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Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161

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Innate lymphoid cells (ILCs) are emerging as a family of effectors and regulators of innate immunity and tissue remodeling. Interleukin 22 (IL-22)- and IL-17-producing ILCs, which depend on the transcription factor RORγt, express CD127 (IL-7 receptor α-chain) and the natural killer cell marker CD161. Here we describe another lineage-negative $CD127^+CD161^+$ ILC population found in humans that expressed the chemoattractant receptor CRTH2. These cells responded *in vitro* to IL-2 plus IL-25 and IL-33 by producing IL-13. $CRTH2^+$ ILCs were present in fetal and adult lung and gut. In fetal gut, these cells expressed IL-13 but not IL-17 or IL-22. There was enrichment for $CRTH2^+$ ILCs in nasal polyps of chronic rhinosinusitis, a typical type 2 inflammatory disease. Our data identify a unique type of human ILC that provides an innate source of T helper type 2 (T_H2) cytokines.

Innate lymphoid cells (ILCs) represent an emerging family of cell types that seem to have crucial roles in tissue remodeling and in innate immunity to pathogenic and nonpathogenic microorganisms[1–3]. These cells are characterized by a lymphoid morphology and an absence of receptors dependent on the RAG recombinase, encoded by recombination activating genes. Natural killer cells (NK cells) and lymphoid tissue–inducer cells (LTi cells) are the prototypical members of this family. Whereas NK cells have a crucial innate defensive role against viral infection, in particular against herpes viruses, LTi cells are essential for the formation of lymph nodes during embryonic development. Initially LTi cells were not considered to have a function in the immune response. However, the discovery that mouse and human LTi cells are able to produce interleukin 17 (IL-17) and IL-22 (refs. 4–6), cytokines known to mediate immunity to microbes, has led researchers to reconsider the functions of these cells in immune responses. Subsequently, cells have been discovered that show characteristics of both NK cells and LTi cells. Like LTi cells, these cells are of lymphoid origin, express the IL-7 receptor α-chain (CD127) and the transcription factor RORγt, but they also share with NK cells expression of the activating receptor NKp46 and, in humans, they express the activating receptor NKp44 and the adhesion molecule CD56 (refs. 7–10). These NK receptor–expressing cells are mostly found at mucosal sites both in mice and humans and produce IL-22. ILCs dedicated to the production of IL-17 and interferon-γ (IFN-γ), either exclusively or in combination, have been described in both mice and humans[11–13]. IL-22-producing ILCs have been shown in several systems to be involved in responses to bacteria. For example, IL-22-producing ILCs are essential for the innate immune response to the enteric bacterium *Citrobacter rodentium* in the gut[14] and *Klebsiella pneumoniae* in the lungs[15]. It seems, therefore, that the ILC family comprises cells that share many characteristics but may have different effector functions that are mediated by distinct cytokines and dependent on their anatomical localization[3].

Another transcription inhibitor Id2–dependent non-T, non-B cell population that produces type 2 cytokines has been discovered in the mouse. These cells have been variously called natural helper lymphocytes[16], multipotent progenitor type 2 cells[17], nuocytes[18] and innate helper type 2 cells[19]. Although differences among these cell types exist, they have in common the production of T helper type 2 (T_H2) cytokines, most notably IL-5 and IL-13, in response to the IL-17 family member IL-25 (IL-17E) and the IL-1 family member IL-33. The ILC-derived T_H2 cytokines mediate eosinophilia and goblet-cell hyperplasia, both of which are critical for anti-helminth reactions. These type 2 ILC populations may be involved in disease, as their signature cytokines IL-5 and IL-13 also have a role in the pathophysiology of type 2 immunity–associated diseases such as asthma and allergic diarrhea.

Like LTi cells and $ROR\gamma t^+$ ILCs[9,20], type 2 ILCs are dependent on the common γ-chain cytokine receptor and IL-7 (refs. 16,21). Type 2 ILCs depend on Id2 (ref. 16), similar to LTi cells and IL-17- and IL-22-producing ILCs[22,23], but in contrast to those cells, type 2 ILCs seem to be independent of RORγt[16]. The common reliance of NK

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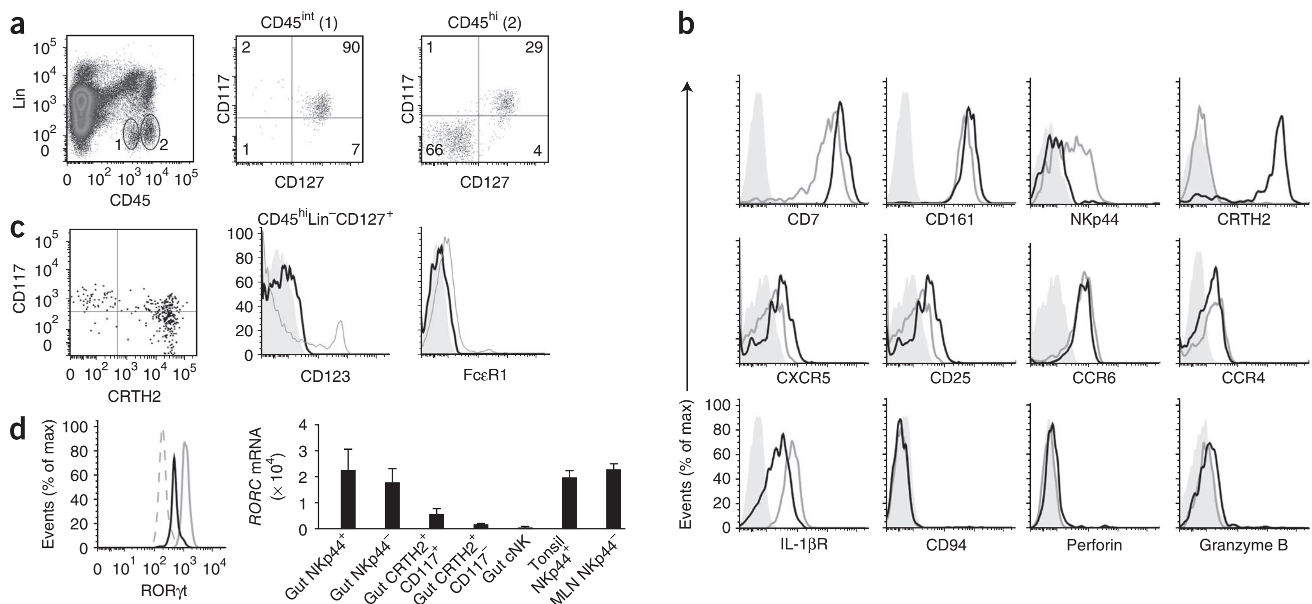

Figure 1 Lin^{-} lymphocytes in the fetal gut include a CRTH2^{+}CD127^{+} ILC population. (**a**) Flow cytometry analysis of the expression of CD127 and CD117 by two distinct ILC populations in the fetal gut: Lin^{-} cells (left; CD1a^{-}CD3^{-}CD11c^{-}CD14^{-}CD19^{-}CD34^{-}CD123^{-}TCR$\alpha\beta^{-}$TCR$\gamma\delta^{-}$BDCA2^{-}FcεR1^{-}) gated as CD45^{int} (1) or CD45^{hi} (2). Numbers in quadrants indicate percent cells in each throughout. (**b**) Flow cytometry characterization of Lin^{-}CD127^{+}CD45^{int} cells (gray lines) and Lin^{-}CD127^{+}CD45^{hi} cells (black lines); light gray shading, isotype-matched control antibody. (**c**) Flow cytometry analysis of the expression of CD117 and CRTH2 (left), CD123 (IL-3R; middle) and FcεR1 (right) on CD45^{hi}Lin^{-}CD127^{+} cells (black lines) and peripheral blood monocytes or basophils (gray lines); light gray shading, isotype-matched control antibody. (**d**) Flow cytometry analysis of RORγt expression (left) in CD56^{int} peripheral blood NK cells (dashed gray line), fetal gut CD45^{hi}Lin^{-}CD127^{+}CRTH2^{+} ILCs (solid black line) and CD45^{int}Lin^{-}CD127^{+}NKp44^{+} ILCs (solid gray line). Right, *RORC* mRNA expression in fetal gut NKp44^{+} or NKp44^{-} ILCs (gated as CD45^{int}Lin^{-}CD127^{+}CD117^{+}), CRTH2^{+} ILCs (CD117^{+} or CD117^{-}; gated as CD45^{hi}Lin^{-}CD127^{+}) and conventional NK cells (cNK; CD45^{hi}CD127^{-}CD56^{+}), tonsil NKp44^{+} ILCs and fetal mesenteric lymph node (MLN) NKp44^{-} ILCs. Data are representative of at least three experiments with one to three donors each (**a**–**c** and **d**, left) or are from one experiment with two to five donors (**d**, right; median and range).

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cells, LTi cells and various ILC populations on Id2 and common γ-chain cytokines support the contention that these cell types have a common origin[3].

A human type 2 ILC population comparable to the one in mice has not yet been described, although innate cells that produce IL-13 have been documented. It has been shown that cord blood CD34^{+} progenitor cells cultured in the presence of IL-2 can develop into a non-T, non-B cell type that expresses the NK cell marker CD161 but lacks CD56 (ref. 24). These cells produce IL-13 but not IFN-γ and include an IL-5-producing subset. However, an equivalent cell type has not been identified *in vivo*. It has since been shown that lineage-negative (Lin^{-}) CD127^{+}CD117^{+} cells from the tonsils are able to produce IL-13 after stimulation with IL-2 and the ligand for Toll-like receptor 2 (ref. 13). These IL-13-producing human ILCs express *RORC* transcripts and RORγt protein (encoded by *RORC*), and they coexpress IL-22. Cloned lines have been established from these cells that mostly coexpress IL-22 and IL-13, although some clones have been identified that produce IL-13 and IL-5 but not IL-22 (ref. 13). Those last clones still express *RORC* transcripts, in contrast to mouse type 2 ILCs. It has also been shown that when stimulated with IL-23 and IL-2, ILCs from the tonsils produce IL-22 but not IL-13, whereas when simulated with IL-2 and agonists for Toll-like receptor 2, these ILCs do produce IL-13 (ref. 13). This suggests that tonsil ILCs are plastic and produce different cytokines dependent on the stimulus they are given. It is also possible, however, that tonsil ILCs include two subsets, one of which produces IL-13 and does not respond to IL-23. Obviously, a marker that distinguishes IL-13-producing ILCs from IL-22-producing ILCs would allow this issue to be addressed.

Here we describe a human Lin^{-}CD127^{+} ILC population characterized by expression of the T_H2 marker CRTH2 (chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes). These ILCs were present in lung, gut and nasal tissues as well as in peripheral blood and expressed CD161 and the lymphoid marker CD7, were of lymphoid size and lacked the mast cell and basophil markers FcεR1 and the IL-3 receptor (IL-3R; CD123). Like mouse natural helper lymphocytes, nuocytes and innate helper type 2 lymphocytes, these human CRTH2^{+} cells responded to IL-25 and IL-33 by producing IL-13 and IL-5, and we therefore suggest that these cells represent the human equivalent of mouse type 2 ILCs.

RESULTS

Lin^{-}CRTH2^{+}CD127^{+} population in fetal gut

In the mouse, type 2 ILCs have been found in the intestine. In search of a human cell population that may represent the equivalent of the type 2 ILCs described in the mouse, we extensively analyzed cells in both fetal and adult human intestines, focusing on cells that were Lin^{-} and CD127^{+}. Thus, we stained single-cell suspensions derived from human intestinal tissues with a 'cocktail' of lineage-specific antibodies, antibody to CD45 (anti-CD45) and a collection of antibodies, including an antibody to CRTH2, which is known to be expressed on T_H2 cells and myeloid cells associated with type 2 immune responses, such as basophils and mast cells. Unexpectedly, we found the largest proportion of Lin^{-}CRTH2^{+} lymphoid cells in the fetal gut and therefore decided to extensively analyze all ILC populations in this organ. In the Lin^{-} population, we were able to distinguish cells with high or intermediate expression of CD45 (CD45^{hi} versus CD45^{int}; **Fig. 1a**).

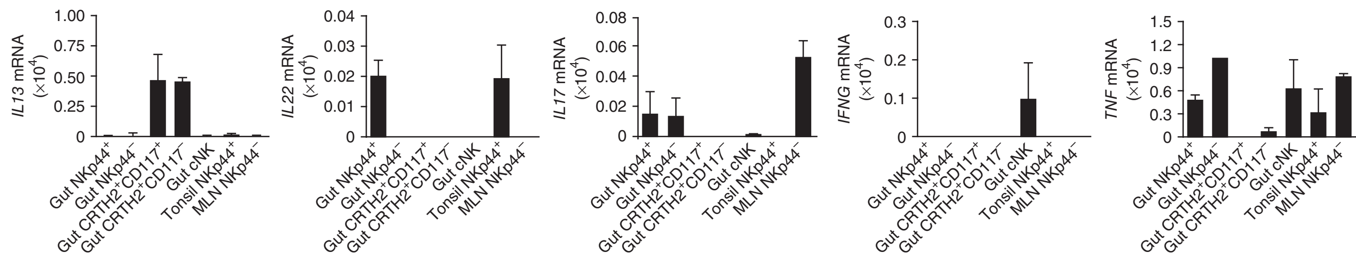

Figure 2 CRTH2+ fetal gut ILCs express *IL13* transcripts *ex vivo*. Real-time PCR analysis of the expression of transcripts of *IL13*, *IL22*, *IL17*, *IFNG* and tumor necrosis factor (*TNF*) in fetal gut NKp44+ or NKp44− ILCs (gated as CD45^{int}Lin−CD127+CD117+), CRTH2+ ILCs (CD117+ or CD117−; purified by flow cytometry and gated as CD45^{hi}Lin−CD127+) and conventional NK cells (CD45^{hi}Lin−CD127−CD56+), tonsil NKp44+ ILCs and fetal mesenteric lymph node NKp44− ILCs. The lineage 'cocktail' included antibodies to CD1a, CD3, CD4, CD11c, CD14, CD19, CD34, CD123, TCRαβ, TCRγδ, BDCA2 and FcεR1. Data are from one experiment with two to five donors (median and range).

Analyzing these two populations for expression of CD117 (c-Kit) and CD127, we observed that the majority of the CD45^{int} cells (>90%) also expressed CD117 and CD127 and this group most probably included LTi cells[4]. In contrast, only around 30% of the CD45^{hi} cells expressed CD127. Most of the CD45+CD127− cells were CD56+ and positive for perforin and were therefore identifiable as NK cells (data not shown). We then gated on CD127+ cells and analyzed the CD45^{hi} Lin− cells in that gate and compared those with CD127+CD45^{int} cells. Both populations expressed CCR6, CCR4, CD7 and CD161, which are also expressed on NK cells and LTi cells (**Fig. 1b**). Thus, the CD45^{hi}CD127+ cells most likely represented an ILC population. The CD45^{int} cells, like LTi cells in the mesenteric lymph nodes, expressed the IL-1β receptor and included two populations, one that expressed NKp44 and the other that lacked this antigen (**Fig. 1b**). In contrast, the fetal gut CD45^{hi} cells all lacked NKp44 and had low expression of the IL-1β receptor (**Fig. 1b**). Most of these CD45^{hi}CD127+ cells were positive for the type 2 marker CRTH2 (**Fig. 1c**). To determine whether these CRTH2+ cells were not mast cells or basophils, which also express CRTH2+, we analyzed the CRTH2+ ILCs for the presence of IL-3R (CD123) and the receptor FcεRI, which normally have high expression on those cell types. The CRTH2+ lymphoid cells did not bind IL-3R- nor FcεRI-specific antibodies (**Fig. 1c**), which indicated that these cells were distinct from both mast cells and basophils.

Fetal LTi cells and postnatal IL-17- and IL-22-producing ILCs express and depend on RORγt, whereas type 2 ILCs in the mouse have been shown to be negative for RORγt. Therefore we analyzed the expression of RORγt on the various ILC populations with anti-RORγt. As this antibody has a high nonspecific binding activity, we used its binding to NK cells, which do not express RORγt, as assessed by real-time PCR analysis, as background. CD45^{int}CD127+ ILCs were RORγt+, whereas NK cells (CD45+CD127−CD56+ cells) were RORγt− (**Fig. 1d**). The CRTH2+ cells expressed more RORγt protein than did NK cells, but this expression was much lower than that on CD45^{int}CD127+ ILCs (**Fig. 1d**). PCR analysis confirmed that the CRTH2+ ILCs had lower expression of *RORC* transcripts than did CD45^{int}CD127+ ILCs, but still had higher expression than did NK cells (**Fig. 1d**). Thus, a CRTH2+ ILC population, distinct from RORγt+ ILCs, mast cells and basophils, was present in fetal gut.

Fetal gut CRTH2+ ILCs respond to IL-25 and IL-33

The signature cytokines of human and mouse RORγt+ ILCs are IL-17 and IL-22 (ref. 3), whereas mouse type 2 ILCs secrete IL-13 (refs. 16,18,19). We therefore analyzed the expression of transcripts for those cytokines and IL-5 in the various ILC populations isolated from fetal gut. Whereas CD45^{int}CD127+NKp44+ cells expressed *IL22* transcripts and some *IL17* transcripts, they did not express IL-13 (**Fig. 2**). In contrast, CRTH2+ ILCs expressed *IL13* transcripts but no *IL17* or *IL22* transcripts. Neither the CRTH2+ population nor the other ILC populations in the gut expressed IL-5 *ex vivo* (data not shown). As CRTH2+ cells express CD25 and IL-13, we stimulated freshly isolated fetal gut CRTH2+ cells with IL-2 alone or with the combination of IL-2 plus IL-25 or of IL-2 plus IL-33. IL-2 in combination with IL-25 or IL-33 stimulated the production of IL-13 protein (**Fig. 3**).

To investigate whether the CRTH2+ population represented a stable cell type, we established cell lines starting from fetal gut CRTH2+ cells through the use of a technique that has also been successfully applied to the generation of lines of NK cells[25] and IL-17- and IL-22-producing ILCs[13,26]. We were able to generate robustly growing cultures, and the cells had stable expression of CRTH2 and CD127 (**Fig. 4a**). Furthermore, these cell lines lacked expression of CD3ε and FcεR1 (**Fig. 4a**), as well as CD123 and CD94 (data not shown), which demonstrated the stability of the cell lines and their inability to differentiate toward the basophil, mast cell, T cell or NK cell lineage (**Fig. 4a**). CRTH2+ ILC populations expanded *in vitro* had intracellular expression of IL-13 but not IL-17 after activation with the polyclonal activator PMA and ionomycin, whereas a small fraction of the IL-13-producing cells also produced IL-22 (**Fig. 4b**). As reported for IL-22-producing NKp44+ ILCs, CRTH2+ ILC lines expressed *AHR* transcripts, encoding the transcription factor aryl hydrocarbon receptor (**Fig. 4c**). However, CRTH2+ ILCs did not produce IL-22 or express *IL17*

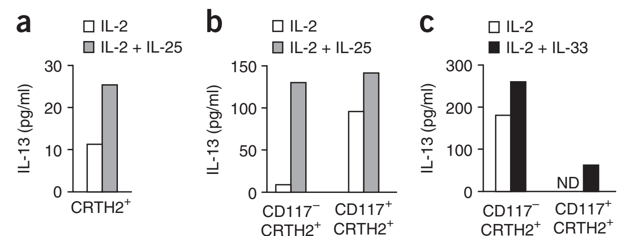

Figure 3 CRTH2+ fetal gut ILCs respond to IL-25 and IL-33 *in vitro* by producing IL-13. (**a**) IL-13 production by fetal gut CD45^{hi}Lin−CD127+CRTH2+ ILCs sorted by flow cytometry and cultured for 4 d with IL-2 (10 U/ml) alone (IL-2) or with a combination of IL-2 (10 U/ml) and IL-25 (50 ng/ml; IL-2 + IL-25). (**b**) IL-13 production by CD45^{hi}Lin−CD127+CRTH2+ ILCs sorted on the basis of CD117 expression and stimulated as in **a**. (**c**) Enzyme-linked immunosorbent of IL-13 in supernatants of fetal gut CD117+CRTH2+ and CD117−CRTH2+ ILCs stimulated with IL-2 (10 U/ml) alone (IL-2) or with a combination of IL-2 (10 U/ml) and IL-33 (50 ng/ml; IL-2 + IL-33). Results (**a**–**c**) were normalized for those of 2,000 cells per 200 μl in a 96-plate well (lineage 'cocktail' as in **Fig. 2**). Data are representative of two experiments with one donor each (**a**,**b**) or one experiment with four donors (**c**).

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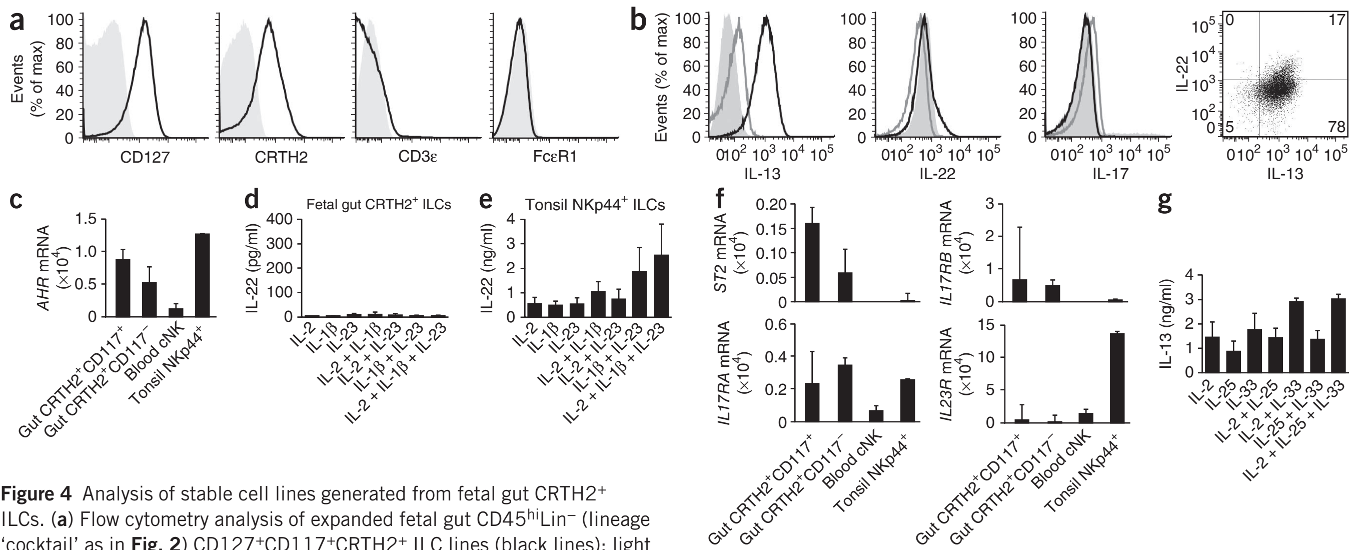

Figure 4 Analysis of stable cell lines generated from fetal gut CRTH2+ ILCs. (**a**) Flow cytometry analysis of expanded fetal gut CD45^{hi}Lin$^-$ (lineage 'cocktail' as in **Fig. 2**) CD127$^+$CD117$^+$CRTH2$^+$ ILC lines (black lines); light gray shading, isotype-matched control antibody. (**b**) Flow cytometry analysis of fetal gut CRTH2$^+$ ILC lines (black lines) and CD56$^+$ conventional NK cell lines (gray lines) stimulated with PMA and ionomycin and stained for intracellular IL-13, IL-22 or IL-17; light gray shading, unstimulated CRTH2$^+$ ILCs. Far right, expression of IL-22 and IL-13 by fetal gut CD127$^+$CD117$^+$CRTH2$^+$ ILCs stimulated with PMA and ionomycin. Numbers in quadrants (far right) indicate percent cells in each (among total cells). (**c**) Expression of *AHR* mRNA in fetal gut CRTH2$^+$ ILCs (CD117$^+$ or CD117$^-$), blood CD56^{int} conventional NK cells and tonsil NKp44$^+$ ILCs. (**d**,**e**) Secretion of IL-22 from fetal gut CRTH2$^+$ ILCs (**d**) or tonsil NKp44$^+$ ILCs (**e**) after stimulation with various combinations (horizontal axes) of IL-2, IL-1β (50 ng/ml) and IL-23 (50 ng/ml). (**f**) Expression of *ST2*, *IL17RB*, *IL17RA* and *IL23R* mRNA in cells as in **c**. (**g**) IL-13 response of fetal gut CRTH2$^+$ ILCs to various combinations (horizontal axes) of IL-2, IL-25 and IL-33. Data are representative of three experiments with one donor each (**a**,**b**) or are from one experiment with three to four donors (**c**; median and range), three to four experiments with one donor each (**d**,**e**; mean and s.e.m.), one experiment with three to five donors (**f**; median and range) or three experiments with one donor each (**g**; mean and s.e.m.).

(data not shown) in response to IL-23 (**Fig. 4d**). In contrast, NKp44$^+$ ILCs isolated from inflamed tonsils responded to IL-23 by producing IL-22 (**Fig. 4e**). Those observations were consistent with the expression of transcripts of *IL23R* (which encodes the IL-23 receptor) by NKp44$^+$ ILCs but not by CRTH2$^+$ ILCs (**Fig. 4f**).

CRTH2$^+$ ILC lines expressed transcripts of *IL1RL1* (also known as *ST2*; encodes a subunit of the IL-33 receptor), *IL17RB* (which encodes a subunit of the IL-25 receptor) and *IL17RA* (which encodes a common subunit of the IL-25 receptor and IL-17 receptor; **Fig. 4f**). Consistent with the expression of *ST2*, most of the cell lines responded to IL-2 in combination with IL-33 and IL-25 plus IL-33 (**Fig. 4g**), whereas the response to IL-25 was lower and was not present in all cell lines (**Fig. 4g** and **Supplementary Fig. 1**, which includes responses of individual cell lines). The CRTH2$^+$ ILC lines never showed any IL-17 expression (data not shown) but, in contrast to the cells isolated *ex vivo*, these cell lines did express *IL5* transcripts (data not shown). Thus, we identified a stable Lin$^-$CRTH2$^+$CD127$^+$ population that expressed *IL13* but not *IL17* or *IL22* transcripts *ex vivo* and responded *in vitro* to IL-25 and IL-33 by producing IL-13.

Enrichment for CRTH2+ ILCs in chronic rhinosinusitis

To gain more insight into the possible functions of CRTH2$^+$ ILCs, we extensively analyzed the presence of Lin$^-$CRTH2$^+$CD127$^+$ cells in a variety of tissues. These cells were located in mucosal tissues at different ontogenic stages, as they were present in both fetal and adult gut and lung (**Fig. 5** and **Table 1**). Given the production of type 2 cytokines and the responsiveness to IL-25 and IL-33, we analyzed the presence of CRTH2$^+$ cells in chronically inflamed airway tissues, specifically

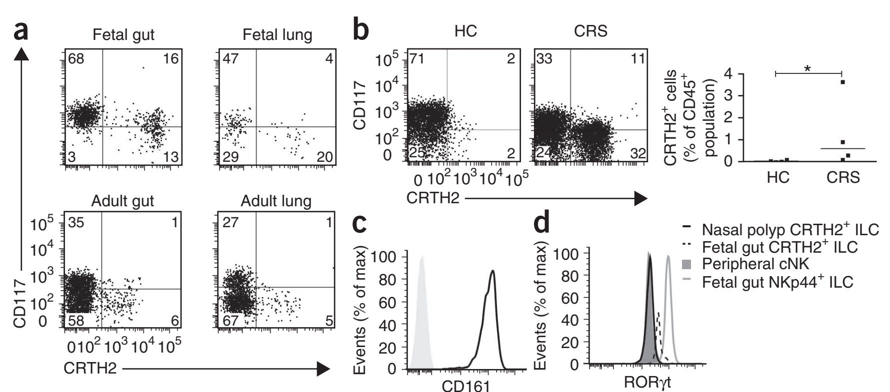

Figure 5 CRTH2$^+$ ILCs are distributed in several fetal and adult tissues and show enrichment in the nasal polyps of patients with chronic rhinosinusitis. (**a**) Expression of CD117 and CRTH2 by mononuclear cells isolated from fetal gut, fetal lung, adult gut and adult lung, stained for ILCs (gated as CD45$^+$, Lin$^-$ (as in **Fig. 1**) and CD127$^+$); numbers in quadrants indicate percent cells of the Lin$^-$CD127$^+$ gate. (**b**) Flow cytometry analysis of the expression of CD117 and CRTH2 (left and middle) in cells from healthy control nasal tissue (HC) and nasal polyps of chronic rhinosinusitis (CRS), and frequency of CRTH2$^+$ ILCs (right). Each symbol represents a separate donor; small horizontal lines indicate the median. $*P < 0.03$ (Mann-Whitney two-tailed test). Numbers in quadrants (left) indicate percent cells of the Lin$^-$CD127$^+$ cell gate. (**c**) Expression of CD161 by nasal polyp ILCs defined as CD45$^+$, Lin$^-$ (as in **Fig. 1**) and CD127$^+$ (black line); gray shading, isotype-matched control. (**d**) Expression of RORγt by nasal polyp ILCs (defined as in **c**), fetal gut (uninflamed) CRTH2$^+$ ILCs, peripheral blood conventional NK cells and fetal gut (uninflamed) NKp44$^+$ ILCs. Data are representative of two to five experiments with one patient each (**a**), four experiments with one donor each (**b**) or four experiments with one patient or donor each (**c**,**d**).

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Table 1 Tissue distribution of CD45⁺Lin⁻CD127⁺CRTH2⁺ ILCs

Tissue	CD45$^+$Lin$^-$CD127$^+$CRTH2$^+$ cells (% of CD45$^+$ cells)
Fetal gut (n = 5)	0.5–2.3
Fetal lung (n = 2)	0.2–0.3
Adult ileum (n = 5)	0.01–0.1
Adult lung (n = 3)	0.02–0.08
Adult blood (n = 3)	0.01–0.03
Adult inflamed nasal polyp (n = 4)	0.1–3.6
Adult non-inflamed nose tissue (n = 4)	0–0.1

Data are representative of one experiment per donor (number of donors in parentheses).

the nasal polyps of patients with chronic rhinosinusitis. This disease is characterized by the presence of very high local titers of immunoglobulin E and large numbers of eosinophils that may be driven by the eosinophil growth factor IL-5 and further supported by IL-13. *IL5* and *IL13* transcripts are indeed more abundant in these patients than in patients with chronic rhinosinusitis without nasal polyps[27]. Indeed, nasal polyps contained larger proportions of CRTH2$^+$CD127$^+$CD161$^+$ ILCs that did uninflamed nose tissue from healthy, nonallergic donors (**Fig. 5** and **Table 1**). These data supported the hypothesis that CRTH2$^+$ cells are type 2 ILCs and that they can contribute to type 2 cell–mediated disease. In contrast to CRTH2$^+$ ILCs in fetal gut, which had low expression of RORγt, the nasal polyp–residing CRTH2$^+$ cells did not seem to express any RORγt protein (**Fig. 5**), an observation that associates these cells with the mouse natural helper cells reported to be completely negative for RORγt[16].

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Presence of CRTH2⁺CCR6⁺ ILCs in peripheral blood

The finding that CRTH2$^+$ ILCs were present in many tissues may mean that they differentiate in these organs from a circulating precursor or that mature CRTH2$^+$ ILCs may circulate. To address this issue, we analyzed adult human peripheral blood for the presence of Lin$^-$CRTH2$^+$ ILCs and other ILCs. For this analysis, we depleted peripheral blood mononuclear cell samples of most T cells (with anti-CD3), B cells (with anti-CD19) and monocytes (with anti-CD14). After gating on Lin$^-$ cells, a population of CD127$^+$ cells distinct from CD56-expressing NK cells was present (**Fig. 6a**). In the Lin$^-$CD127$^+$ population, we were able to distinguish subsets of CD117$^+$ and CD117$^-$ cells.

To investigate how the peripheral Lin$^-$CRTH2$^+$ cells may have been related to the cells we found in other human tissues, we analyzed the expression of a comprehensive panel of markers. CD127$^+$CD117$^+$ cells had abundant expression of the lymphoid markers CD7, CD161, CD25 and CD62L (**Fig. 6b**). They were negative for NKp44, which suggested they were different from the previously described human IL-22-producing NKp44$^+$ cells, and they had low expression of HLA-DR. The CD127$^+$CD117$^-$ population was very similar to the CD127$^+$CD117$^+$ population in its expression of those markers, although some markers, such as CD25 and CD161, were present in a bimodal distribution (**Fig. 6b**), which suggested that this population was heterogeneous. Neither CD127$^+$CD117$^+$ cells nor CD127$^+$CD117$^-$ cells expressed CD34 (data not shown), which indicated that these cell populations did not include immature hematopoietic progenitors. Both the CD127$^+$CD117$^+$ and the CD127$^+$CD117$^-$ populations expressed CCR6 and CRTH2 (**Fig. 6c**), two receptors also present on fetal gut type 2 ILCs (**Fig. 1b**). In contrast, CRTH2 was not present on either CD56^{hi} or CD56$^+$ NK cells (**Supplementary Fig. 2**). The expression of RORγt protein by CRTH2$^+$ cells was lower than that by IL-17-producing helper T cells, but in contrast to NK cells, which lack RORγt, the CRTH2$^+$ cells expressed some RORγt (**Fig. 6d**), in agreement with the results obtained with fetal gut CRTH2$^+$ cells. Together these data indicated that human peripheral blood contains a cell population that shares the main phenotypic features of tissue-resident type 2 ILCs.

Response of blood CRTH2⁺ ILCs to IL-25 and IL-33

Lin$^-$CD127$^+$CD117$^+$ circulating cells stimulated with PMA plus ionomycin produced a wide range of cytokines, including IL-2, IL-13 and tumor necrosis factor and small amounts of IL-22 and IL-17 (**Supplementary Fig. 3**), which suggested that these cells were functionally heterogeneous. Indeed, when we stimulated freshly isolated cells with IL-2 only or with a combination of IL-2 plus IL-25 or of IL-2 plus IL-33, IL-13 was induced only in the CRTH2$^+$ subset but not in the CRTH2$^-$ subset (**Fig. 7**). To determine the stability of the CRTH2$^+$ cells, we generated cell lines from blood CRTH2$^+$ ILCs. All expanded cell populations expressed CRTH2 and CD127 (**Fig. 8a**) and had low expression of *RORC* transcripts (**Fig. 8b**), similar to CRTH2$^+$ ILCs isolated *ex vivo*; this confirmed the stability of this phenotype. The cultured cells had high expression of IL-13 when stimulated with ionomycin plus PMA, but they did not express IL-17 (**Fig. 8c**). Furthermore, these cell lines expressed *ST2*, *IL17RB* and *IL17RA* (**Fig. 8d**). CD117$^-$ cells expressed more *ST2* and *IL17RB* than did CD117$^+$ cells, in agreement with the greater responsiveness of CD117$^-$ ILC lines to IL-25 and IL-33 (**Supplementary Fig. 4**). As noted for fetal gut, most blood CRTH2$^+$ ILC lines responded

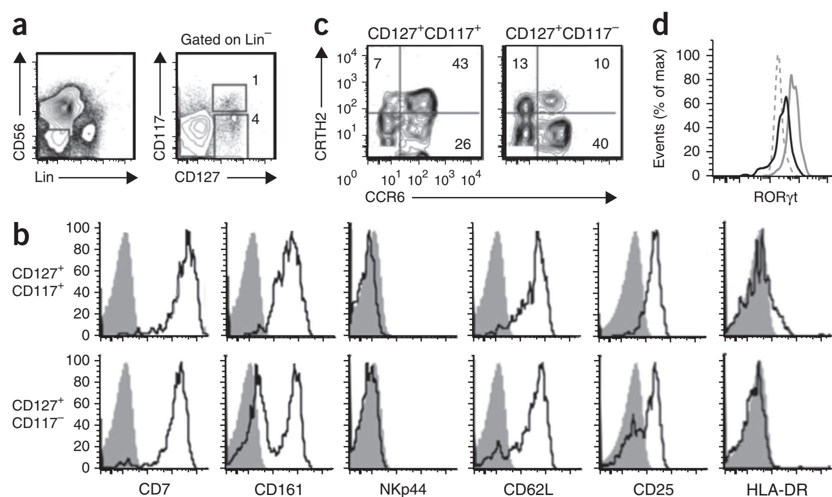

Figure 6 CRTH2$^+$CD127$^+$CCR6$^+$ innate lymphoid cells are present in peripheral blood. (**a**) Flow cytometry analysis of peripheral blood cell samples depleted of most T cells, B cells and monocytes; right, gating showing the presence of a Lin$^-$CD127$^+$CD117$^+$ population (1) and Lin$^-$CD127$^+$CD117$^-$ population (4). (**b**) Flow cytometry analysis of various markers (horizontal axes) in Lin$^-$CD127$^+$CD117$^+$ and Lin$^-$CD127$^+$CD117$^-$ populations as in **a**; gray shading, isotype-matched control antibody. (**c**) Flow cytometry analysis of the expression of CRTH2 and CCR6 in Lin$^-$CD127$^+$CD117$^+$ (top) and Lin$^-$CD127$^+$CD117$^-$ (bottom) cells as in **a**. Numbers in quadrants indicate percent cells of gate above plot. (**d**) Expression of RORγt in Lin$^-$CD127$^+$CRTH2$^+$ ILCs (black line), conventional CD56$^+$ NK cells (dashed gray line) and IL-17-producing helper T cells (CD3$^+$CD4$^+$CCR4$^+$CCR6$^+$; solid gray line). Data are representative of at least three experiments with one donor each.

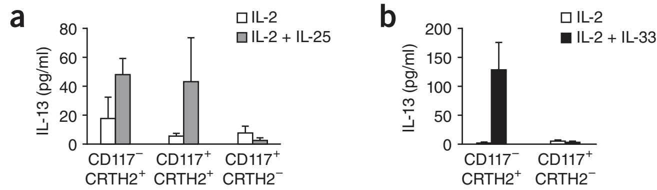

Figure 7 CRTH2+ peripheral blood ILCs respond to IL-25 and IL-33 by producing IL-13 protein. Enzyme-linked immunosorbent assay of IL-13 in supernatants of peripheral blood mononuclear cell samples depleted of T cells (with anti-CD3), B cells (with anti-CD19), NK cells (with anti-CD16) and monocytes (with anti-CD14) through the use of magnetic beads, followed by sorting as Lin−CD127+CRTH2+ and Lin−CD127+CRTH2− cells and culture for 4 d with IL-2 (10 U/ml) alone or a combination of IL-2 (10 U/ml) and IL-25 (50 ng/ml; **a**) or with IL-2 (10 U/ml) alone or a combination of IL-2 (10 U/ml) and IL-33 (50 ng/ml; **b**); concentrations were normalized to those of 2,000 cells in 200 μl. The lineage 'cocktail' used for sorting included antibodies to CD1a, CD3, CD4, CD11c, CD14, CD19, CD34, CD56, CD94, CD123, TCRαβ, TCRγδ, BDCA2 and FcεR1. Data are from two to six experiments with one donor each (mean and s.e.m.).

to IL-25, but the response to IL-33 and to combinations of these two cytokines was more pronounced (**Fig. 8e**; responses of individual cell lines, **Supplementary Fig. 4**). IL-22 was expressed in a minority of the cells stimulated with PMA plus ionomycin, but all IL-22-producing cells also produced IL-13 (**Fig. 8c**). The CRTH2+ ILCs expressed *AHR*, which encodes a transcription factor associated with IL-22 production (**Fig. 8f**). However, stimulation with IL-23 or IL-1β did not further enhance IL-22 secretion (**Fig. 8g**), in contrast to results obtained with tonsil NKp44+ cells (**Fig. 4e**). Together our data indicate that some of the CRTH2+ cells from peripheral blood had the ability to produce IL-22.

DISCUSSION

Here we have described CRTH2+ ILCs that were distinct from basophils and mast cells. Lin−CRTH2+ ILCs were present in intestinal tissue during the fetal stage and persisted in adults. Fetal gut CRTH2+ ILCs isolated *ex vivo* expressed IL-13 but not IL-17 or IL-22. *In vitro*, these cells responded to IL-25 and IL-33 by producing the type 2 cytokine IL-13. Our data suggest, therefore, that the CRTH2+ ILCs described here are similar to the non-B, non-T, common γ-chain–dependent IL-25-responsive cell type initially identified[21] and then rediscovered by several other groups who called them, variously, nuocytes[18], natural helper lymphocytes[16] and innate helper type 2 cells[19].

The type 2 ILCs in the mouse have essential roles in the innate response to helminthes and are responsible for goblet-cell hyperplasia and eosinophilia[16,18,19]. Given those features it is possible that type 2 ILCs are also involved in the pathophysiology of type 2 immunity–driven inflammatory diseases in other tissues, such as the airways. Indeed, we found type 2 ILCs in healthy human lungs. Furthermore, IL-13-producing ILCs have been shown to mediate influenza-induced airway hyper-reactivity[28], which supports the idea of a crucial role for type 2 ILCs in airway inflammation driven by the IL-13–IL-33 axis. In this context, our observation of relatively high proportions of type 2 ILCs in nasal polyps of patients with rhinosinusitis is relevant, as this disease is characterized by eosinophilia, most probably caused by IL-5. Furthermore, those patients have a greater abundance of *IL5* and *IL13* transcripts than do patients with chronic rhinosinusitis without nasal polyps[27].

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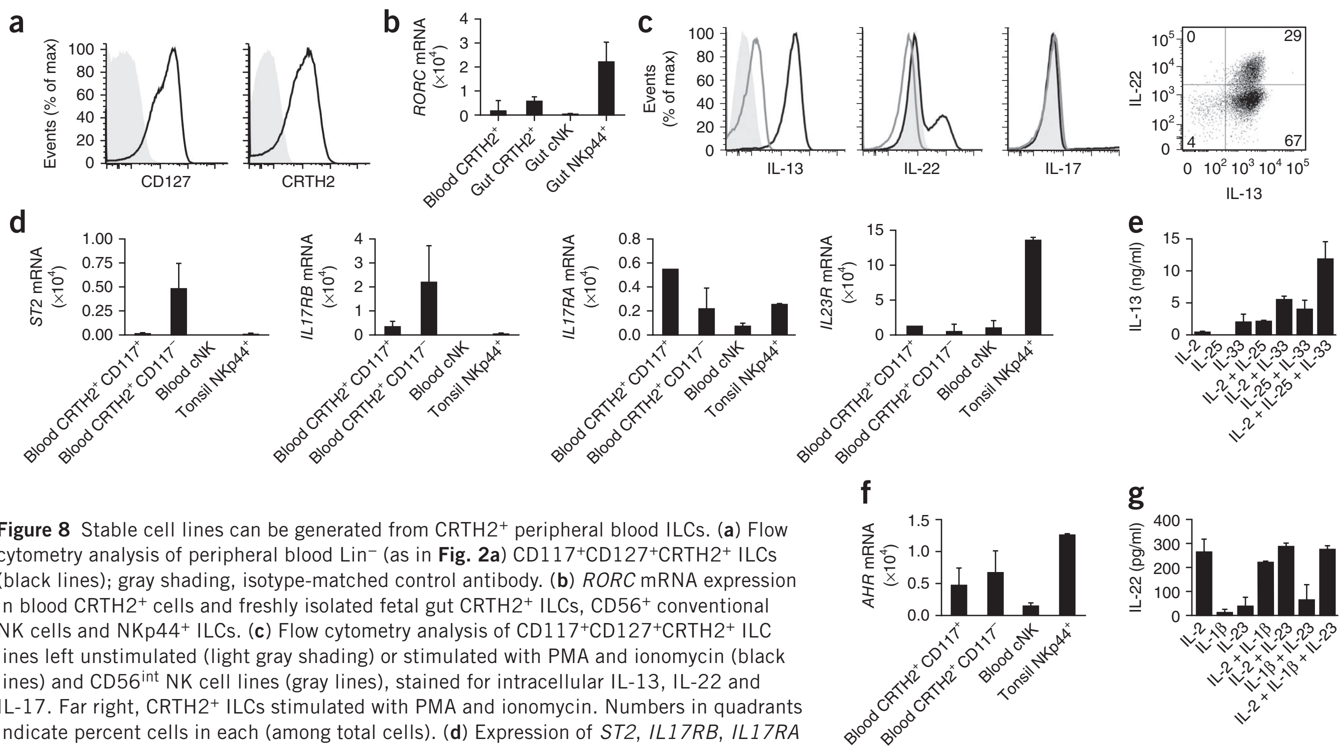

Figure 8 Stable cell lines can be generated from CRTH2+ peripheral blood ILCs. (**a**) Flow cytometry analysis of peripheral blood Lin− (as in **Fig. 2a**) CD117+CD127+CRTH2+ ILCs (black lines); gray shading, isotype-matched control antibody. (**b**) *RORC* mRNA expression in blood CRTH2+ cells and freshly isolated fetal gut CRTH2+ ILCs, CD56+ conventional NK cells and NKp44+ ILCs. (**c**) Flow cytometry analysis of CD117+CD127+CRTH2+ ILC lines left unstimulated (light gray shading) or stimulated with PMA and ionomycin (black lines) and CD56int NK cell lines (gray lines), stained for intracellular IL-13, IL-22 and IL-17. Far right, CRTH2+ ILCs stimulated with PMA and ionomycin. Numbers in quadrants indicate percent cells in each (among total cells). (**d**) Expression of *ST2*, *IL17RB*, *IL17RA* and *IL23R* mRNA in blood CRTH2+ ILCs (CD117+ or CD117−), blood CD56int conventional NK cells and tonsil NKp44+ ILCs. (**e**) IL-13 response of CRTH2+ ILCs to various combinations of IL-2, IL-25 and IL-33 (horizontal axis). (**f**) Expression of *AHR* mRNA in blood CRTH2+ ILCs (CD117+ or CD117−), blood CD56int conventional NK cells and tonsil NKp44+ ILCs. (**g**) Secretion of IL-22 from CRTH2+ ILC lines after stimulation with various combinations of IL-2, IL-1β and IL-23 (horizontal axis). Data are representative of three experiments with one donor each (**a**,**c**) or are from one experiment with two to three donors (**b**), one experiment with one to five donors (**d**; median and range), three experiments with one donor each (**e**,**g**; mean and s.e.m.) or one experiment with two to five donors (**f**; median and range).

As CRTH2+ ILCs were also present in the gut, it is conceivable that these cells may have a role in chronic gut inflammation with an IL-13-linked etiology, such as ulcerative colitis[29]. Notably, treatment of lamina propria cells from patients with ulcerative colitis with anti-CD161 results in 90% less IL-5 and IL-13 (ref. 30). It was concluded that the CD161+ NKT cells were the main producers; however, it may be possible that type 2 ILCs that also express CD161 contribute to IL-13 production in ulcerative colitis tissues. Future work should investigate the possible role of CRTH2+ cells in other type 2 immunity–mediated inflammatory diseases, such as asthma, allergic diarrhea and atopic skin disorders, which might provide new avenues for therapeutic intervention directed at these cells.

The presence of CRTH2+ ILCs in fetal tissues, particularly in fetal gut, raises the question of whether these cells are involved in tissue generation. Perhaps fetal type 2 ILCs and LTi cells exert their tissue-generating activity in different tissues during embryonic development. Studies of mice should be done to test this possibility. For example, the identification of cell surface markers specific for type 2 ILCs in the mouse may permit the demonstration of effects of specific deletion of these cells during development. LTi cells have also been linked to the regeneration of lymphoid tissue after acute viral infection[31], and it is possible that type 2 ILCs also have such a function as well.

The presence of CRTH2+ cells in a variety of tissues raised the question of whether these were derived from circulating mature CRTH2+ ILCs. To address this issue, we observed that IL-25- and IL-33-responsive CRTH2+ cells with phenotypes similar to those found in the tissues were present in the peripheral blood. However, the type 2 ILCs in peripheral blood did not express *IL13* or other transcripts of genes encoding other cytokines *ex vivo*, in contrast to results we obtained with fetal gut, which indicated they were in an inactivated state. Most CRTH2+ peripheral blood cells coexpressed CCR6, which may be instrumental in the homing of these cells to the tissues. It is possible that type 2 ILCs homing to various tissues such as lung and gut become activated *in situ* and therefore adopt slightly different features, including expression of IL-13 and perhaps certain cell surface receptors, which might explain why type 2 ILCs found in different tissues in the mouse are not identical to each other[32]. In further support of that idea, we observed a distinct IL-13-producing profile for gut tissue type 2 ILCs, whereas a minority of the circulating CRTH2+ ILCs also produced some IL-22, which was not regulated by IL-1β or IL-23. This further supports the proposal that the circulating ILC population is distinct from the previously described IL-22 producing ILCs but has more functional plasticity than do tissue-resident type 2 ILCs. Both fetal gut and blood type 2 ILCs responded to IL-25 and responded even more to IL-33, consistent with results obtained with mice[16,18]. Accordingly, type 2 ILCs specifically expressed transcripts for the receptors for IL-25 (*IL17RA* and *IL17RB*) and IL-33 (*ST2*).

In contrast to NK cells, which do not express *RORC* transcripts or RORγt protein, CRTH2+ type 2 ILCs expressed some RORγt, as determined by flow cytometry and analysis of *RORC* transcripts. This observation was unexpected, as mouse type 2 ILCs are reported to lack *RORC* transcripts[16]. In contrast to fetal LTi cells and postnatal IL-17- or IL-22-producing ILCs, type 2 ILCs are also independent of RORγt for their development in the mouse[16]. The expression of RORγt in human CRTH2+ cells does not necessarily mean that these cells depend on this transcription factor. It is possible that type 2 ILCs and IL-17- and IL-22-producing ILCs have a common RORγt+ precursor; although differentiation of the common precursor to IL-17- and IL-22-producing ILCs requires RORγt, this might not be the case for type 2 ILCs. An analogous situation exists in the T cell system. RORγt is expressed on all double-positive (CD4+CD8+) thymocytes[33]. Although RORγt is required for the optimal survival of double-positive thymocytes, mature helper T cells do develop in RORγt-deficient mice, except for IL-17-producing helper T cells[34]. Published evidence suggests that RORγt expression is not always stable; in RORγt reporter mice, downregulation of RORγt is associated with a functional shift from IL-22 production to IFN-γ production[35]. Perhaps an analogous situation exists for CRTH2+ ILCs in that they may develop from RORγt+ ILCs, whereby downregulation of the production of RORγt and IL-22 parallels the upregulation of CRTH2 and IL-13. The question of whether or not IL-17- and IL-22-producing ILCs and type 2 ILCs derive from a common RORγt+ precursor should be addressed in mouse models by cell fate–mapping experiments.

In summary, we have described here a unique human ILC population that was responsive to IL-25 and IL-33 and produced large amounts of IL-13. In contrast to what we observed in lung, gut and healthy nasal tissues, relatively large proportions of CRTH2+ cells were present in inflamed nasal polyps of chronic rhinosinusitis, an inflammatory disease mediated by the type 2 immune response[27]. These cells may therefore be the human equivalent of cells found in the mouse that have been called natural helper cells, nuocytes or innate helper type 2 cells.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

J.M.M. designed the study, did experiments, analyzed the data and wrote the manuscript; S.T. designed the study, did experiments, analyzed the data and wrote the manuscript; N.K.C. did experiments and analyzed the data; C.P.P. did experiments, and provided and processed gut tissue; C.M.v.D. and W.J.F. provided inflamed and uninflamed nasal tissue; B.P. provided and processed lung tissue; T.C. designed the study; and H.S. designed the study and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Fetal and adult tissues. 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.

Uninflamed nose conchae tissue was obtained from healthy adults. Inflamed nasal polyps were from patients with chronic rhinosinusitis. Lung tissues were obtained (after receipt of informed consent) from adult patients undergoing lung tumor surgery; tissues were obtained at an appropriate distance from the tumor. Collection of lung and nose tissue was approved by the Medical Ethical Commission of the Academic Medical Center, Amsterdam. Inflamed ileum tissue was collected from patients with Crohn's disease undergoing resection surgery. Uninflamed ileum was obtained from patients undergoing colon tumor–resection surgery; ileal tissue was collected at an appropriate distance from the tumor. Both inflamed and uninflamed ileal tissue was obtained as residual material after clinical procedures according to ethical guidelines of the Academic Medical Center, Amsterdam, the Netherlands.

Buffy coats were provided by the blood bank at Sanquin, Amsterdam, or by the Blood Centers of the Pacific (California) after receipt of written informed consent.

Isolation of cells. All solid tissues were rinsed of connective tissue, fat and muscle. Fetal and adult intestinal tissues were also cleared of meconium and feces, respectively, after which adult ileal tissues were incubated with dithiothreitol (154 µg/ml), 0.1% (vol/vol) β-mercaptoethanol and 5 mM EDTA for elimination of epithelial cells and mucus. Tissues were cut into fine pieces and digested for 30–45 min at 37 °C with Liberase TM (125 µg/ml) and DNase I (200 µg/ml; both from Roche). 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 Ficoll-Paque PLUS (GE Healthcare). Peripheral blood mononuclear cells were isolated on Lymphoprep (Nycomed) or Ficoll-Paque (GE Healthcare).

Flow cytometry analysis and sorting. The following antibodies to human proteins were used: fluorescein isothiocyanate (FITC)-conjugated anti-CD1a (HI149), anti-CD3 (OKT3), anti-CD11c (3.9), anti-CD94 (DX22), anti-CD123 (6H6), anti-FcER1α (AER-37), phycoerythrin (PE)-conjugated anti-CD7 (CD7-6B7), anti-CD94 (HP-3D9), anti-CD161 (HP-3G10), anti-KIR3DL1 (DX9), anti-perforin (DG9), anti-NKp44 (P44-8), peridinin chlorophyll protein–cyanine 5.5–conjugated anti-CD117 (104D2), Alexa Fluor 647–conjugated anti-CD25 (BC96), anti-NKp46 (9E2), anti-NKp44 (P44-8), anti-NKp30 (P30-15), anti–granzyme B (GB11), anti-CCR7 (TG8/CCR7), anti-CCR6 (TG7/CCR6), allophycocyanin (APC)-conjugated anti-NKG2D (1D11), Alexa Fluor 700–conjugated anti-CD56 (HCD56; all from BioLegend); biotinylated anti-CCR6 (11A9) in combination with streptavidin–Horizon V450 (BD); FITC-conjugated anti-CD4 (RPA-T4), anti-CD14 (MφP9), anti-CD16 (3G8), anti-CD19 (HIB19), anti-CD34 (581), anti-CD56 (NCAM16.2), anti-TCRαβ (IP26), TCRγδ (B1), PE-conjugated anti-CD16 (3G8), anti-CCR4 (1G1), anti-CXCR3 (IC6/CXCR3), Alexa Fluor 647–conjugated anti-CRTH2 (CD294; BM16), anti-CXCR5 (RF8B2), APC-indotricarbocyanine (Cy7)–conjugated anti-CD45 (2D1), and isotype-matched control antibodies conjugated to Alexa Fluor 700 (MOPC-21), PE, APC or PE-Cy7 (X40; all from Beckton Dickinson); APC-conjugated anti-CD4 (S3.5; Invitrogen); PE-conjugated anti-CD45RA (ALB11; Beckman Coulter); PE-Cy7–conjugated anti-CD127 (R34.34; Beckman Coulter); PE-conjugated anti-RORγt (AFKJS-9; eBioscience); PE-conjugated goat polyclonal anti-IL-1R1 (R&D Systems); and FITC-conjugated anti-BDCA2 (CD303; AC144; Milenyi).

For phenotype analysis by flow cytometry, data were acquired on an LSRFortessa or LSR II (BD) and were analyzed with FlowJo software (TreeStar).

For sorting by flow cytometry, peripheral blood mononuclear cell samples were depleted of T cells, B cells, NK cells and monocytes by labeling with FITC-conjugated anti-CD3, anti-CD14, anti-CD16 and anti-CD19 (described above) plus anti-FITC microbeads (Miltenyi) or the corresponding EasySep antibodies plus beads (StemCell Technologies). $Lin^-CD127^+CRTH2^+$ ILCs from fetal gut and peripheral blood were sorted on a FACSAria (BD) to a purity of ≥98%.

Establishment of $CD127^+CRTH2^+$ cell lines and analysis of cytokine production. $Lin^-CD127^+CRTH2^+$ ILC and NK cell 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 or Genentech; made 'in-house') supplemented with 1% (vol/vol) human AB serum. Fresh and expanded $Lin^-CD127^+CRTH2^+$ ILC and NK cell populations were stimulated for 3–4 d with IL-2 (10 U/ml; Novartis), IL-25 (50 ng/ml; R&D Systems) or IL-33 (50 ng/ml; R&D Systems) or combinations of these cytokines. Fresh cells were stimulated for 24 h with PMA (phorbol 12-myristate 13-acetate) plus ionomycin. Expanded populations were stimulated for 3–4 d with IL-2, IL-1β (Miltenyi) or IL-23 (R&D Systems) or various combinations of these cytokines. IL-13 and IL-22 were measured in supernatants by enzyme-linked immunosorbent assay (Sanquin and Genentech-Roche 'in-house' or R&D Systems, respectively). Multiple cytokines were detected in some experiments by Luminex technology (Bio-Rad).

Intracellular cytokine staining. Cell lines expanded *ex vivo* were stimulated for 6 h with PMA (10 ng/ml; Sigma) and ionomycin (500 nM; Merck) in the presence of GolgiPlug (BD) or brefeldin A (5 µg/ml) 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: APC-conjugated anti-IL-13 (JES10-5A2; BioLegend), APC-conjugated IL-17 (BL168; BioLegend) and PE-conjugated anti-IL-22 (142928; R&D Systems) or Alexa Fluor 647–conjugated anti-IL-22 (3F11; Genentech-Roche[36]), anti-IFN-γ (B27; BD Bioscience) or anti–tumor necrosis factor (MAb11; BD Bioscience). Data were acquired on an LSRFortessa or LSR II (BD) and were analyzed with FlowJo software (TreeStar).

Quantitative real-time PCR. Total RNA was extracted with an RNeasy Micro kit (Qiagen) or NucleoSpin RNA XS kit (Macherey-Nagel) according to the manufacturer's instructions. Then, cDNA was produced with a High-Capacity cDNA Archive kit (Applied Biosystems). SYBR Green I Master Mix (Roche) and a LightCycler 480 (Roche) were used for PCR (primers designed 'in-house'; **Supplementary Table 1**). Primers for *IL17*, *IL22* and *IFNG* have been published[4]. LinRegPCR software[37,38] was used for quantification of expression. All samples were normalized to the expression of 18S rRNA and results are presented in arbitrary units.

Statistical analysis. Differences between healthy controls and patients with chronic rhinosinusitis in the frequency of nasal tissue $CRTH2^+$ ILCs were calculated with the two-tailed Mann-Whitney *U*-test.

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