421 anti-human CD45, Alexa Fluor 647 anti-CRTH2, PE/Cy7 anti-CD127 and APC/Cy7 anti-CD161. We then sorted ILC2s as CD45⁺, Lin (FITC)⁻, CD4⁻, CD127⁺, CRTH2⁺, CD161⁺ cells with a BD FACS Aria SORP-5-laser cell sorter (BD Biosciences) at the Robert H Lurie Comprehensive Cancer Center at Northwestern University.

We also sorted ILC2 from NP tissues from patients with CRSwNP, a disease characterized by type 2 inflammation with high levels of eosinophilia, ILC2 infiltration and type 2 cytokines including IL-5 and IL-13.^{E1} After the isolation of cells from NP tissues, cells were first treated with Aqua dead cell staining reagent. Cells were blocked by Fc Block reagent and then incubated with FITC anti-human Lineage Cocktail (CD3, CD14, CD16, CD19, CD20, CD56), FITC anti-CD11c, FITC anti-CD303, PE/Cy7 anti-FcεRIa, BUV395 anti-CD4, Alexa Fluor 700 anti-CD45, Alexa Fluor 647 anti-CRTH2, BV421 anti-CD127 and APC/Cy7 anti-CD161. We sorted ILC2 as Aqua⁻, CD45⁺, Lin (FITC)⁻, CD4⁻, FcεRIa⁻, CD127⁺, CRTH2⁺, CD161⁺ cells with a BD FACS Aria SORP-5-laser cell sorter. The purity of ILC2s was always greater than 95% (not shown).

Cell culture

PBMC were stained with FITC anti-human Lineage Cocktail, FITC anti-FcεRIα, FITC anti-CD11c, FITC anti-CD303, Alexa Fluor 647 anti-CRTH2, Brilliant Violet 421 anti-CD127 and Alexa Fluor 700 anti-CD161, and stained PBMC were equilibrated in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) for 2 hours before stimulation. Stained PBMC were stimulated with 10 ng/ml IL-10 (R&D systems) for 15 minutes and the reaction was stopped by adding BD Cytofix Fixation Buffer (BD Biosciences). Fixed cells were permeabilized by Phosflow Perm Buffer III (BD Biosciences) for 30 minutes on ice in the dark. After washing, cells were stained with PE/Cy7 anti-CD45 (HI30), PerCP/Cy5.5 anti-CD20 (H1) and PE anti-STAT3 (pY705) (4/P-STAT3, BD Biosciences) for 30 minutes at 4°C in the dark. The level of phospho-STAT3 in ILC2s was detected by a CytoFLEX. Data analysis was performed with FlowJo software.

Sorted blood ILC2s and NP ILC2s (10,000 cells/ml) were suspended in RPMI 1640 medium supplemented with 25 IU/ml IL-2 (Prometheus, San Diego, CA), 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin and were cultured in the presence or absence of 10 ng/ml IL-33 (BioLegend), 10 ng/ml TSLP (R&D systems), 10 ng/ml IL-10 (R&D systems), 20 ng/ml TGF-β1 (R&D systems), 0.01% DMSO (Sigma-Aldrich, St. Louis, MO), 100 nM dexamethasone (Sigma-Aldrich) and 100 nM RU-486 (Sigma-Aldrich) for 4 days. We did dose-dependent experiments using 1-20 ng/ml cytokines (n=2, not shown) and then selected these concentrations for this study. The concentrations of IL-4, IL-5, IL-9 and IL-13 in cell-free supernatants were measured using a MILLIPLEX MAP Human Cytokine/Chemokine Panel from EMD Millipore (Billerica, MA). The minimal detection limits for IL-4, IL-5, IL-9 and IL-13 are 3.2 pg/ml.

115

116 **Statistics**

117 All data are reported as the as the mean $\pm$ SEM. Differences between groups were analyzed
118 using the Paired *t* test or the RM 1-way ANOVA Tukey's multiple comparison test. A *p* value of
119 less than 0.05 was considered significant.

120

Table E1 Subject characteristics

	Tonsil (n=6) n (%)	NP (IL-10R) (n=10) n (%)	NP (TGFB2) (n=10) n (%)	NP (ILC2 culture) (n=8) n (%)
Female	4 (67)	4 (40)	4 (40)	2 (25)
Atopy	1 (17)	8 (80)	4 (40)	4 (50)
Asthma	3 (50)	7 (70)	2 (20)	5 (63)
Aspirin sensitivity	0 (0)	1 (10)	1 (10)	0 (0)
Nasal steroid	2 (33)	3 (30)	1 (17)	2 (25)
Inhaled steroid	0 (0)	3 (30)	1 (10)	3 (38)
Oral steroid	0 (0)	3 (30)	2 (20)	1 (13)
Age (y), median (range)	22* (18-38)#	58 (42-71)	51 (28-80)	39 (28-70)

*median, #(range).

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Supplemental figure legends

Figure E1. Gating strategy to identify ILC2s in human peripheral blood.

Representative flow cytometric plots for ILC2s in peripheral blood are shown. We gated on single, live CD45 positive cells and excluded granulocytes (side scatter high). We then assessed ILC2s in the lineage (CD3, CD11c, CD14, CD16, CD19, CD20, CD56, FcεRIα, CD303) negative population as CD127, CRTH2 and CD161 triple positive cells.

Figure E2. Presence of IL-10 receptor on human ILC2s.

Representative histograms of flow cytometric plots for IL-10RA (A) and IL-10RB (A-C) in ILC2s from a tonsil (A), a blood sample (B) and a NP (C) are shown. Levels of cell surface expression of IL-10 receptors on ILC2s from tonsils (A, n=6), blood (B, n=10) and NPs (C, n=10) are shown by geometric mean fluorescence intensity (gMFI). ** p<0.01, *** p<0.001, by the Paired *t* test.

Figure E3. Presence of receptors for IL-10 and TGF-β on human Th2 cells.

Representative flow cytometric plots for Th2 cells and ILC2s in NP are shown within the singlets, Aqua- and granulocytes (SSChigh) excluded population (A). We gated on single, live CD45 positive cells and excluded granulocytes (side scatter high). We then assessed ILC2s as lineage-, CD127⁺, CRTH2⁺ CD161⁺ cells and Th2 cells as CD4⁺, CRTH2⁺ cells (A). Levels of cell surface expression of IL-10RA and TGFBR2 on Th2 cells from blood (B, n=4) and NPs (C, n=4) are shown by geometric mean fluorescence intensity (gMFI). We normalized the data by calculating the gMFI ratio of receptor to isotype IgG and compared expression of receptors

between Th2 cells and ILC2s in blood (n=4) and NP tissue (n=4) (D). * $p < 0.05$, by the Paired t test.

Figure E4. Effect of IL-10 and TGF- β on IL-33 stimulated human ILC2s.

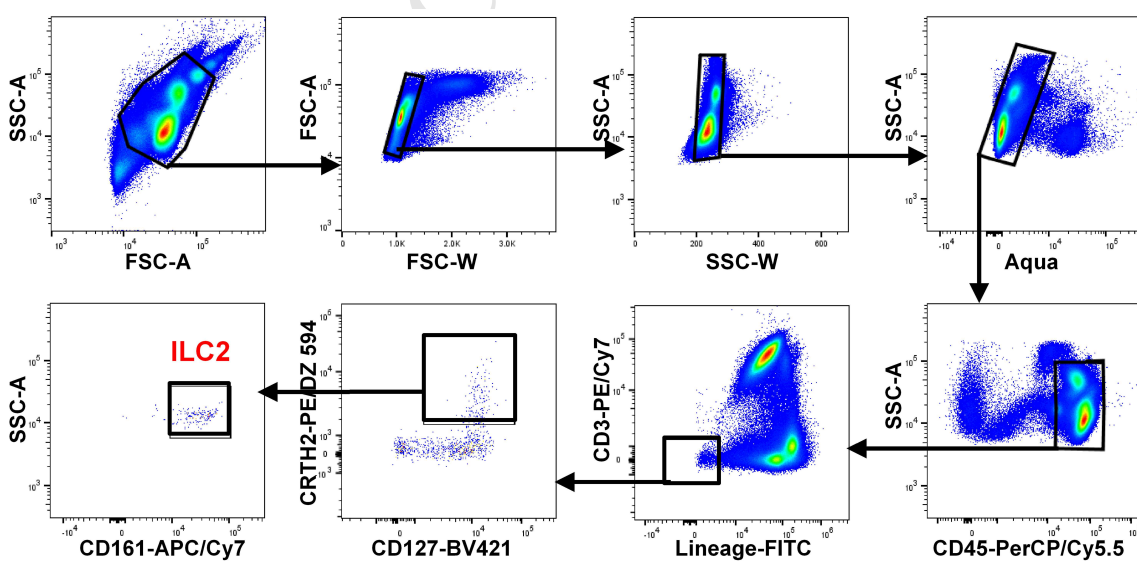
Sorted blood ILC2s were cultured in the presence or absence of 10 ng/ml IL-33, 10 ng/ml IL-10 (A, n=8) and 20 ng/ml TGF- β 1 (B, n=5) for 4 days. The concentrations of IL-4, IL-5, IL-9 and IL-13 were measured by Luminex. * $p < 0.05$, ** $p < 0.01$, by one-way ANOVA.

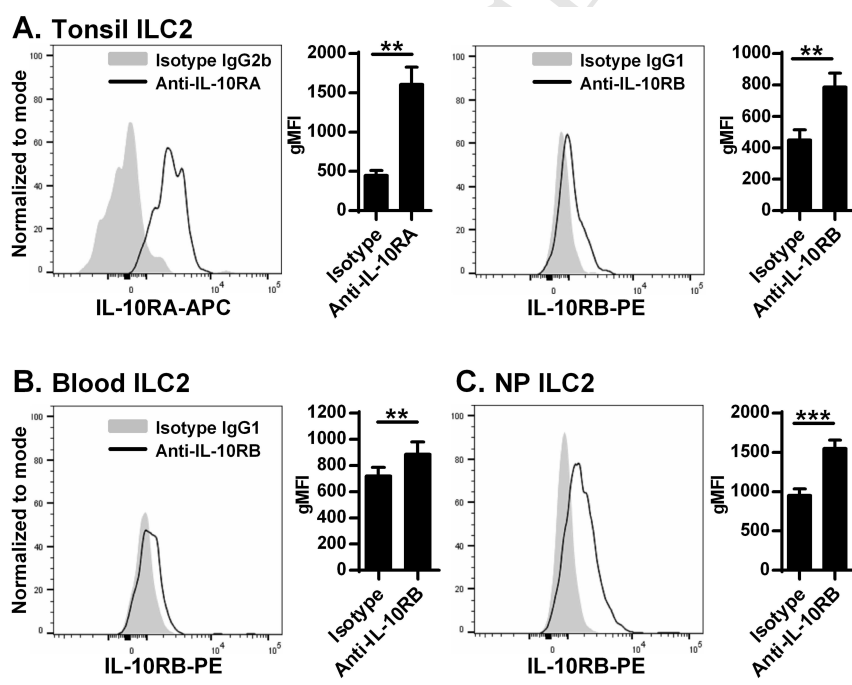
Figure E5. RU-486 inhibited dexamethasone-mediated suppression in human ILC2s.

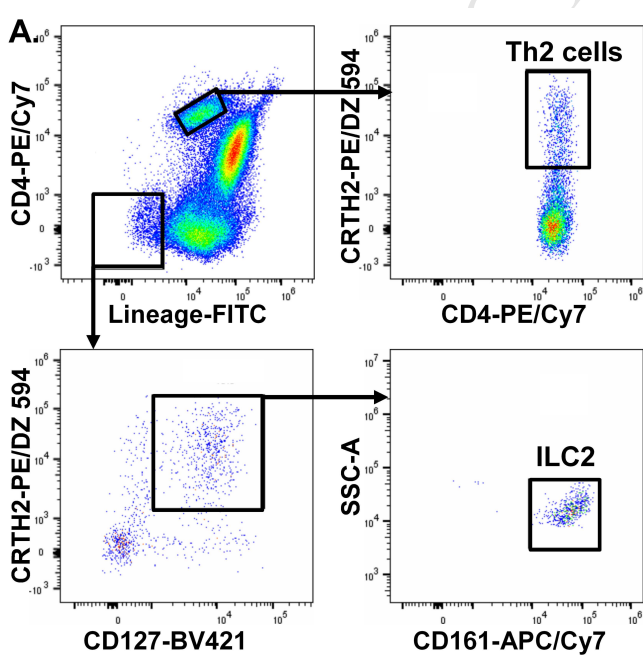
Sorted blood ILC2s were cultured in the presence or absence of 10 ng/ml IL-33, 10 ng/ml TSLP, 0.01% DMSO (vehicle control), 100 nM dexamethasone (Dex) and 100 nM RU-486 (RU) for 4 days (n=3). The concentrations of IL-5 and IL-13 were measured by Luminex. * $p < 0.05$, by the Paired t test.

Figure E6. IL-10, TGF- β and dexamethasone inhibit cytokine production in NP ILC2s.

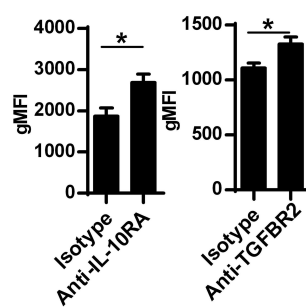
Sorted NP ILC2s were cultured in the presence or absence of 10 ng/ml IL-10 (n=8), 20 ng/ml TGF- β 1 (n=8) and 100 nM dexamethasone (Dex, n=4) for 4 days. The concentrations of IL-5 were measured by Luminex. Four NP donors were excluded from the Dex study because these patients were taking corticosteroids before the surgery.



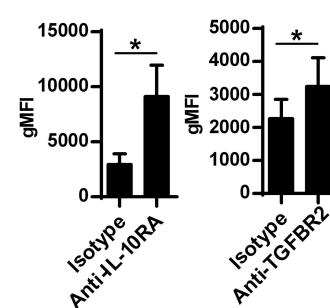




B. Blood Th2 cells



C. NP Th2 cells



D.

