

IL-1 β , IL-4 and IL-12 control the fate of group 2 innate lymphoid cells in human airway inflammation in the lungs

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Group 2 innate lymphoid cells (ILC2s) secrete type 2 cytokines, which protect against parasites but can also contribute to a variety of inflammatory airway diseases. We report here that interleukin 1 β (IL-1 β) directly activated human ILC2s and that IL-12 induced the conversion of these activated ILC2s into interferon- γ (IFN- γ)-producing ILC1s, which was reversed by IL-4. The plasticity of ILCs was manifested in diseased tissues of patients with severe chronic obstructive pulmonary disease (COPD) or chronic rhinosinusitis with nasal polyps (CRSwNP), which displayed IL-12 or IL-4 signatures and the accumulation of ILC1s or ILC2s, respectively. Eosinophils were a major cellular source of IL-4, which revealed cross-talk between IL-5-producing ILC2s and IL-4-producing eosinophils. We propose that IL-12 and IL-4 govern ILC2 functional identity and that their imbalance results in the perpetuation of type 1 or type 2 inflammation.

At steady state, the human respiratory tract is protected against airborne pathogens, pollutants and other challenges from the environment through crosstalk between epithelial cells and tissue-resident immune cells. However, perpetual exposure to certain microbial pathogens, toxins or other environmental agents often results in respiratory diseases, such as asthma, CRS, COPD and cancer^{1,2}.

Whereas CRSwNP and allergic asthma are type 2 inflammatory diseases^{3,4}, COPD is a heterogeneous airway disease with elements of type 1 inflammation, including elevated production of IL-12, IL-18 and IFN- γ ^{5,6}. Although airway epithelial cells and innate immune cells are essential components in the process that drives chronic airway inflammatory diseases⁷, the mechanisms by which these cells contribute to disease development remain poorly understood.

ILCs are a family of effector cells that are important for protection against infiltrating pathogens and restoration of tissue integrity⁸. Three major subsets have been defined on the basis of their phenotype and functional similarities to helper T cells: group 1 ILCs, which include the prototypical natural killer cell and IFN- γ -secreting ILC1 subsets; ILC2s, which are endowed with the ability to secrete the type 2 helper T cell (T_H2 cell)-associated cytokines (IL-4, IL-5, IL-9 and IL-13); and group 3 ILCs, which include lymphoid-tissue-inducer cells and ILC3s able to produce IL-22, IL-17 and the cytokine GM-CSF⁹.

At steady state, ILC1s are present only at a low frequency within tissues, whereas ILC2s and ILC3s represent the most prominent subsets that contribute to protective innate immune responses, tissue homeostasis and repair. However, when dysregulated, ILCs can promote inflammation-driven pathology^{10,11}. ILC1s accumulate in the lamina propria of patients with Crohn's disease¹²; patients with type 2 diseases show enrichment for ILC2s, such as in blood, sputum and bronchoalveolar lavage (BAL) samples of allergic asthmatic patients^{13,14}, in polyps from patients with CRS¹⁵, and in the skin of patients with atopic dermatitis¹⁶; and NKp44⁺ ILC3s are higher in frequency in psoriatic skin lesions and obesity-induced asthma^{17,18}.

The composition of ILCs might be altered in diseased tissues by the recruitment of ILC subsets from the periphery. However, it has been reported that ILCs are tissue-sessile cells, with limited migration of hematogenic ILCs into tissue¹⁹. Another mechanism by which the ILC composition might change is the transdifferentiation of one ILC subset into another. Indeed, bidirectional transdifferentiation between ILC1s and ILC3s in the intestinal lamina propria has been reported^{12,20,21}: ILC3s differentiate into IFN- γ -secreting CD127⁺ ILC1s under the influence of the type 1 cytokine IL-12, whereas ILC1s are reverted by IL-23, IL-1 β and retinoic acid into IL-22-secreting ILC3s.

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Here we observed that IFN- γ -producing ILC1s accumulated in lung tissue from patients with the inflammatory disease severe COPD. Investigating the possible origin of these ILC1s, we found that ILC2s had the ability to transdifferentiate into ILC1s in an IL-12 dependent manner. The conversion of ILC2s into ILC1s was reversed by IL-4. The importance of IL-4 in maintaining ILC2 phenotype and function was emphasized by the observation that both the expression of *IL4* mRNA and the frequency of ILC2s were greater in nasal polyps from patients with CRS than in control turbinates of healthy subjects. Eosinophils were an important source of IL-4 in nasal polyps from patients with CRS. Overall, our data reveal considerable plasticity of ILC2s.

RESULTS

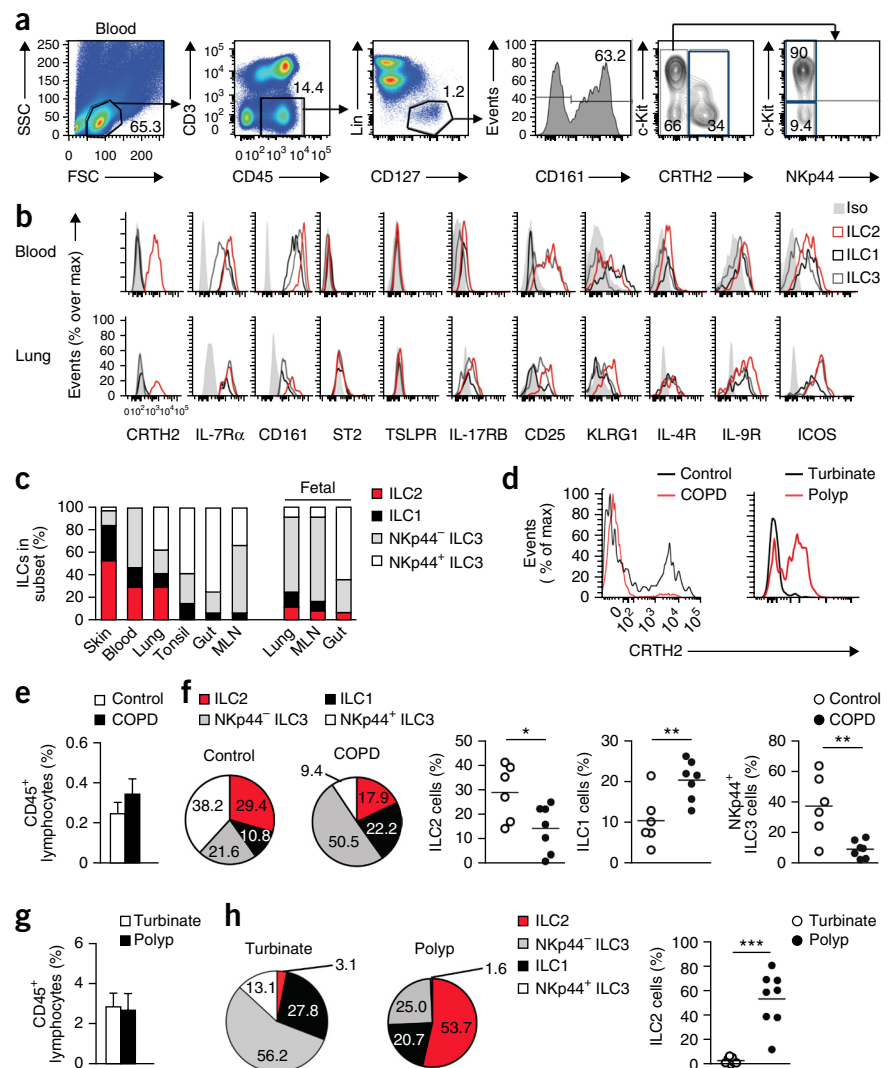
Altered ILC tissue composition in chronic airway inflammation

Human ILC2s are characterized by expression of the marker CRTH2. To explore whether additional markers could help to further define human ILC2s, we performed a comprehensive phenotypic analysis of all ILC subsets using a previously established gating strategy²² (Fig. 1a

and Supplementary Fig. 1a,b). All human ILC subsets in peripheral blood and lung tissue expressed the cytokine receptor subunit IL-7R α (CD127) and the natural killer cell receptor CD161, although this expression was highest on ILC2s (Fig. 1b). ILC2s also expressed the costimulatory receptor ICOS, the cytokine receptor subunit IL-2R α (CD25)²³, the cytokine receptors IL-4R and IL-9R, the senescence marker KLRG1 and the IL-25 receptor IL-17RB, but expression of these was not specific (Fig. 1b). Furthermore, and in contrast to findings published elsewhere²⁴, expression of the cytokine receptor IL-33R (ST2) and the receptor for thymic stromal lymphopoietin (TSLP) was undetectable on the cell-surface membrane of freshly isolated ILCs from blood or lung tissues (Fig. 1b), although transcripts encoding these have been detected in published studies^{15,25}. Thus, the only surface marker tested that in our analyses uniquely identified human ILC2s among CD161⁺CD127⁺ ILCs was CRTH2.

Although ILCs are found in many organs throughout the human body, their composition varies considerably among tissues²². Side-by-side comparison of ILC ratios in peripheral blood, skin, tonsil,

Figure 1 Phenotype and tissue distribution of human ILCs, and changes in ILC ratios in COPD and nasal polyps. (a) Gating strategy for the detection, by flow cytometry, of ILCs in human blood, with ILCs defined as negative for lineage markers (Lin⁻: CD1a⁻CD3⁻CD14⁻CD16⁻CD19⁻CD34⁻CD94⁻CD123⁻BDCA2⁻Fc ϵ R1 α ⁻TCR $\alpha\beta$ -TCR $\gamma\delta$ -) and CD45⁺CD127⁺CD161⁺. Numbers adjacent to outlined areas (left half) indicate percent lymphocytes (far left), CD3⁻CD45⁺ cells (middle left) or Lin⁻CD127⁺ cells (left); number above bracketed line (middle) indicates percent CD161⁺ cells; and numbers in outlined areas (right) indicate percent CRTH2⁻ (ILC1 or ILC3) cells (left half) and CRTH2⁺ (ILC2) cells (right half) (middle right plot), and c-Kit⁺NKp44⁻ (ILC3) cells (top) or c-Kit⁺NKp44⁺ (ILC1) cells (bottom) (far right plot) among the CRTH2⁺ cells at left. SSC, side scatter; FSC, forward scatter. (b) Flow cytometry characterization of ILC1, ILC2 and ILC3 subsets in blood (top row) and non-inflamed lungs (bottom row). Iso, isotype-matched control antibody. (c) Quantification of ILC1, ILC2, and NKp44⁺ and NKp44⁻ ILC3 cells (defined as in a) in adult skin ($n = 5$ donors), blood ($n = 20$ donors), lungs ($n = 6$ donors), tonsils ($n = 3$ donors), gut ($n = 6$ donors) and mesenteric lymph nodes (MLN) ($n = 4$ donors) (left), and in fetal lungs ($n = 6$ donors), mesenteric lymph nodes ($n = 5$ donors) and gut ($n = 6$ donors) (right). (d) Flow cytometry analyzing CRTH2 expression in total ILCs (CD45⁺Lin⁻CD127⁺CD161⁺) from lungs of patients with COPD at GOLD stage IV (COPD) and those of healthy subjects and patients with COPD at GOLD stage I and II (Control) (left) or in nasal polyps from patients with CRS (Polyp) and nasal tissue from healthy control subjects (Turbinate) (right). (e) Frequency of total ILCs in lungs from control subjects and patients with COPD as in d, left (key). (f) Frequency of ILC subsets in lungs from healthy control subjects and patients with COPD, collectively (left) and separately (right). Each symbol (right) represents an individual donor; small horizontal lines indicate the mean. (g) Frequency of total ILCs in healthy nasal tissue and nasal polyps as in d, right (key). (h) Frequency of ILC subsets in turbinate and nasal polyps as in g (presented as in f). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (t -test). Data are from one experiment representative of three (a,b) or six (d) experiments with similar results, one experiment with three to twenty donors (c), or one experiment with six (e-g) or seven (h) donors (mean $\pm$ s.e.m. in e,g).



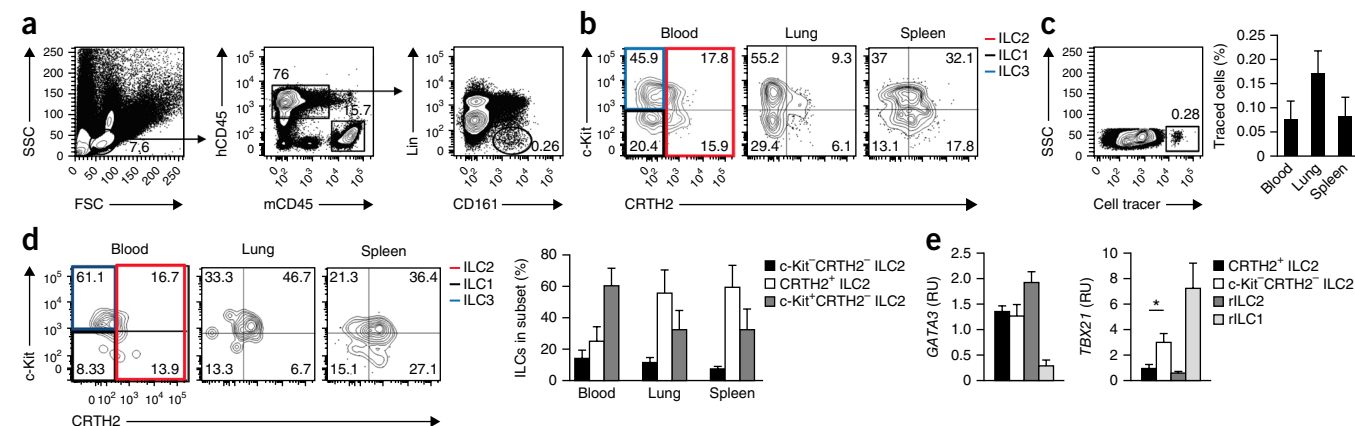


Figure 2 Transferred ILC2s alter their phenotype and transcription-factor profile in *Il2rg*^{-/-} NOD-SCID mice engrafted with human immune cells. (a) Gating strategy for the detection, by flow cytometry, of human ILCs in the spleen of humanized *Il2rg*^{-/-} NOD-SCID mice. Numbers adjacent to outlined areas indicate percent lymphocytes (far left), cells positive for human (h) CD45 and negative for mouse (m) CD45 (hCD45⁺mCD45⁻) or vice versa (hCD45⁻mCD45⁺) (middle), or Lin⁻CD161⁺ cells among the hCD45⁺ cells at left (human ILCs) (right). (b) Reconstituted human ILC subsets (defined as in **Fig. 1a**) in the blood, lungs and spleen of humanized mice (as in **a**), categorized as ILC1, ILC2 and ILC3 (outlined areas, far left; key, above) on the basis of expression of c-Kit and CRTH2. Numbers in quadrants indicate percent cells in each throughout. (c) Flow cytometry of cell-tracer-labeled human ILC2s from an *Il2rg*^{-/-} NOD-SCID mice 4 d after retro-orbital inoculation (1×10^5 to 1×10^6 donor cells per host mouse) (left), and the frequency of cell-tracer-positive ILCs among total hCD45⁺ cells in the blood, lungs and spleen of such mice (right). Number adjacent to outlined area (left) indicates cell-tracer-positive ILCs. (d) Flow cytometry analyzing transferred human ILC subsets in the blood, lungs and spleen of mice as in **c** (defined and presented as in **b**) (left), and frequency of c-Kit⁺CRTH2⁻, CRTH2⁺ or c-Kit⁺CRTH2⁺ ILC2s (key) in the blood, lungs and spleen of those mice (right). (e) Expression of *GATA3* and *TBX21* in CRTH2⁺ or c-Kit⁺CRTH2⁻ ILC2s pooled from various organs and in reconstituted (r) ILC1s and ILC2s (key), presented (in relative units (RU)) relative to expression of the control gene *ACTB*. **P* < 0.05 (*t*-test). Data are from one experiment representative of five experiments with one to two mice in each (one to three donors per reconstitution; **a–d**; mean + s.e.m. in **c,d**) or are pooled from three experiments with four mice (**e**; mean + s.e.m.).

lungs, intestine and mesenteric lymph nodes revealed that ILC3s were the predominant population in all tissues analyzed, whereas CD127⁺ ILC1s were present only at a low frequency (**Fig. 1c**). Adult mucosa-associated tissues showed selective enrichment for NKp44⁺ ILC3s, relative to the abundance of these cells in peripheral blood and fetal tissues. ILC2s represented approximately 10% of the total ILC population in fetal lungs, and this value rose to a frequency of greater than 25% in adult lungs. In contrast, the frequency of ILC2s was diminished in adult mesenteric lymph nodes and intestinal tissues. Thus, lungs were the only tissue investigated in which both ILC2s and NKp44⁺ ILC3s coexisted at a substantial frequency at steady state.

The frequency of ILC2s was lower in lung tissue from patients with severe COPD (The Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV) than in that from healthy subjects or from patients with less-severe COPD (GOLD stages I and II) (**Fig. 1d,f**; patient characteristics, **Supplementary Table 1**), whereas the total frequency of CD45⁺ lymphocytes was unaltered (**Fig. 1e**). In parallel, we observed marked enrichment for ILC1s and NKp44⁺ ILC3s in lung tissue from patients with severe COPD (GOLD stage IV) relative to their abundance in lung tissue from healthy subjects and from patients

with less-severe COPD (GOLD stages I and II) (**Fig. 1f**). The nasal polyps of patients with CRS show enrichment for ILC2s relative to their abundance in healthy turbinate tissue¹⁵. Here we observed that whereas the total frequency of CD45⁺ lymphocytes was unchanged in nasal polyps of patients with CRS relative to their frequency in healthy turbinate tissue (**Fig. 1g**), the frequency of both ILC1s and ILC3s was diminished in parallel with an increased frequency of ILC2s in the nasal polyps (**Fig. 1h**). Together these data suggested that an inflammatory environment altered ILC composition.

Change in ILC2 phenotype *in vivo*

To evaluate whether the microenvironment determines the stability of the ILC2 phenotype, we employed a mouse model of the human immune system. At 8 weeks after injection of human hematopoietic stem cells into sublethally irradiated newborn mice of the non-obese diabetic (NOD)–severe combined immunodeficiency (SCID) strain that were deficient in the common γ -chain of the IL-2 receptor (*Il2rg*^{-/-})²⁶, 80% of the immune cells of these mice were of human origin (**Fig. 2a** and **Supplementary Fig. 2a**), and the blood, spleen and lungs were repopulated with human ILCs, including ILC2s (**Fig. 2b**).

Figure 3 IL-1 β activates ILC2s. (a) Flow cytometry analyzing intracellular IL-5 and production of IFN- γ in Lin⁻CD127⁺CD161⁺ CRTH2⁺ ILC2s isolated from peripheral blood and cultured for 6 d with IL-2 alone, IL-2 plus IL-1 β , or IL-2 plus IL-33 plus TSLP (left), and frequency of IL-5-producing cells among those ILC2s (middle right) and ELISA of IL-13 in those ILC2s (far right). (b) IL-5 production by ILC2s stimulated for 6 d with various combinations of cytokines (below plot). **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (one-way analysis of variance (ANOVA)). Data are from one experiment with four biological replicates (**a**, left) or four pooled biological replicates (**a** (right), **b**; mean + s.e.m.).

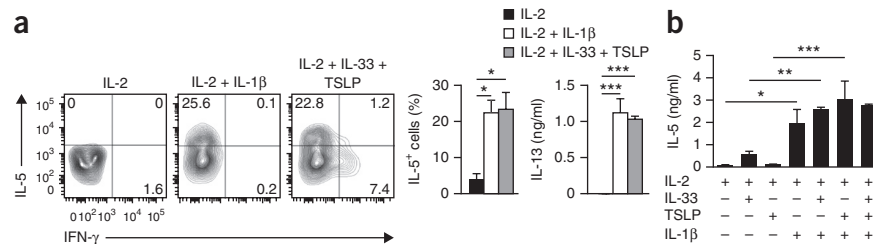
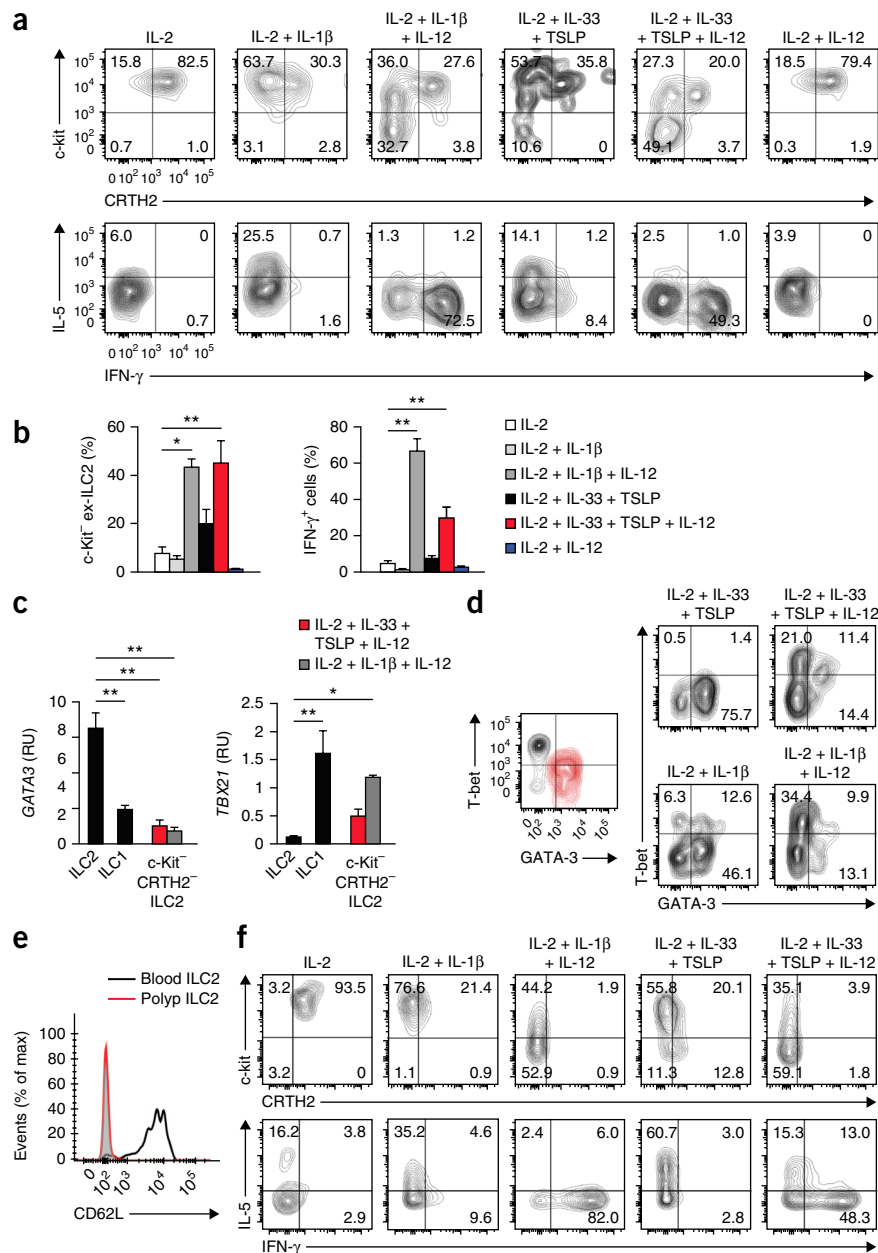


Figure 4 IL-12 governs the transdifferentiation of ILC2s into IFN- γ -producing cells.

(a) Expression of c-Kit and CRTH2 (top) and intracellular IL-5 and secreted IFN- γ (bottom) of ILC2s purified from blood (gating strategy, Fig. 1a) and activated for 6 d with IL-2 alone (far left) or with IL-2 and various combinations (above plots) of IL-1 β , IL-12, IL-33 and TSLP. (b) Frequency of c-Kit⁺CRTH2⁺ ILC2s ('ex-ILC2') (left) and IFN- γ -producing cells (right) generated in the activation conditions in a (key). (c) Expression of *GATA3* and *TBX21* mRNA by ILC2s and ILC1s or by c-Kit⁺CRTH2⁺ ILC2s sorted after stimulation with IL-2, IL-33, TSLP and IL-12 or with IL-2, IL-1 β and IL-12 (key) (presented as in Fig. 2e). (d) Intracellular staining of T-bet and GATA-3 in ILC2s (red) and ILC1s (black) (left) and in ILC2s stimulated as in c (right). (e) Expression of CD62L on ILC2s in the peripheral blood of healthy subjects and nasal polyps from patients with CRS. Light gray shading, isotype-matched control antibody. (f) Expression of c-Kit and CRTH2 (top row) and of intracellular IL-5 and secreted IFN- γ (bottom row) of Lin⁺CD127⁺CD161⁺CRTH2⁺ ILC2 sorted from nasal polyp tissue and stimulated for 6 d as in a. * $P < 0.01$ and ** $P < 0.001$ (one-way ANOVA). Data are from one experiment representative of four (a,f) or three (d,e) independent experiments, are pooled from three experiments (b; mean + s.e.m.) or are from one experiment with four donors (c; mean + s.e.m.).



These observations indicated that these host mice were able to support the development and maintenance of human ILC2s.

Next we expanded populations of purified blood CRTH2⁺ ILC2s and subsequently resorted the resultant cells to a purity of >98% (ref. 15) (Supplementary Fig. 2b). We labeled the purified ILC2s with a cell-tracer dye and injected them intravenously into mice of the human immune system model described above (*Il2rg*^{-/-} NOD-SCID mice engrafted with human hematopoietic stem cells) to monitor the fate of the ILC2s *in vivo*. Analysis of blood, lungs and spleen 4 d after injection revealed a small but distinct population of labeled cells that 'preferentially' homed to the lungs (Fig. 2c). Whereas labeled cells cultured *in vitro* remained CRTH2⁺ (Supplementary Fig. 2c), we observed partial loss of CRTH2 expression in all tissues in our *in vivo* model (Fig. 2d), which mimicked the phenotype of NKp44⁺ ILC3s and ILC1s. Transcriptional analysis of the labeled cells revealed that although c-Kit⁺CRTH2⁺ ILC2s and CRTH2⁺ ILC2s had similar expression of *GATA3*, which encodes the transcription factor GATA-3, the former had higher expression of *TBX21*, which encodes the transcription factor T-bet (Fig. 2e). No cytokine-encoding transcripts were detectable (data not shown). These results suggested that a proportion of ILC2s might convert to other subsets under the influence of an altered microenvironment.

Control of the fate of IL-1 β activated ILC2s by IL-12

IL-1 β activates ILC3s, particularly in the presence of IL-23 (refs. 12,27,28). IL-1 β also acts together with IL-23 and retinoic acid

to transdifferentiate ILC1s into ILC3s²⁰. As ILC2s also express the receptor for IL-1 β ¹⁵, we assessed the effect of IL-1 β on ILC2s. We observed that IL-1 β in combination with low concentrations of IL-2 (which by itself does not activate ILC2s) strongly stimulated the production of IL-5 and IL-13 by ILC2s (Fig. 3a). The IL-5 production induced by IL-2 and IL-1 β was even greater than that induced by the combination of IL-2 and IL-33 or IL-2 and TSLP and was boosted by the combination of IL-1 β with either IL-2 and IL-33 or IL-2 and TSLP (Fig. 3b). These data indicated that IL-1 β was a strong ILC2-stimulating cytokine.

People with COPD exhibit elevated titers of the inflammatory cytokines IL-1 β , IL-12 and IFN- γ ^{5,29}, which raises the possibility that these cytokines contribute to the transdifferentiation of ILC3s into ILC1s¹². As ILC2 frequency, like that of NKp44⁺ ILC3s, was diminished in patients with COPD (Fig. 1e), we sought to determine whether IL-12 was also able to drive differentiation of ILC2s into ILC1s. We cultured freshly isolated human peripheral blood ILC2s,

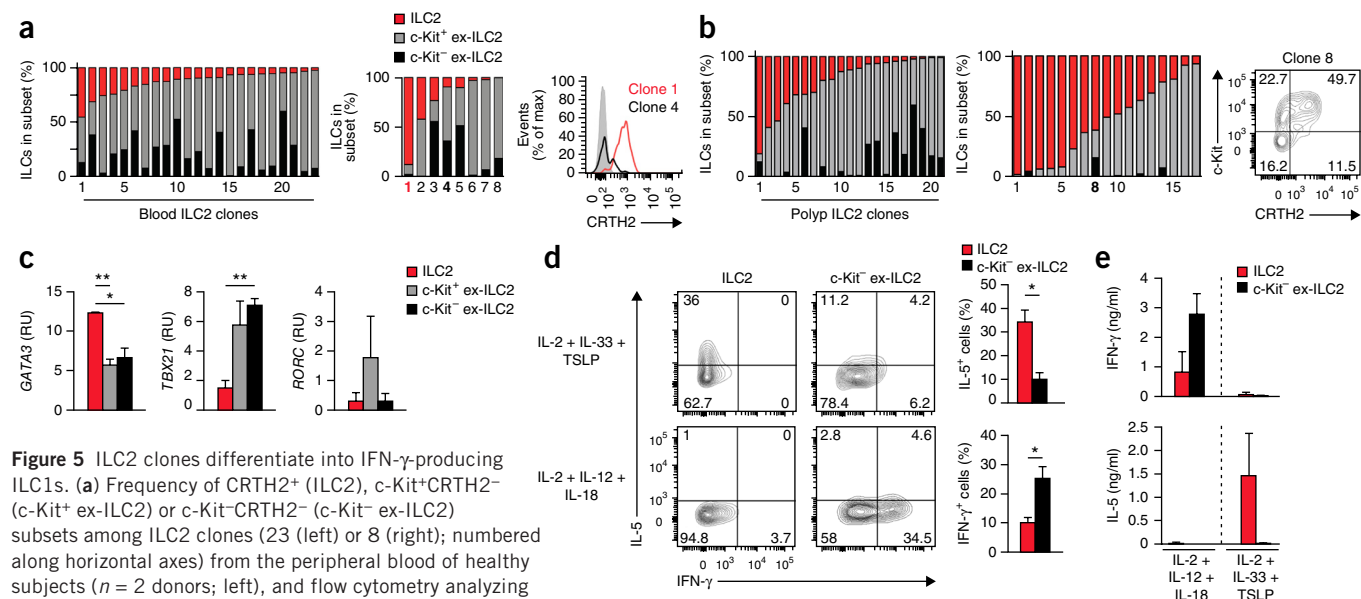


Figure 5 ILC2 clones differentiate into IFN- γ -producing ILC1s. (a) Frequency of CRTH2⁺ (ILC2), c-Kit⁺CRTH2⁻ (c-Kit⁺ ex-ILC2) or c-Kit⁻CRTH2⁻ (c-Kit⁻ ex-ILC2) subsets among ILC2 clones (23 (left) or 8 (right); numbered along horizontal axes) from the peripheral blood of healthy subjects ($n = 2$ donors; left), and flow cytometry analyzing CRTH2 expression on two of those ILC2 clones (1 and 4; bold at left) after 3 weeks of culture (right). (b) Frequency of ILC2 subsets (as in a) among ILC2 clones (numbered as in a) from nasal polyps of patients with CRS ($n = 2$ donors; left), and flow cytometry analyzing the expression of c-Kit and CRTH2 on clone 8 (bold at left) (right). (c) Expression of *GATA3*, *TBX21* and *RORC* mRNA by ILC2 subsets (as in a; sorted by expression of CRTH2 and c-Kit) derived from ILC2 clones (presented as in Fig. 2e). (d) Flow cytometry (left) of intracellular IL-5 in and production of IFN- γ by CRTH2⁺ ILC2s (left) and c-Kit⁻CRTH2⁻ ILC2s (c-Kit⁻ ex-ILC2; right) sorted from peripheral blood clones and activated for 2–3 d with IL-2, IL-33 and TSLP (top row) or with IL-2, IL-12 and IL-18 (bottom row). Right, frequency of IL-5⁺ cells (top) or IFN- γ ⁺ cells (bottom) among those subsets (key). (e) Production of IFN- γ and IL-5 by CRTH2⁺ and c-Kit⁻CRTH2⁻ ILC2 fractions (as in d) sorted from nasal polyp clones and activated for 4 d with IL-2, IL-33 and TSLP (left) or with IL-2, IL-12 and IL-18 (right). * $P < 0.05$ and ** $P < 0.01$ (one-way ANOVA). Data are from two experiments representative of at least three independent experiments with one donor in each (a,b), or are pooled from three independent experiments (c–e; mean $\pm$ s.e.m.).

with or without IL-12, in combination with IL-33 plus TSLP or with IL-1 β (Fig. 4a). The combination of IL-33 plus TSLP induced ILC2s to secrete IL-5 and IL-13 (Fig. 4a,b), and this was accompanied by some downregulation of CRTH2 expression (Supplementary Fig. 3a). The decrease in CRTH2 expression did not affect the capacity of these cells to produce IL-5 and IL-13 upon subsequent activation with IL-33 plus TSLP (Supplementary Fig. 3a). The addition of IL-12 to IL-33 and TSLP caused substantial downregulation of CRTH2 expression and cell-surface-marker c-Kit (CD117) expression; this resulted in cells with a c-Kit⁻CRTH2⁻ phenotype (Fig. 4a,b), which is a phenocopy of ILC1s¹². We observed a similar change in phenotype after stimulation with IL-1 β plus IL-12, whereas IL-12 alone did not induce this change (Fig. 4a,b), even though all human ILC subtypes expressed transcripts encoding both subunits of the IL-12 receptor¹² (Supplementary Fig. 3b). In agreement with those findings, we observed robust production of IFN- γ in ILC2s stimulated with IL-1 β plus IL-12 or IL-33, and TSLP plus IL-12 (Fig. 4a,b). Stimulation of ILC2s in the presence of IL-12 resulted in a substantial reduction in expression of *GATA3* transcripts and *GATA-3* protein and upregulation of T-bet expression relative to that of cells stimulated without IL-12 (Fig. 4c,d). Moreover, ILC2s stimulated in the presence of IL-12 were no longer responsive to IL-33 and TSLP but became responsive to IL-12 and IL-18, in a manner similar to that of true ILC1s (Supplementary Fig. 3c,d).

Blood-derived ILC2s from healthy subjects had high expression of the adhesion molecule L-selectin (CD62L), which was absent in ILC2s from nasal polyps of patients with CRS (Fig. 4e); this indicated that ILC2s in nasal polyps were more mature than those in blood. Both polyp-derived ILC2s and human fetal-lung-derived ILC2s downregulated their expression of CRTH2 and c-Kit in response to ILC1-inducing cytokines and produced IFN- γ but not IL-5 (Fig. 4f

and Supplementary Fig. 3e,f). These results indicated that ILC2s had the potential to transdifferentiate into ILC1s when exposed to a type 1 inflammatory environment, such as that present in the lungs of patients with COPD.

To rule out the possibility that the phenotypic and functional changes observed in ILC2s were a consequence of the 'preferential' outgrowth of a few contaminating ILC1s, we performed clonal analyses of ILC2s isolated from the peripheral blood of healthy subjects and from the nasal polyps of patients with CRS. We cultured single ILC2s with irradiated allogeneic lymphocytes²⁵, which provided a source of IL-12 and IL-1 β (Supplementary Table 2). The cloning efficiencies (number of positive wells relative to total wells seeded, for samples from two different donors) were 23% and 8% (peripheral blood) and 21% and 17% (nasal polyps). In all experiments, we observed clones that maintained CRTH2 expression, clones that partly maintained CRTH2 expression, and clones with undetectable CRTH2 expression (Fig. 5a,b). The proportion of clones that retained their CRTH2 expression was highest among those isolated from nasal polyps (Fig. 5b). From selected clones we isolated CRTH2⁺ cells and c-Kit⁻CRTH2⁻ cells and analyzed their expression of transcription factors and production of cytokines after stimulation. We observed that the CRTH2⁺ fraction of peripheral-blood-derived ILC2 clones had higher expression of *GATA3* mRNA than that of the CRTH2⁻ ILC2 clones (Fig. 5c). Conversely, both c-Kit⁺CRTH2⁻ cells and c-Kit⁻CRTH2⁻ cells isolated from the same heterogeneous clone had high expression of *TBX21* mRNA, and the c-Kit⁺CRTH2⁻ ILC2s had higher expression of *RORC* mRNA (which encodes the transcription factor ROR γ t) than that of the c-Kit⁻CRTH2⁻ cells (Fig. 5c). The CRTH2⁺ ILC2s responded to IL-33 plus TSLP by secreting IL-5 and IL-13, whereas the c-Kit⁻CRTH2⁻ cells responded only modestly to these cytokines (Fig. 5d). The c-Kit⁻CRTH2⁻ progeny of clones from

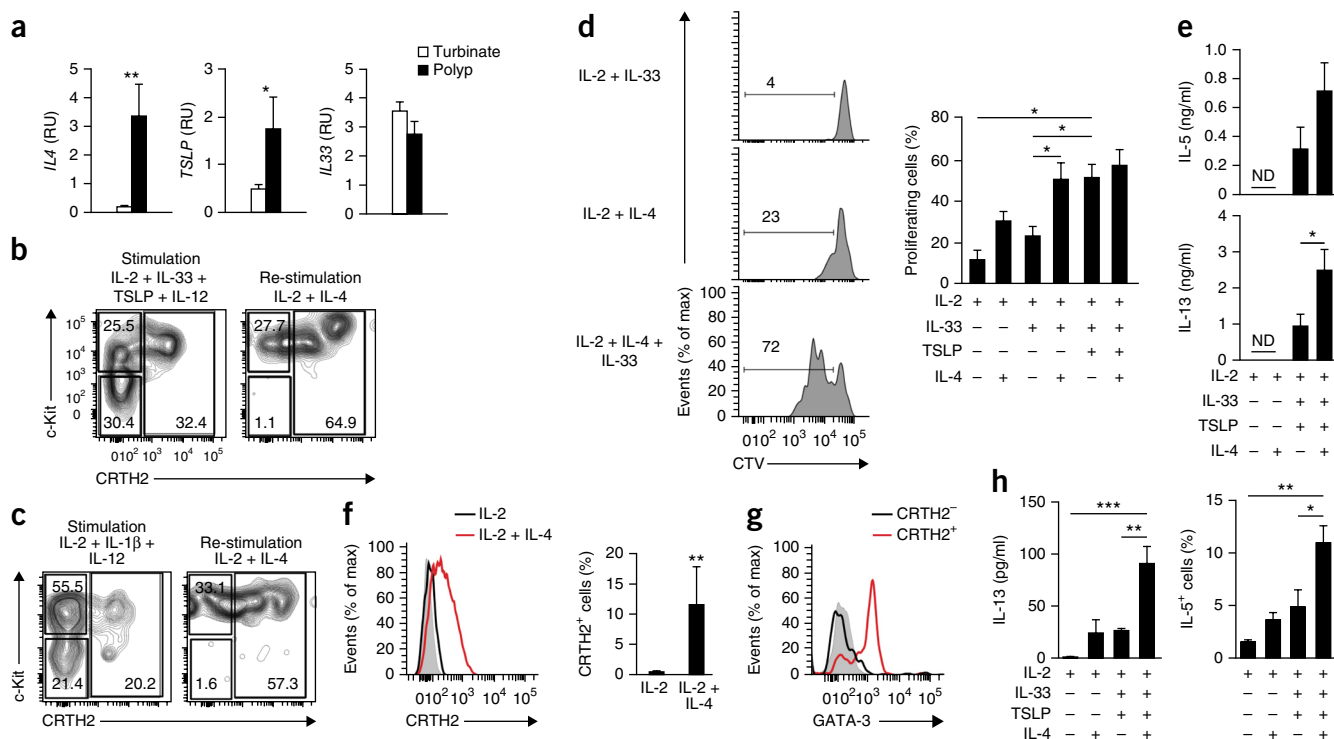


Figure 6 IL-4 is essential for maintenance of CRTH2 expression and expansion of ILC2 populations. **(a)** Expression of *IL4*, *TSLP* and *IL33* mRNA in healthy turbinate tissue and nasal polyps (presented as in **Fig. 2e**). **(b,c)** Flow cytometry of blood ILC2s stimulated for 5 d with IL-2, IL-33, TSLP and IL-12 (**b**, left) or with IL-2, IL-1 β , and IL-12 (**c**, left) and then successively re-stimulated for 5 d with IL-2 plus IL-4 (right). Numbers in outlined areas indicate percent c-Kit⁺CRTH2⁻ (ILC3) cells (top left), c-Kit⁺CRTH2⁻ (ILC1) cells (bottom left) or CRTH2⁺ (ILC2) cells (right). **(d)** Flow cytometry (left) of blood ILC2s labeled with CellTrace Violet (CTV) and stimulated for 5 d with various combinations of cytokines (left margin), and frequency of proliferating cells among those cells (right). Numbers above bracketed lines (left) indicate percent proliferating cells. **(e)** Production of IL-5 and IL-13 by blood ILC2s stimulated for 5 d with IL-2 in the presence (+) or absence (-) of IL-33, TSLP or IL-4 (below plot); results are normalized per 5×10^3 cells. **(f)** Flow cytometry (left) analyzing the expression of CRTH2 on total BAL-fluid ILCs after 2 weeks of population expansion with feeder cells in the presence of IL-2 alone or IL-2 plus IL-4 (key), and frequency of CRTH2⁺ cells among those cells (right). **(g)** GATA-3 expression in the CRTH2⁺ and CRTH2⁻ fractions (key) of BAL-fluid ILCs after population expansion. Light gray shading, isotype-matched control antibody. **(h)** Production of IL-13 by (left) and frequency of IL-5⁺ cells among (right) total BAL-fluid ILCs after population expansion and 6 d of stimulation with various combinations of cytokines (below plot). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (*t*-test (**a,f**) or one-way ANOVA (**d,e,h**)). Data are from one experiment with at least four donors (**a**; mean + s.e.m.) or one experiment representative of three (**b,c,g**), five (**f**) or six (**d**) independent experiments (mean + s.e.m. in **d,f**) or are from four (**e,h**), five (**f**) or six (**d**) independent experiments (mean + s.e.m. in **e,h**).

peripheral blood and nasal polyps were much more responsive to type 1 stimuli than were CRTH2⁺ ILC2s, and they secreted IFN- γ in response to IL-12 and IL-18 (**Fig. 5d,e**). Together these data indicated that ILC2s isolated from either peripheral blood or nasal polyps were able to acquire features of IFN- γ -producing ILC1s.

IL-4 promotes ILC2 maintenance and proliferation

Although CRSwNP and COPD are both inflammatory airway diseases^{3,4,6}, ILC2s were present at an increased frequency only in nasal polyps from patients with CRS (**Fig. 1h**). As the opposing roles of IL-4 and IL-12 in helper T cell differentiation are well documented³⁰, we compared the expression of transcripts encoding these cytokines in nasal polyps with that in healthy turbinate tissue and observed that the amount of *IL4* mRNA was elevated in polyp tissues relative to that in healthy turbinate tissue, while *IL12* mRNA was undetectable in polyp tissues (**Fig. 6a** and data not shown). We did not detect differences between nasal polyps and healthy turbinate tissue in their expression of *IL33* transcripts³¹, but expression of *TSLP* mRNA was higher in total polyp tissue from patients with CRS than in healthy, non-inflamed turbinate tissue (**Fig. 6a**).

To investigate whether IL-4 was involved in inducing or maintaining CRTH2 expression, we first cultured peripheral-blood-derived

ILC2s under ILC1-polarizing conditions. We then re-stimulated these cells in the presence of IL-4 and observed that the expression of CRTH2 and that of c-Kit was partly restored and completely restored, respectively (**Fig. 6b,c**). Of note, stimulation of true CD127⁺ ILC1s or ILC3s from peripheral blood with IL-4 did not result in the upregulation of CRTH2 expression (**Supplementary Fig. 4a**), which indicated that the transdifferentiating influence of IL-4 was restricted to ILC2-derived ILC1s (former 'ex-' ILC2s). IL-4 acted in synergy with IL-33 to induce ILC2 proliferation, along with the production of IL-5 and IL-13, to a level comparable to that induced by IL-33 plus TSLP (**Fig. 6d,e**). IL-4 by itself, however, was insufficient to induce production of IL-5 and IL-13 (**Fig. 6d,e**).

In cells from patients undergoing bronchoscopy for diagnostic purposes for various indications, including asthma (patient characteristics, **Supplementary Table 3**), we found that the total ILC pool in BAL fluid lacked CRTH2⁺ ILC2s (**Supplementary Fig. 4b**). To test the hypothesis that the BAL-fluid ILC populations included ex-ILC2s, we expanded those populations *in vitro* in the presence of IL-4 and observed a distinct increase in CRTH2 expression and upregulation of GATA-3 expression (**Fig. 6f,g**). This suggested that a considerable proportion of the BAL-fluid ILCs were derived from ILC2s that had lost CRTH2 expression. In line with those findings, BAL-fluid ILCs

Figure 7 Anti-IL-12p70 treatment abrogates the conversion of ILC2s into ILC1-like cells. (**a–h**) Flow cytometry analyzing the expression of c-Kit and CRTH2 (**a,c,e,g**) and intracellular IL-13 and production of IFN- γ (**b,d,f,h**) by ILC2s and ILC3s sorted from peripheral blood and incubated for 5 d with supernatant from an MLR (MLR sup) alone (**a,b**) or with the MLR supernatant plus anti-IL-12p70 (α -IL-12p70) (5 μ g/ml) (**c,d**) or IL-4 (50 ng/ml) (**e,f**) or a combination of IL-4 and anti-IL-12p70 (**g,h**). (**i**) Frequency of newly generated c-Kit⁺CRTH2⁺ ex-ILC2s and ex-ILC3 cells (top) and IFN- γ -producing cells (bottom) among ILC2s and ILC3s activated as in **a–h** (key). (**j**) Flow cytometry of cells from a polyp homogenate treated for 6 d with IL-12 and anti-IL-4 (α -IL-4) (left) or with IL-4 and anti-IL-12p70 (right). Numbers adjacent to outlined areas (top) indicate percent Lin⁺CD127⁺ cells (numbers in or adjacent to outlined areas (bottom), as in **Fig. 6b**). Data are from one experiment representative of three (**a–i**) or four (**j**) independent experiments; mean $\pm$ s.e.m. in **i**).

stimulated with IL-33 and TSLP in the presence of IL-4 produced much larger amounts of IL-5 and IL-13 than the amounts produced after stimulation without IL-4 (**Fig. 6h**). Together these data indicated that the functional identity of ILC2s was restored by IL-4.

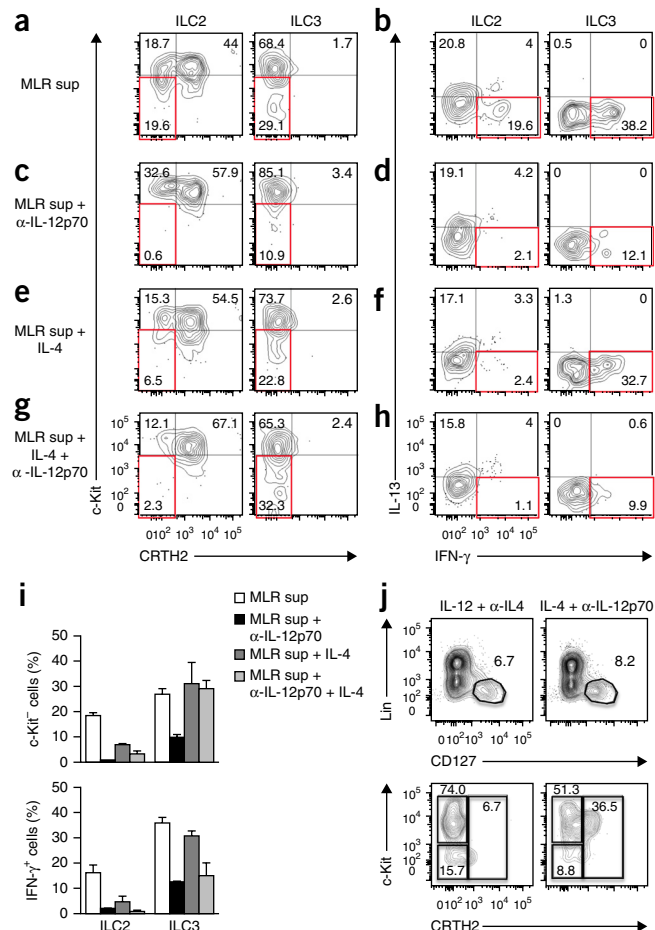
Targeting of IL-12p70 and IL-4 regulates ILC2 transdifferentiation

Next we sought to determine whether the neutralization of IL-12 or the addition of IL-4 would prevent the transdifferentiation of ILC2s into ILC1s. After exposure of blood-derived ILC2s and ILC3s to the supernatant of a mixed-lymphocyte reaction (MLR) that contained IL-12 (**Supplementary Table 2**), both ILC subsets downregulated their c-Kit expression, while ILC2s also downregulated their CRTH2 expression (**Fig. 7a**). Both ILC subsets secreted large amounts of IFN- γ after being cultured with the MLR supernatant followed by re-stimulation with the phorbol ester PMA and ionomycin (**Fig. 7b**). These findings suggested that factors in the MLR supernatant contributed to the differentiation of both ILC subsets into ILC1s. Neutralization of IL-12 mediated by antibody to IL-12p70 (anti-IL-12p70) in this setting inhibited the transdifferentiation of ILC2s, and also of ILC3s, into ILC1s (**Fig. 7c,d**). IL-4 dampened the polarization of ILC2s toward ILC1s to an extent similar to that achieved by neutralization of IL-12p70 but did not affect the transdifferentiation of ILC3s into ILC1s (**Fig. 7e,f**). The combined administration of anti-IL-12p70 and IL-4 did not result in a greater reduction in IFN- γ or an enhanced IL-13 response by ILC2s (**Fig. 7g–i**).

To investigate whether the administration of neutralizing antibodies and recombinant cytokines was also effective in a pathophysiological setting, we obtained polyp cells from patients with CRS and cultured total polyp cells in the presence of IL-4 and anti-IL-12p70 and observed that the CRTH2⁺ population was maintained (**Fig. 7j**). In contrast, culture of ILC2s in the presence of IL-12 and anti-IL-4 resulted in substantially downregulated CRTH2 expression (**Fig. 7j**). These findings confirmed critical roles for IL-12 and IL-4 in determining the ratio of ILC2s to ILC1s in inflamed tissues.

Cross-talk between eosinophils and ILC2 in nasal polyps

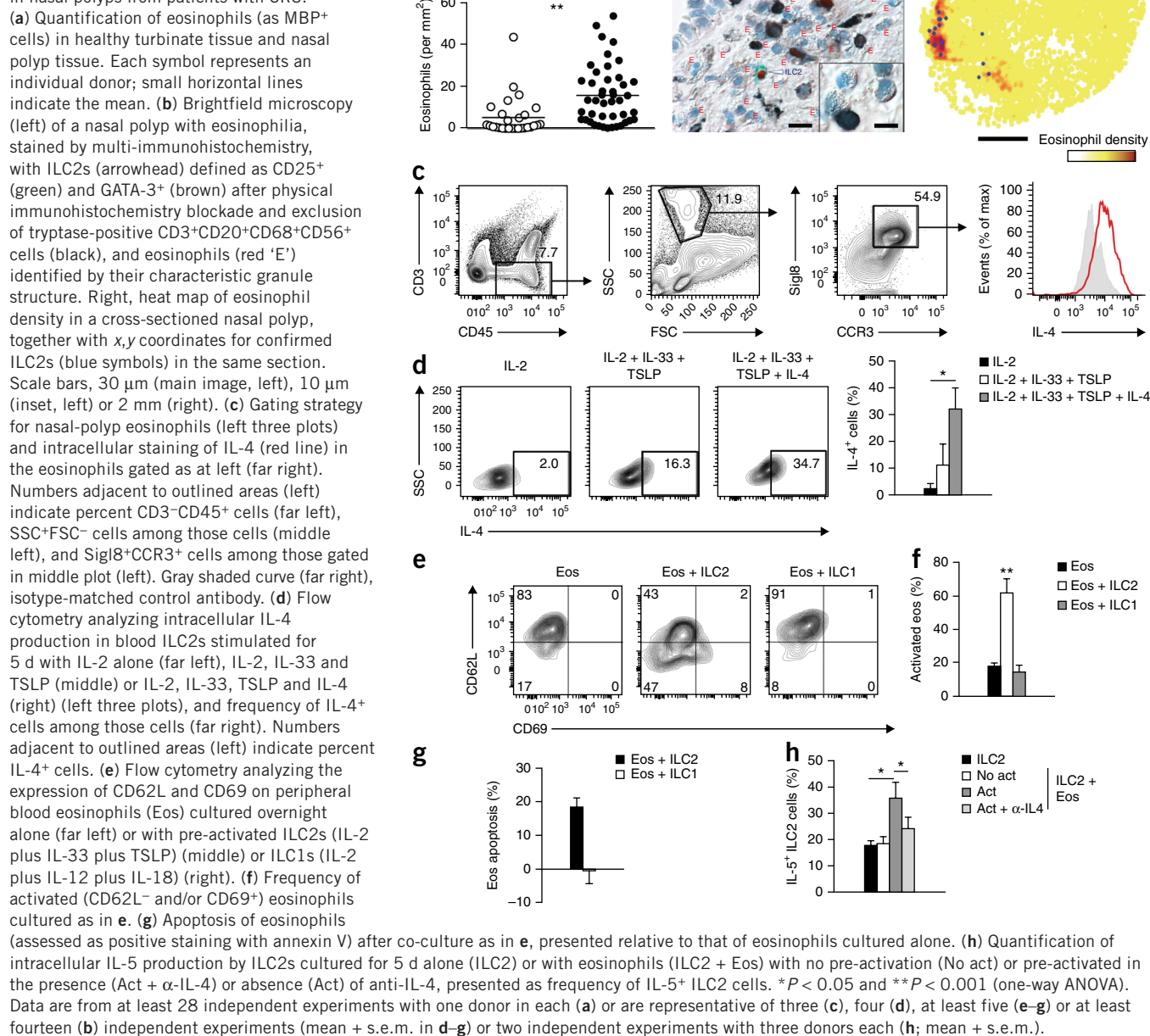
The observation of increased transcripts encoding IL-4 in nasal polyps of patients with CRS (**Fig. 6a**) raised the question of which cell type was the source of this cytokine. IL-4 production has been ascribed to mast cells, basophils and eosinophils³². As expected, we found a much greater abundance of eosinophils in nasal polyps of patients with CRS than in healthy turbinate tissue (**Fig. 8a** and **Supplementary Fig. 5**), whereas we detected a lower and only slightly greater number of basophils and mast cells, respectively, in nasal polyps than in healthy turbinate tissue (**Supplementary Table 4**). To study the spatial



relationship between ILC2s and eosinophils, we subjected nasal polyp tissues to an immunohistochemistry protocol that allows simultaneous visualization of ILC2s and eosinophils. We visualized eosinophils as positive for eosinophil cationic protein (EG2⁺) and visualized ILC2s as lineage-marker-negative, CD25⁺GATA-3⁺ cells (**Fig. 8b**, left). We categorized polyps as having tissue eosinophilia or no or a few scattered eosinophils. No ILC2s were present in the polyps with no or a few scattered eosinophils (data not shown). In contrast, we were able to identify ILC2s in 12 of the 14 polyps with eosinophilia (86%), and in these cases they had an average tissue density ranging between 0.03 ILC2s per mm² and 0.6 ILC2s per mm² (data not shown). Within the individual tissue sections, the eosinophilia had a patchy and heterogeneous distribution that co-localized spatially with presence of ILC2s (**Fig. 8b**, right). In such eosinophil-rich foci, the ILC2 density was greater than 2 ILC2s per mm² (data not shown). The spatial link between ILC2s and eosinophils was further illustrated by computerized Monte-Carlo-simulation-based methodology (**Supplementary Fig. 6**), which confirmed the proposal that ILC2s did not follow a pattern of random distribution but were instead embedded in eosinophilic areas.

Intracellular staining of freshly isolated polyp cells confirmed that in an inflammatory setting, eosinophils produced IL-4 (**Fig. 8c**). Eosinophils were not the only source of IL-4, as ILC2s themselves also produced IL-4 upon stimulation with IL-33 and TSLP, and this production was enhanced in the presence of exogenous IL-4 (**Fig. 8d**). Co-culture of eosinophils with ILC2s led to activation of the eosinophils, as indicated by the downregulation of CD62L expression and upregulation of CD69 expression on the eosinophils (**Fig. 8e,f**). This

Figure 8 Eosinophils and ILC2s activate each other and perpetuate type 2 inflammation in nasal polyps from patients with CRS.



stimulation resulted in prolonged survival of eosinophils (Fig. 8g). In contrast, ILC1s were unable to stimulate eosinophils and did not promote their survival (Fig. 8f,g). The eosinophil-ILC2 interplay resulted in the activation of not only the eosinophils but also the ILC2s, as co-culture of ILC2s with pre-activated eosinophils enhanced the IL-5 production of the ILC2s in an IL-4-dependent manner (Fig. 8h). Together these data suggested that in a type 2 inflammatory setting, eosinophil-produced IL-4 was able to act as a mediator for continued crosstalk between ILC2s and eosinophils.

DISCUSSION

COPD is a heterogeneous disease associated with elevated production of IL-1 β , IL-12, IL-33, IL-17 and IFN- γ in the inflamed lung tissue^{5,6}. Similarly, elevated concentrations of IL-12, IFN- γ and IL-33 have been measured in a COPD mouse model and were associated with a T_H1-like

inflammatory response⁶. We observed in lung specimens of patients with severe COPD that the frequency of ILC2s and Nkp44+ ILC3s was diminished, whereas the frequency of ILC1s was increased, relative to their frequency in lung samples from healthy (control) subjects. The overall ILC frequency was unaltered in patients with severe COPD relative to that of healthy (control) subjects, which suggested that the change in the ratio of ILC2s to ILC1s resulted from transdifferentiation rather than from the recruitment of ILC1s from peripheral blood. This would be consistent with published data showing that ILC2s, like other ILC subsets, are tissue-resident cells^{19,33}. Here we found that stimulation of ILC2 with IL-1 β or IL-33 plus TSLP in the presence of IL-12 induced a substantial increase in the expression of T-bet, which was associated with a reciprocal downregulation in the expression of GATA-3 and of its target, CRTH2 (ref. 25). The acquisition of T-bet expression resulted in the ability to produce high titers of IFN- γ and

the loss of production of IL-5 and IL-13. These findings indicated that IL-12 mediated the transdifferentiation of not only ILC3s but also ILC2s into ILC1s. The concentration of IL-1 β protein is elevated in sputum from patients with COPD⁵. Although IL-1 β has been thus far associated mostly with the activation of ILC3s, our studies presented here indicated that IL-1 β was also a major stimulator of ILC2s, being even more potent than IL-33. IL-12 was found to substantially modulate the effect of IL-1 β on ILC2s, changing IL-1 β from an activator of ILC2s into an inducer of ILC1s. Therefore, it is likely that chronic exposure to pro-inflammatory cytokines such as IL-12, in combination with IL-1 β and IL-33, contributes to the pathology of COPD by perpetuating type 1 immune responses. A related study showing that mouse ILC2s can transdifferentiate into IFN- γ ⁺ ILC1s following infection with influenza virus suggests that acquisition of IFN- γ production by ILC2s is of relevance to tailoring an optimal immune response to viral pathogens or intracellular bacteria³⁴. Of note, COPD is a heterogeneous disease, and therefore phenotypes may exist that are not characterized by an enhanced ILC1 frequency³⁵.

Loss of ILC2 identity as a consequence of activation in the presence of IL-12 raised the question of how ILC2 identity might be maintained. We observed that activation of ILC2s in the presence of IL-4 resulted in robust proliferation, which was accompanied by secretion of the type 2 cytokines IL-5 and IL-13. Moreover, freshly isolated ILCs in BAL fluid lacked phenotypic ILC2 markers, but culturing these ILCs in the presence of IL-4 resulted in the expression of GATA-3 and CRTH2, along with the ability to secrete large amounts of type 2 cytokines. In addition, IL-4 inhibited the IL-12-induced transdifferentiation of ILC2s. These findings indicated that IL-4 favored the maintenance of ILC2 function and phenotype. Given that IL-4 is expressed in nasal polyps of patients with CRSwNP, our findings suggest that this cytokine is responsible for the enhanced frequency of ILC2s in the inflamed nasal tissue in this disease. That hypothesis is supported by the finding that antibody-mediated neutralization of IL-4 in the presence of IL-12 decreased the frequency of ILC2s in homogenates of polyps from patients with CRS.

As has been reported, ILC2s are important regulators of eosinophil recruitment^{36,37}. We found that ILC2s co-localized with the accumulated eosinophils in nasal polyps. We identified eosinophils as a cellular source of IL-4, and when activated, the eosinophils were able to induce the secretion of IL-5 by ILC2s. Co-culture of IL-4-secreting eosinophils with IL-5-secreting ILC2s conferred a survival advantage to the eosinophils, most probably as result of the IL-5 secretion by the ILC2s³⁷. As ILC2s in nasal polyp tissues also produce IL-4, cross-talk between ILC2s and eosinophils might be critical for the amplification of type 2 inflammation in CRSwNP.

The identification of modulators of ILC function might be of interest for future therapeutic interventions. Specific targeting of IL-12 might result in a reduction in IFN- γ -mediated pathology in the lungs, such as the progressive emphysema in patients with COPD^{38,39}. This could be achieved by the therapeutic monoclonal antibodies (mAbs) ustekinumab and briakinumab, directed against IL-12p40, although these antibodies target both IL-12 and IL-23. No specific anti-IL-12 drugs have been developed for clinical application. Dupilumab, a mAb directed against the IL-4R α chain that is shared by IL-4 and IL-13, has shown promising results in reducing pathology in patients with CRSwNP⁴⁰, persistent moderate-to-severe asthma⁴¹ and atopic dermatitis, and this treatment might target several groups of type 2 effector cells, including ILC2s⁴². Treatment with mepolizumab or reslizumab, two mAbs directed against IL-5, was able to reduce polyp size in half of the patients with CRSwNP in clinical studies, which would suggest that targeting eosinophils might be a promising treatment

strategy^{43,44}. Given our data revealing an ILC2-eosinophil activation loop, it might be possible that simultaneous targeting of ILC2s with anti-IL-4 or anti-IL-4R might increase the therapeutic efficacy of anti-IL-5 in CRSwNP. In addition, lebrikizumab, a mAb directed against IL-13 that can improve lung function in asthma⁴⁵, might diminish the pathological function of ILC2s in CRSwNP and act in synergy with anti-IL-5 to improve its therapeutic action.

In summary, our data here and the results of published studies^{12,20,46} indicate that a high degree of plasticity is common to all ILC populations. This allows ILCs to quickly adapt to changes in their microenvironment without the need for the recruitment of circulating ILCs. As a result, upon mucosal infection, ILC plasticity could help in the prompt control of pathogens and could induce the rapid regeneration of damaged tissue. Once dysregulated, this ILC plasticity could contribute to the worsening of chronic diseases. Our findings provide an impetus for the generation of drugs that target ILC2s, which could be relevant for the treatment of chronic airway diseases such as asthma, CRSwNP and COPD.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.M.B. and J.H.B. designed the study, did experiments, analyzed the data and wrote the manuscript; M.N., J.G. and M.M.S. did experiments and analyzed the data; K.G., M.B., J.V. and J.M.M. did experiments; C.M.v.D. and W.F. provided nasal tissue; R.L. provided BAL fluid cells and analyzed data; R.E.J. and P.H. provided lung tissue; J.S.E. performed histological assessments; H.S. designed the study, analyzed data and wrote the manuscript; X.R.R. designed the study, did experiments, analyzed the data and wrote the manuscript; and all authors critically read the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Tissue collection. All tissues were collected after subjects provided informed consent, with the approval of tissue-specific protocols by the Medical Ethical Committee of the Academic Medical Centre, Amsterdam. Uninflamed nose turbinate tissue was obtained from healthy adults. Inflamed nasal polyps were from patients with chronic rhinosinusitis. Lung tissues were obtained from adult patients undergoing lung tumor surgery or lung transplantation surgery; tissues were obtained at an appropriate distance from the tumor. Bronchoalveolar lavage samples were obtained from patients undergoing a bronchoscopy for diagnostic purposes. Human fetal tissues were obtained from elective abortions at the Stichting Bloemenhove clinic in Heemstede, the Netherlands, after receipt of informed consent. The use of human abortion tissues was approved by the Medical Ethical Commission of the Academic Medical Center, Amsterdam. Gestational age was determined by ultrasonic measurement of the diameter of the skull or femur and ranged from 14 to 17 weeks. Buffy coats were provided by the blood bank at Sanquin, Amsterdam.

Isolation of cells. All solid tissues were rinsed of connective tissue, fat and muscle. Tissues were cut into fine pieces and digested for 30–45 min at 37 °C with Liberase TM (125 µg/ml). Lung cells were isolated by incubation of tissues with DNase I (50 U/ml; Sigma-Aldrich) and collagenase type 1 (300 U/ml; Worthington). Cell suspensions were filtered through a 70-µm nylon mesh or steel strainer, and mononuclear cells were isolated with Lymphoprep (Axis-Shield). Peripheral blood mononuclear cells were isolated on Lymphoprep (Axis-Shield).

Flow cytometry analysis and sorting. The following antibodies to human proteins were used at the concentrations recommended by the manufacturers. From BioLegend: fluorescein isothiocyanate (FITC)-conjugated anti-CD1a (HI149), anti-CD3 (OKT3), anti-CD14 (HCD14), Anti-CD16 (3G8), Anti-CD19 (HIB19), Anti-CD34 (581), anti-CD94 (DX22), anti-CD123 (6H6), anti-FcER1α (AER-37); phycoerythrin (PE)-conjugated anti-CD161 (HP-3G10), anti-NKp44 (P44-8), anti-TSLPR (1B4), anti-IL4α (CD124, G077F6), anti-IL9R (CD129, AH9R7), anti-KLRG1 (2F1/KLRG1), anti-CD25 (BC96), anti-ICOS (CD278, C398.4A), anti-Siglec-8 (7C9); Alexa Fluor 647-conjugated anti-CCR3 (5E8), anti-NKp44 (P44-8); allophycocyanin (APC)-conjugated anti-GATA3 (16E10A23); Alexa Fluor (AF) 700-conjugated anti-CD3 (UCHT1), anti-CD45 (HI30); brilliant violet (BV) 421-conjugated anti-CD161 (HP-3G10). From Beckman Dickinson: FITC-conjugated anti-CD34 (581), anti-TCRαβ (IP26), TCRγδ (B1); PE-CF594-conjugated anti-CD3 (UCHT1), anti-CRTH2 (BM16); AF647 or PE-CF594-conjugated anti-CRTH2 (CD294; BM16); APC-indotricarbocyanine (Cy7)-conjugated anti-CD45 (2D1); peridinin chlorophyll protein-cyanine 5.5-conjugated (PerCP-Cy5.5) anti-CD94 (HP-3D9). From other manufacturers: phycoerythrin-Cy7-conjugated anti-CD127 (R34.34; Beckman Coulter), anti-T-bet (4B10; eBioscience); PE-conjugated anti-RORγt (AFKJS-9; eBioscience), anti-IL17RB (170220; R&D systems), anti-GATA3 (TWAJ; eBioscience); FITC-conjugated anti-BDCA2 (CD303; AC144; Miltenyi), anti-ST2L (B4E6); and PE Cy5.5-conjugated anti-CD117 (104D2D1; Beckman Coulter). The cell proliferation dye was CellTrace Violet (c34557; Life Sciences).

For phenotypic analysis by flow cytometry, data were acquired on an LSRFortessa or FACSCanto II instrument (BD Biosciences) and analyzed with FlowJo software (TreeStar). For sorting by flow cytometry, lung, nasal polyp and peripheral blood mononuclear cell samples were depleted of T cells, B cells and monocytes by labeling with FITC-conjugated anti-CD3, anti-CD14 and anti-CD19 (described above) plus anti-FITC microbeads (Miltenyi) or positively selected by labeling with PE-conjugated anti-CD161 (described above) plus anti-PE microbeads. Cells were sorted on a FACSARIA (BD).

Establishment of CD127⁺CRTH2⁺ cell lines and analysis of cytokine production. Lin[−]CD127⁺CD161⁺CRTH2⁺ ILC populations were expanded with irradiated allogeneic peripheral blood mononuclear cells (25 Gy), irradiated Epstein-Barr virus-transformed JY human B cells (50 Gy), phytohemagglutinin (1 µg/ml; Oxoid) and IL-2 (100 U/ml; Novartis) in Yssel's medium (AMC; made 'in-house') supplemented with 1% (vol/vol) human AB serum.

Fresh and expanded Lin[−]CD127⁺CD161⁺ ILC populations were stimulated for 5–6 d with IL-2 (10 U/ml; Novartis), IL-33, TSLP, IL-4, IL-12, IL-18, IL-1β (all at a concentration of 50 ng/ml; R&D Systems) or combinations of these cytokines. In some experiments, anti-IL-4 (5 µg/ml; BioLegend), anti-IL-12p70 (5 µg/ml; R&D systems), or anti-IL-1β (5 µg/ml; BioLegend) were added to block the respective cytokines. IL-5, IL-13 and IFN-γ were measured in supernatants by enzyme-linked immunosorbent assay (eBioscience). Multiple cytokines were detected in some experiments by Luminex technology (Millipore).

Intracellular cytokine staining. Fresh or expanded ILC populations were stimulated for 6 h with PMA (10 ng/ml; Sigma) and ionomycin (500 nM; Merck) in the presence of GolgiPlug (BD) for the final 4 h of culture. A Cytofix/Cytoperm kit (BD) was used for cell permeabilization, staining and subsequent washing. The following antibodies were used at the concentrations recommended by the manufacturers: AF488-conjugated IL-4 (8D4-8; eBioscience); APC-conjugated anti-IL-13 (JES10-5A2; BioLegend), anti-IL-5 (JES1-39D10; BioLegend), PE-conjugated anti-IL-5 (JES1-39D10; BioLegend); PE-Cy7-conjugated anti-IFN-γ (B27; BD Bioscience); BV421-conjugated anti-IL-4 (MP4-25D2; BioLegend). Data were acquired on an LSRFortessa or FACSCanto II instrument and were analyzed with FlowJo software.

Quantitative real-time PCR. Total RNA was extracted with a NucleoSpin RNA XS kit (Macherey-Nagel) according to the manufacturer's instructions. cDNA was synthesized with a High-Capacity cDNA Archive kit (Applied Biosystems). PCRs were performed in BioRad iCycler (BioRad, France) with a mRNA-specific TaqMan gene expression assay (Applied Biosystems, USA) for IL-4 (HS00174122_m1) or with IQ SYBR Green Supermix (Bio-Rad, France) using the primers described in **Supplementary Table 5**. Bio-Rad CFX Manager 3.1 software was used for quantification of expression. All samples were normalized to the expression of control genes encoding GAPDH and β-actin and results are presented in arbitrary units.

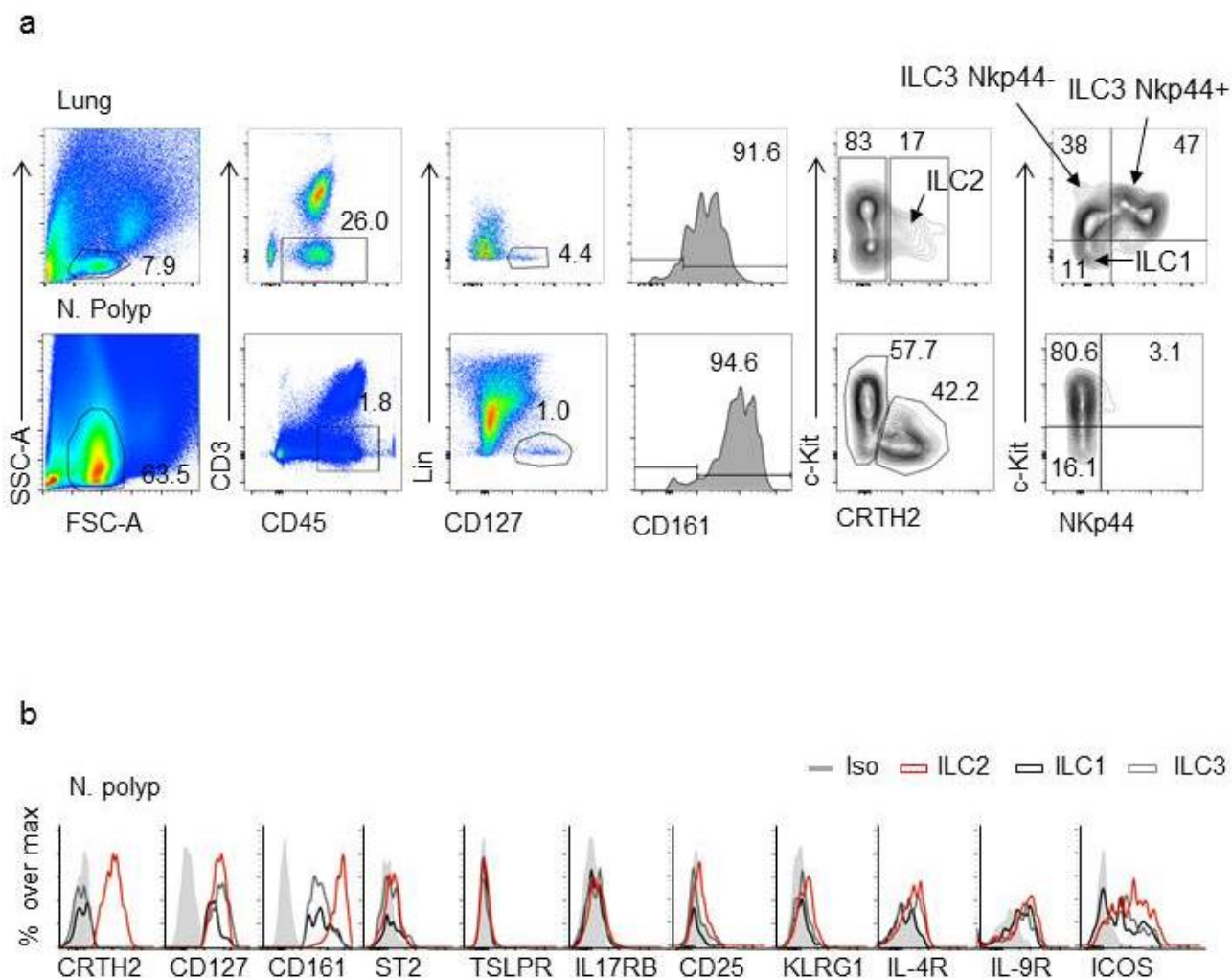
Human immune system mice. CD34⁺CD38[−] HSCs isolated from human fetal liver ($0.2 \times 10^5 - 2 \times 10^5$ cells) were transplanted intrahepatically into eight sublethally irradiated (1.0 Gy) newborn *Il2g^{−/−}* NOD-SCID female mice (younger than 1 week of age). Peripheral blood was collected from a facial vein every 3–4 weeks after transplantation to determine the kinetics of human cell engraftment. At 2 months of age, mice were given retro-orbital inoculation of approximately 1×10^5 to 5×10^5 expanded and cell-tracker-labeled CRTH2⁺ ILC2 populations²⁰. After 4 d, mice were sacrificed and organs were harvested for analysis.

Histological analysis. Snap-frozen biopsies from 28 healthy patients and 47 patients with nasal polyposis were cut into 6-µm-thick serial sections and transferred to amino-phosphate-ethylsilane coated slides (Starfrost, MarketLab, Caledonia, MI, USA). Slides were stained for eosinophils by detecting major basic protein (Clone BMK13 at 0.02 µg/ml, Monosan) using Brightvision (Immunologic) as per manufacturer's instructions. All sections were examined by two independent observers blinded to the experimental conditions. The numbers of positively stained cells were counted in the epithelium (per mm) and adjacent lamina propria (per mm²) at a final magnification of 200×. Results are expressed as the median number of positive cells per mm or mm². In separate experiments, tissue ILCs in histological sections were identified by immunohistochemistry as cells double-positive for CD25 (Vina green HRP chromogen) and GATA3 (DAB HRP chromogen) after physical chromogen exclusion (deep space black chromogen) of cells positive of CD3, CD20, tryptase, CD68 or CD56. In the same section, the eosinophils were identified by positive immunohistochemistry staining for eosinophil cationic protein (using the monoclonal antibody EG2 (Pharmacia-Upjohn Diagnostics AB, Sweden; concentration 1:1,000) and Permanent red chromogen). The multi-stained slides were digitalized in an Olympus VS120 slide scanner. The x,y coordinates for individual ILCs and eosinophils were generated in ImageJ (v 1.47v, NIH, USA). The hypothesis that the ILC2 cells tend to occur in regions with a high density of eosinophils was tested using Monte Carlo simulations. A small circular neighborhood of radius 300 pixels was defined around each of the 20 ILC2 locations (**Fig. 8b**). The eosinophils

in each of these neighborhoods were counted and added to produce a count for the entire group of cells. The distribution of the group eosinophil counts under the null hypothesis of random placement was determined by simulation of 10^6 groups of 20 locations randomly placed within the study area and calculation of the group eosinophil count measure for each simulated group. A simple proportional edge correction was applied to neighborhood counts when part of the circular neighborhood fell outside the study area. The null hypothesis could be rejected with a significance level of $P < 0.001$ showing that

the density of eosinophils around the ILC2 locations is indeed significantly higher than would be expected by chance. The simulations were repeated for ten neighborhood search radii between 50 and 500 pixels, and the results were found to be consistent across all the radii. All simulations were performed using MATLAB and Statistics Toolbox Release R2015b (MathWorks).

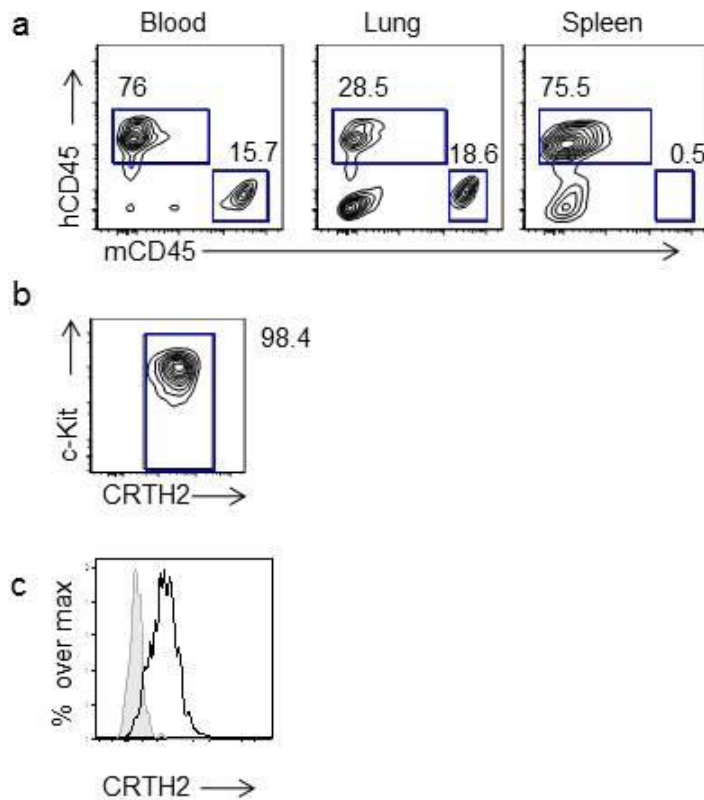
Statistical analysis. Statistical significance was determined with ANOVA, Student's *t*-test, or Mann-Whitney *U*-test. Prism GraphPad software was used.



Supplementary Figure 1

Gating strategy for the detection of ILCs in human lungs and nasal polyps, and the expression of cell-surface proteins on nasal-polyp ILCs.

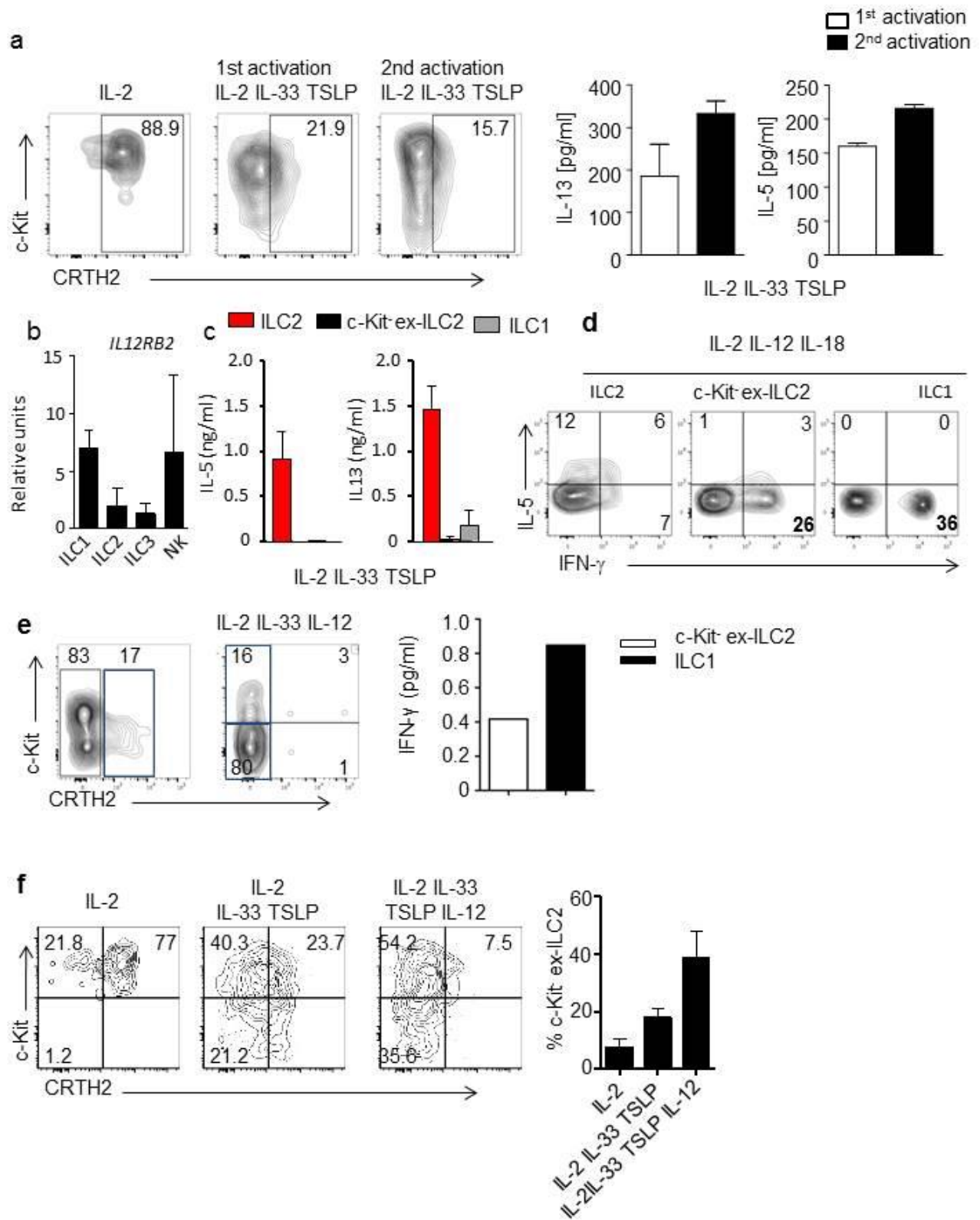
(a) Flow cytometry analysis of human lung and nasal polyp tissue of CD45, Lin (CD1a, CD3, CD14, CD16, CD19, CD34, CD123, BDCA2, FcεR1α, TCRαβ, TCRγδ), CD127, CD161, c-Kit, CRTH2, and NKp44 to detect ILC1 (CRTH2⁻ c-Kit⁻ NKp44⁻), ILC2 (CRTH2⁺ c-Kit⁺ NKp44⁻), and ILC3 (CRTH2⁻ c-Kit⁺ NKp44⁺). (b) Flow cytometry analyzing the expression of CRTH2, IL7Rα, CD161, ST2, TSLPR, IL17RB, CD25, KLRG1, IL4R, IL9R and ICOS in ILC1 (black), ILC2 (red) and ILC3 (grey line) from nasal polyp tissue. Isotype-matched control antibody is shown as grey shaded curve. Data are representative of at least three different donors (a,b).



Supplementary Figure 2

Repopulation of *Il2rg*^{-/-} NOD-SCID mice with human hematopoietic stem cells and purity of expanded ILC2 populations.

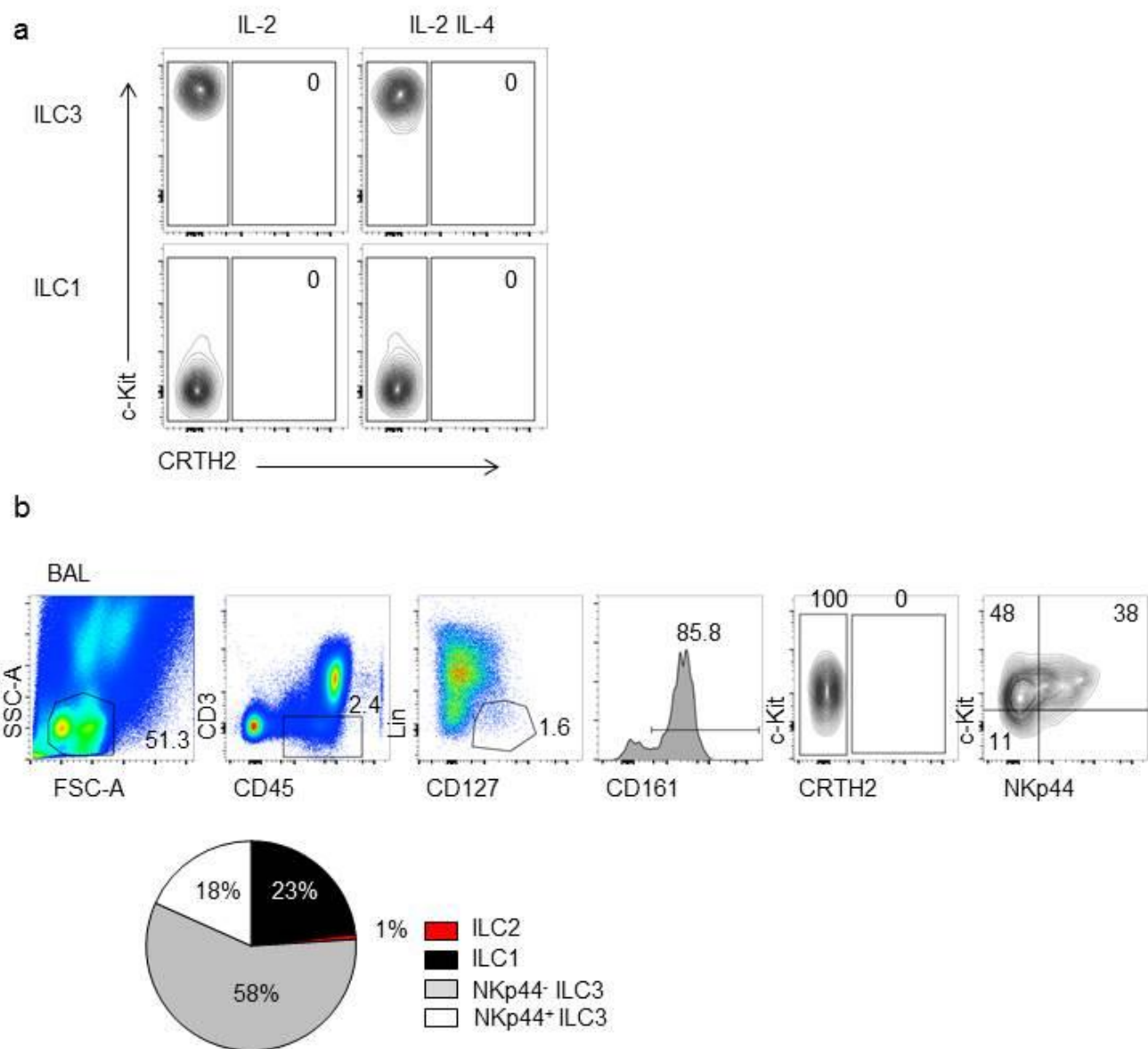
(a) Flow cytometry analysis of human and murine CD45 expression in blood, lung and spleen of *Il2rg*^{-/-} NOD-SCID mice eight weeks after engraftment with human hematopoietic stem cells. (b) c-Kit and CRTH2 expression after resorting of ILC2 that were expanded for four weeks with irradiated allogeneic peripheral blood mononuclear cells, irradiated Epstein-Barr virus-transformed JY human B cells, phytohemagglutinin and IL-2. (c) CRTH2 expression after culturing CTV labeled ILC2 for 4 days with IL-2. Data are representative of six mice (a) or five different experiments (b).



Supplementary Figure 3

Reactivation of ILC2 cells with IL-33 and TSLP and transdifferentiation of lung ILC2 cells.

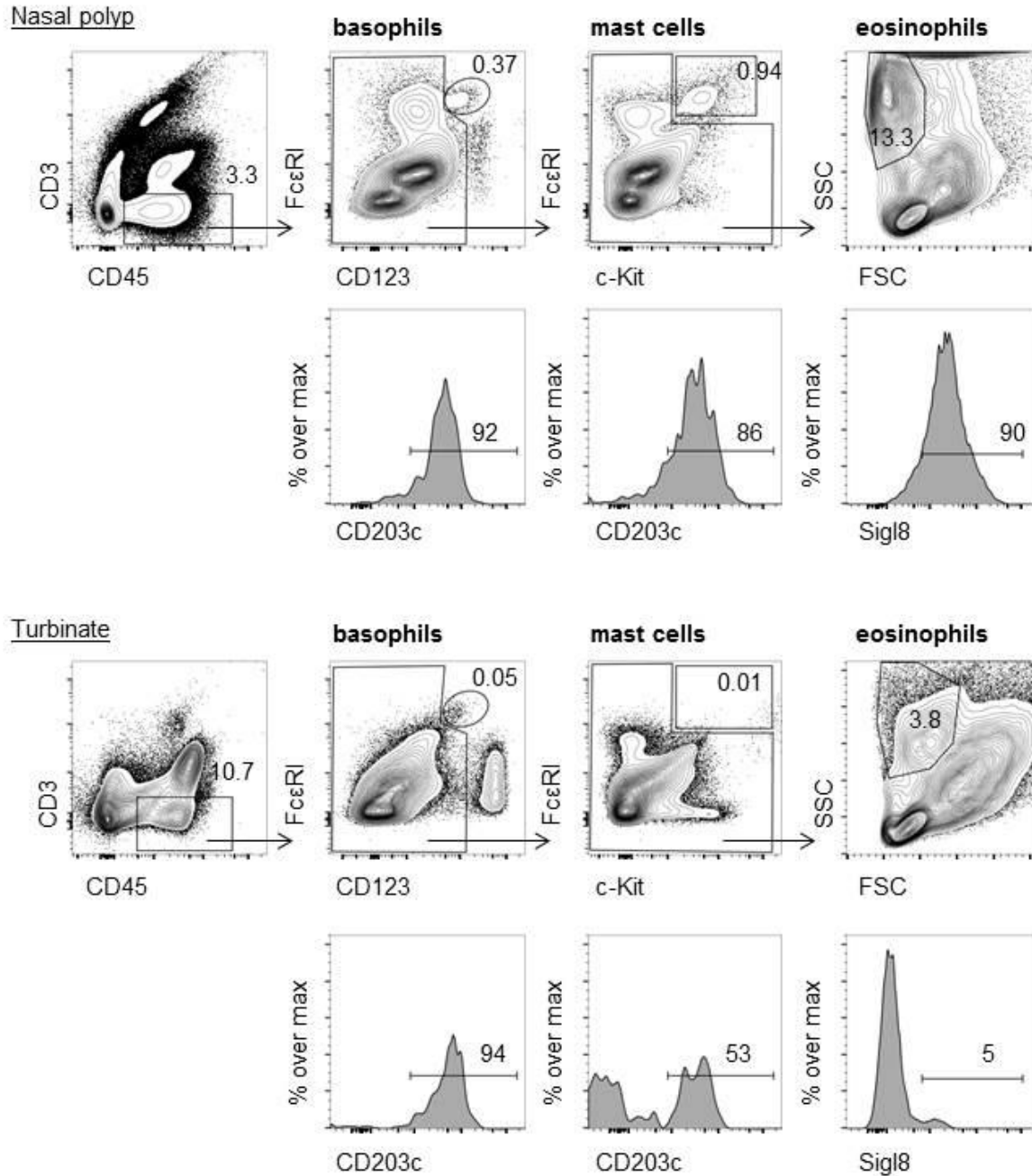
(a) Flow cytometry analysis of c-Kit and CRTH2 expression on blood ILC2 (left panel) and IL-5 and IL-13 production (right panel) upon stimulation for five days with IL-2, IL-2 + IL-33 + TSLP. Cells were washed and stimulated for another four days with IL-2 + IL-33 + TSLP. (b) Expression of *IL12RB2* on ILC1, ILC2, ILC3 and NK cells as measured by qPCR. (c) IL-5 and IL-13 production by blood ILC2, ILC1, and ILC2 expanded with IL-2, IL-1 β and IL-12 after culture for 4 days with IL-2, IL-33, and TSLP, (d) intracellular IL-5 and IFN- γ production by cells as under after culture for 4 days with IL-2, IL-12, and IL-18. (e) Flow cytometry analysis of c-Kit and CRTH2 expression on lung ILC2 directly after isolation and upon stimulation with IL-2, IL-33, and IL-12 for seven days and IFN- γ production of lung c-Kit⁺ ex-ILC2 obtained upon stimulation with IL-2, IL-33, and IL-12 for seven days. (f) Isolated ILC2 from lung cultured for 6 days either with IL-2, or IL-2, IL-33, and TSLP, or with IL-2, IL-33, TSLP, and IL-12. Cells were analyzed for c-Kit and CRTH2 expression (left panels), percentage of c-Kit⁺ ex-ILC2 generated in above mentioned activation conditions (right panel). Data are representative of three (a,c,d,e,f) or four (b) different donors.



Supplementary Figure 4

Stimulation of ILC1 and ILC3 cells with IL-4, and gating strategy for BAL-fluid ILCs.

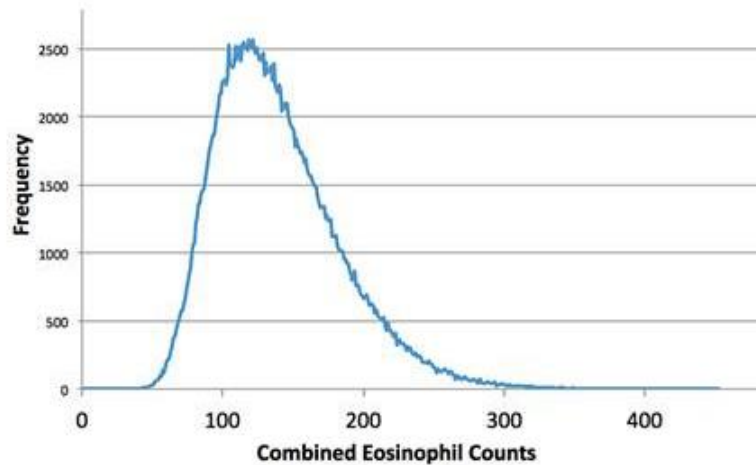
(a) Flow cytometry analysis of c-Kit and CRTH2 expression on blood ILC1 and ILC3 upon stimulation for five days with IL-2 or IL-2 and IL-4. (b) Gating strategy for ILC detection and ILC distribution in bronchoalveolar lavage (BAL) fluid. Data are representative of three individual experiments (a) or five different samples (b).



Supplementary Figure 5

Gating strategy for basophils, mast cells and eosinophils in nasal-polyp and turbinate tissue.

Polyp and turbinate tissue was analyzed by flow cytometry for CD45, CD3, CD123, FcεRI, c-Kit, CD203c and Siglec8. Basophils were gated as CD3⁻ CD123⁺ FcεRI⁺ CD203c⁺, mast cells as c-Kit⁺ FcεRI⁺ CD203c⁺, and eosinophils as SSC^{high} Siglec8⁺. Data are representative of five polyp and two turbinate samples.



Supplementary Figure 6

ILC2 spatial distribution.

Using the known x,y coordinates for each eosinophil, the true observed accumulated eosinophil counts within a fixed close space surrounding each ILC2 cell were calculated and compared to computer simulations of random ILC distributions. The true accumulated eosinophils count within a 300 μ m radius around each of the 20 ILC in figure 8b (n=668 ILC-associated eosinophils) was compared to the combined eosinophil counts calculated for simulated 250 000 cases of computer-generated random distributions of the same number of fictive ILCs (mean combined eosinophil count = 139, range 35-452).

Suppl. Table 1

Supplementary table 1: Clinical characteristics of COPD patients.

	Patient	Source	Smoke status
COPD	Female, 58 yrs	Lung transplant (GOLD stage IV)	Ex
	Female, 63 yrs	Lung transplant (GOLD stage IV)	Ex
	Male, 60 yrs	Lung transplant (GOLD stage IV)	Ex
	Female, 60 yrs	Lung transplant (GOLD stage IV)	Ex
	Male, 58 yrs	Lung transplant (GOLD stage IV)	Ex
	Male, 58 yrs	Lung transplant (GOLD stage IV)	Ex
	Male, 60 yrs	Lung transplant (GOLD stage IV)	Ex
Control	Male, 74 yrs	Lung resection	Ex
	Male, 68 yrs	Lung resection (GOLD stage I)	Ex
	Male, 63 yrs	Lung resection (GOLD stage II)	Current
	Female, 63 yrs	Lung resection	No
	Male, 46 yrs	Lung resection	Current
	Female, 54 yrs	Cancelled donor lung	Current

Suppl. Table 2

Supplementary table 2: Cytokines present in supernatant of mixed lymphocyte reaction measured by multiplex.

Cytokine	Concentration
IL-12p70	31 ± 12 pg/ml
IL-1β	332 ± 175 pg/ml
TNF-α	4 ± 0.5 ng/ml
IFN-γ	629 ± 44 pg/ml
IL-8	29 ± 2 ng/ml
IL-6	2 ± 1 ng/ml
IL-13	715 ± 181 pg/ml

IL-33, TSLP and IL-23 were undetectable

Suppl. Table 3

Supplementary table 3: Clinical characteristics of BAL patients.

Patient	Diagnosis
Male, 70 yrs	Atypic pneumonia
Male, 71 yrs	Extrinsic allergic alveolitis
Male, 65 yrs	Non-specific interstitial pneumonia
Male, 42 yrs	Sarcoidosis
Female, 39 yrs	Corticosteroid resistant asthma

Suppl. Table 4

Supplementary table 4: Cellular composition of nasal polyps and turbinate tissue.

	T cells	Eosinophils	Basophils	Mast cells
Nasal polyp (% CD45 ⁺ cells $\pm$ SEM) n=6	44.5 $\pm$ 4.7	3.7 $\pm$ 2.1	0.3 $\pm$ 0.07	0.9 $\pm$ 0.1
Turbinate (% CD45 ⁺ cells $\pm$ SEM) n=2	56.1 $\pm$ 6.4	0.4 $\pm$ 0.2	0.1 $\pm$ 0.00	0.7 $\pm$ 0.6

Supplementary table 5: Sequences of real-time PCR primers designed in-house

mRNA	Primer	Sequence (5'-3')
GAPDH	Forward primer	GTC TCC TCT GAC TTC AAC AGC G
	Reverse primer	ACC ACC CTG TTG CTG TAG CCA A
Beta Actin	Forward primer	CAC CAT TGG CAA TGA GCG GTT C
	Reverse primer	AGG TCT TTG CGG ATG TCC ACG T
GATA3	Forward primer	ACC ACA ACC ACA CTC TGG AGG A
	Reverse primer	TCG GTT TCT GGT CTG GAT GCC T
RORC	Forward primer	AAT CTG GAG CTG GCC TTT CA
	Reverse primer	CTG GAA GAT CTG CAG CCT TT
TBX21 (T-bet)	Forward primer	ATT GCC GTG ACT GCC TAC CAG A
	Reverse primer	GGA ATT GAC AGT TGG GTC CAG G
TSLP	Forward primer	TAT CTG GTG CCC AGG CTA TTC G
	Reverse primer	TGA AGC GAC GCC ACA ATC CTT G
IL-12p35	Forward primer	TGC CTT CAC CAC TCC CAA AAC C
	Reverse primer	CAA TCT CTT CAG AAG TGC AAG GG
IL-33	Forward primer	GCC TGT CAA CAG CAG TCT ACT G
	Reverse primer	TGT GCT TAG AGA AGC AAG ATA CTC
IL-5	Forward primer	AGC TGC CTA CGT GTA TGC CA
	Reverse primer	CAG GAA CAG GAA TCC TCA GA
IL-22	Forward primer	CCC ATC AGC TCC CAC TGC
	Reverse primer	GGC ACC ACC TCC TGC ATA TA
IL-13	Forward primer	ATT GCT CTC ACT TGC CTT GG
	Reverse primer	GTC AGG TTG ATG CTC CAT ACC
CRTH2	Forward primer	AAT CCT GTG CTC CCT CTG TG
	Reverse primer	ATG TAG CGG ATG CTG GTG TT
IL1RL1 (IL33R)	Forward primer	ATG TTC TGG ATT GAG GCC AC
	Reverse primer	GAC TAC ATC TTC TCC AGG TAG CAT
IL17RB(IL25R)	Forward primer	CCA ACA CAG CAC TAT CAT CG
	Reverse primer	ATA TGG AGT CAG CTG CAC CG
TSLPR	Forward primer	GAG TGG CAG TCC AAA CAG GAA
	Reverse primer	ACA TCC TCC ATA GCC TTC ACC
IL-12Rb2	Forward primer	AGA CCT CAG TGG TGT AGC AGA G
	Reverse primer	TAG ATG ACC AGC GGT TCA GGA TC
IL-4Ra	Forward primer	GAC CTG GAG CAA CCC GTA TC
	Reverse primer	GAT GCG GAG GGA GGG TTC TA