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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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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 µg/ml DNase I and 1 mg/ml type I collagenase containing media at 4^{o}C overnight. Following this, tissues were minced using a gentleMACS dissociator (Miltenyi Biotec, Auburn, CA) and the cells were

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

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

References

E1. Poposki JA, Klingler AI, Tan BK, Soroosh P, Banie H, Lewis G, et al. Group 2 innate lymphoid cells are elevated and activated in chronic rhinosinusitis with nasal polyps. *Immun Inflamm Dis* 2017; **5**:233-243

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.

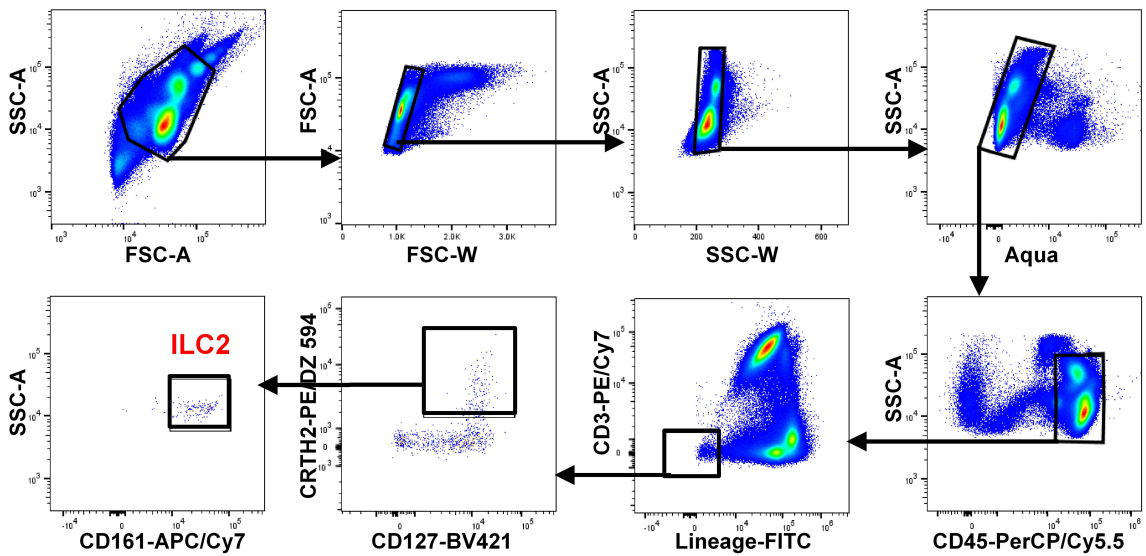
Sorted blood ILC2s were cultured in the presence or absence of 10 ng/ml IL-33, 10 ng/ml IL-10 (A, n=8) and 20 ng/ml TGF-β1 (B, n=5) for 4 days. The concentrations of IL-4, IL-5, IL-9 and IL-13 were measured by Luminex. * $p<0.05$, ** $p<0.01$, by one-way ANOVA.

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-β1 (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.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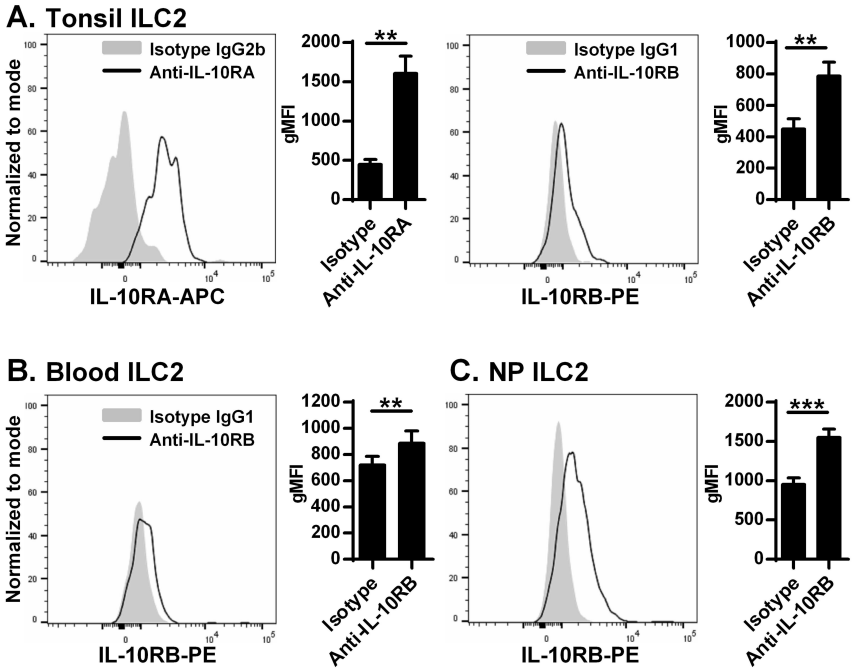
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**
Isotype
Anti-IL-10RA
Isotype IgG1
Anti-IL-10RB
IL-10RB-PE
gMFI
**
Isotype
Anti-IL-10RB
B. Blood ILC2
Isotype IgG1
Anti-IL-10RB
Normalized to mode
IL-10RB-PE
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***
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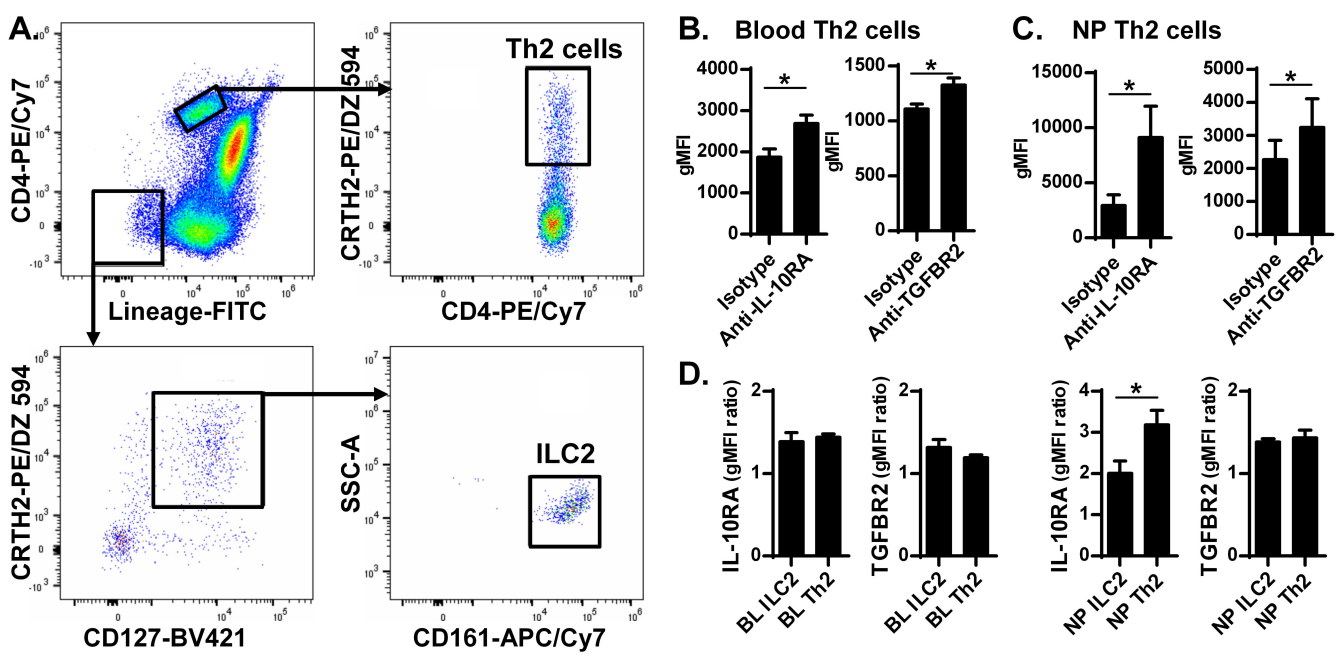
Anti-IL-10RB


A.
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SSC-A
CD161-APC/Cy7
ILC2
B. Blood Th2 cells
gMFI
Isotype
Anti-IL-10RA
Isotype
Anti-TGFBR2
C. NP Th2 cells
gMFI
Isotype
Anti-IL-10RA
Isotype
Anti-TGFBR2
D.
IL-10RA (gMFI ratio)
BL ILC2
BL Th2
TGFBR2 (gMFI ratio)
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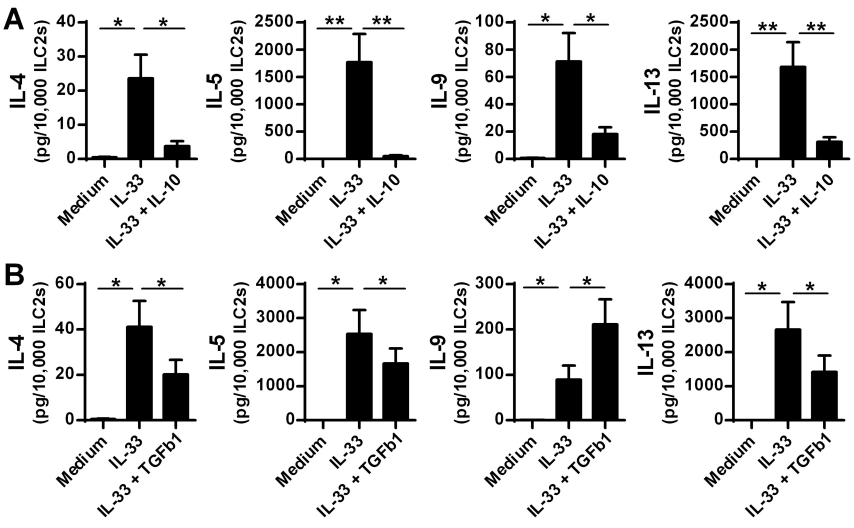
A
IL-4
(pg/10,000 ILC2s)
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30
20
10
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*
*
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IL-33
IL-33 + IL-10
IL-5
(pg/10,000 ILC2s)
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2000
1500
1000
500
0
**
**
Medium
IL-33
IL-33 + IL-10
IL-9
(pg/10,000 ILC2s)
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80
60
40
20
0
*
*
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IL-33
IL-33 + IL-10
IL-13
(pg/10,000 ILC2s)
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2000
1500
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500
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**
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IL-33
IL-33 + IL-10
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IL-33 + TGFb1
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(pg/10,000 ILC2s)
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*
*
Medium
IL-33
IL-33 + TGFb1

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10
1
*
DMSO
Dex
Dex + RU
IL-33 + TSLP
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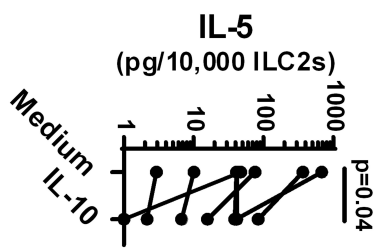
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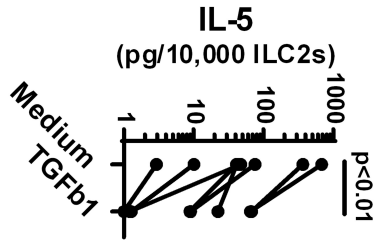
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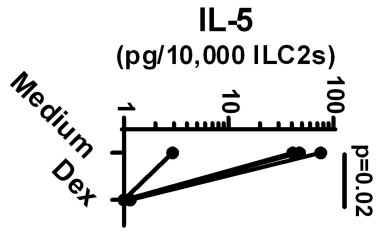
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