

IL-12 drives functional plasticity of human group 2 innate lymphoid cells

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Group 2 innate lymphoid cells (ILC2) include IL-5- and IL-13-producing CRTh2⁺CD127⁺ cells that are implicated in early protective immunity at mucosal surfaces. Whereas functional plasticity has been demonstrated for both human and mouse ILC3 subsets that can reversibly give rise to IFN- γ -producing ILC1, plasticity of human or mouse ILC2 has not been shown. Here, we analyze the phenotypic and functional heterogeneity of human peripheral blood ILC2. Although subsets of human CRTh2⁺ ILC2 differentially express CD117 (c-kit receptor), some ILC2 surface phenotypes are unstable and can be modulated in vitro. Surprisingly, human IL-13⁺ ILC2 can acquire the capacity to produce IFN- γ , thereby generating plastic ILC2. ILC2 cultures demonstrated that IFN- γ ⁺ ILC2 clones could be derived and were stably associated with increased T-BET expression. The inductive mechanism for ILC2 plasticity was mapped to the IL-12-IL-12R signaling pathway and was confirmed through analysis of patients with Mendelian susceptibility to mycobacterial disease due to IL-12R β 1 deficiencies that failed to generate plastic ILC2. We also detected IL-13⁺IFN- γ ⁺ ILC2 ex vivo in intestinal samples from Crohn's disease patients. These results demonstrate cytokine production plasticity for human ILC2 and further suggest that environmental cues can dictate ILC phenotype and function for these tissue-resident innate effector cells.

Innate lymphoid cells (ILCs) represent a novel family of hematopoietic effectors that serve essential roles in the early immune response by their prompt cytokine and chemokine secretion. Three distinct groups of ILCs have been described that functionally correspond to innate counterparts of CD4⁺ Th cells. Group 1 ILCs (ILC1) express the transcription factor (TF) T-BET and produce the Th1-associated cytokines IFN- γ and TNF. Group 2 ILCs (ILC2) secrete the Th2-associated cytokines IL-5 and IL-13 via a GATA-3- and ROR α -dependent pathway. Group 3 ILCs (ILC3) use ROR γ t to drive production of the Th17-associated cytokines IL-17 and -22 (Spits et al., 2013; Serafini et al., 2015). These different ILC subsets are found in diverse lymphoid and nonlymphoid tissues, and enriched at mucosal sites, where they play essential roles in barrier function and innate immune defense (Artis and Spits, 2015; Eberl et al., 2015).

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Abbreviations used: ILC, innate lymphoid cell; iono, ionomycin; Lin, Lineage; TF, transcription factor.

The ILC2 subset in mice was originally identified in fat-associated lymphoid clusters or as systemically dispersed IL-13-producing non-B, non-T lymphoid cells (Moro et al., 2010; Neill et al., 2010; Price et al., 2010). ILC2 lack lineage (Lin) markers, express IL-7R (CD127), c-Kit (CD117), Sca-1, and T1/ST2 (IL-33R), and can be triggered to produce copious amounts of IL-5, -6, and -13. Human ILC2 were first characterized in the fetal intestine and were defined as Lin⁻CD127⁺CD161⁺ cells expressing the chemoattractant receptor CRTh2, which has been shown to mark Th2 cells (Mjösberg et al., 2011). CRTh2⁺ ILC2 respond to IL-25 and -33 stimulation by rapidly producing IL-13 and -5. ILC2 have also been identified in various human tissues, including lung, skin, and adipose tissues, where they are implicated in a variety of physiological processes and disease conditions (Barlow and McKenzie, 2014; Kim and Artis, 2015; Roederer and Weninger, 2015). Circulating ILC2 in human blood

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share main phenotypic features of tissues-resident ILC2, such as CRTh2 expression (Mjösberg et al., 2011; Bartemes et al., 2014; Xue et al., 2014); however, their properties have not been extensively characterized. Interestingly, a small subset of CRTh2⁺ ILC2 can produce IL-22 (Mjösberg et al., 2011), although the relevance of this finding remains obscure.

In recent years, the ILC nomenclature system has been challenged by the discovery that cytokines and TF expression profiles in some ILC subsets are not stable. For example, both human and mouse intestinal ROR γ T ILC3 can reversibly differentiate into T-BET⁺ IFN- γ -producing ILC1 (Vonarbourg et al., 2010; Bernink et al., 2015). More recently, a potential IL-25-responsive ILC2 precursor in mouse has been reported to acquire IL-17-producing ability (Huang et al., 2015). Whether human ILC2 exhibit plastic phenotypic or functional features remains largely unexplored.

In this study, we have characterized the phenotypic, molecular, and functional attributes of circulating ILC2. We uncover a novel functional plasticity in these cells with the capacity to coexpress the Th1 cytokine IFN- γ driven by the IL-12–IL-12R pathway. Our results suggest that environmental cues can elicit novel ILC phenotypes and functions within tissues.

RESULTS

Phenotypic and functional heterogeneity of human peripheral blood ILC2

Previous studies (Mjösberg et al., 2011; Bartemes et al., 2014; Xue et al., 2014) identified ILC2 at low frequency (0.01–0.03% of total CD45⁺ cells) in the blood of normal healthy humans and in patients suffering from atopic dermatitis and asthma, but did not provide a detailed phenotypic analysis of these cells. We thus performed a multiparametric flow cytometric analysis of blood ILC2 to better characterize this circulating ILC subset (Fig. 1, A and B). Human blood ILC2 (defined as Lin[−]CD127⁺CD161⁺CRTh2⁺ cells; see Fig. S1 for gating strategy) uniformly expressed CD7 and CD11a, but were negative for CD2, CD5, and intracellular CD3 (Fig. 1 C and Fig. S1 A). CD90 has been used extensively in mouse to identify ILCs (Monticelli et al., 2011) and identifies a small subset of memory CD4⁺ and CD8⁺ T cells in human blood (Guillot-Delost et al., 2012). Interestingly, human peripheral blood ILC2s were CD90[−] suggesting a species divergence for this molecule. Evidence for potentially distinct human blood ILC2 subsets was observed with some markers (CD117, CCR6, and KLRG1) that generated heterogeneous staining patterns (Fig. 1 C). Two parameter correlations showed that CCR6⁺ ILC2 were somewhat enriched for CD117 expression, although this varied among individuals tested, whereas KLRG1⁺ ILC2 were not enriched for either CCR6 or CD117 (unpublished data). Concerning TF expression, ILC2 were strongly positive for GATA-3, as expected, but lacked T-BET or ROR γ T protein (Fig. 1 D). Curiously, when stimulated with cytokines (combinations of IL-25 and IL-33) known to activate ILC2, no clear evidence of IL-5 or

IL-13 production for up to 12 h after stimulation was found. In contrast, pharmacological activators (PMA/ionomycin [iono]) were able to elicit IL-13 production (Fig. 1 E). All IL-13⁺ ILC2 expressed CRTh2, although only a fraction of CRTh2⁺ ILC2 produced IL-13 (Fig. 1 E).

To further explore potential functional heterogeneity associated with ILC2 subsets, we sorted single CD117⁺ and CD117[−] CRTh2⁺ ILC2 and assessed expression of a selected set of transcripts using Biomark (Fluidigm). Targets included cytokines, chemokines, cell surface proteins, TFs, and cytokine receptors that have been shown to be important in ILC differentiation or function (Table S1). After hierarchical clustering, we found that freshly isolated CD117⁺ and CD117[−] ILC2 subsets were not clearly distinguished and their expression profiles appeared to overlap (Fig. 2 A). Moreover, we found that whereas all single ILC2 exhibited the expected TF signature (*GATA3*, *RORA*, *MAF*, and *ID2* without expression of *TBX21* and *RORC*), the frequency of *IL13*- or *IL5*-expressing cells was surprisingly low (~11.5% of ILC2 expressed *IL13* and only 4.4% expressed *IL5*). Transcripts for other genes, such as *AREG*, *CSF2*, and *IL4*, whose effector functions are associated with ILC2 biology, were also infrequently expressed (<10%). Comparing single ILC2 that expressed these different effector molecules showed that most were monofunctional (positive for *IL13*, *IL5*, *IL4*, *AREG*, or *CSF2* only; Fig. 2 A). Finally, expression of cytokine receptors that are involved in ILC2 activation (IL-25R and -33R) was only detected in a fraction of cells (41% for *IL17RB* and 7.7% for *IL1RL1*), and coexpression was rare (4.3%; Table 1), providing a potential explanation for the absence of IL-25 and -33 responsiveness observed in freshly stimulated ILC2 (Fig. 1 E).

We hypothesized that effector functions in circulating ILC2 might be rapidly up-regulated after entry into tissues where cellular activators would be present as a result of infection, inflammation, or cellular stress. We next examined changes in the expression pattern of ILC2 at the single-cell level after activation. For this series of experiments, we stimulated Lin[−]CD127⁺CRTh2⁺ ILC2 with an activation cocktail, including IL-25, IL-33, and TL1A, a TNF superfamily member reported to trigger human blood-derived ILC2 (Yu et al., 2014). Importantly, this cytokine combination should be able to activate >90% blood ILC2 based on the frequency of expression of the cognate receptors (Table 1).

After 3-h exposure to IL-25, IL-33, and TL1A, a dramatic change in the ILC2 transcriptional profile was observed (Fig. 2, A–C). Two-dimensional hierarchical clustering clearly identified a novel ILC2 cluster with increased expression of cytokines (*IL13* and *CSF2*), TFs (*ZEB2*), and cell surface molecules (*ICOS*). We also observed the up-regulation of ILC2-related factors, including *IL4*, *IL5*, *AREG*, and *PPARG*, with notable clonal heterogeneity after activation (Fig. 2, A and C). The majority (76%) of stimulated ILC2 expressed *IL13*, *IL5*, *IL4*, *AREG*, or *CSF2* with a dominance of polyfunctional cells (expressing two to four targets). ILC2

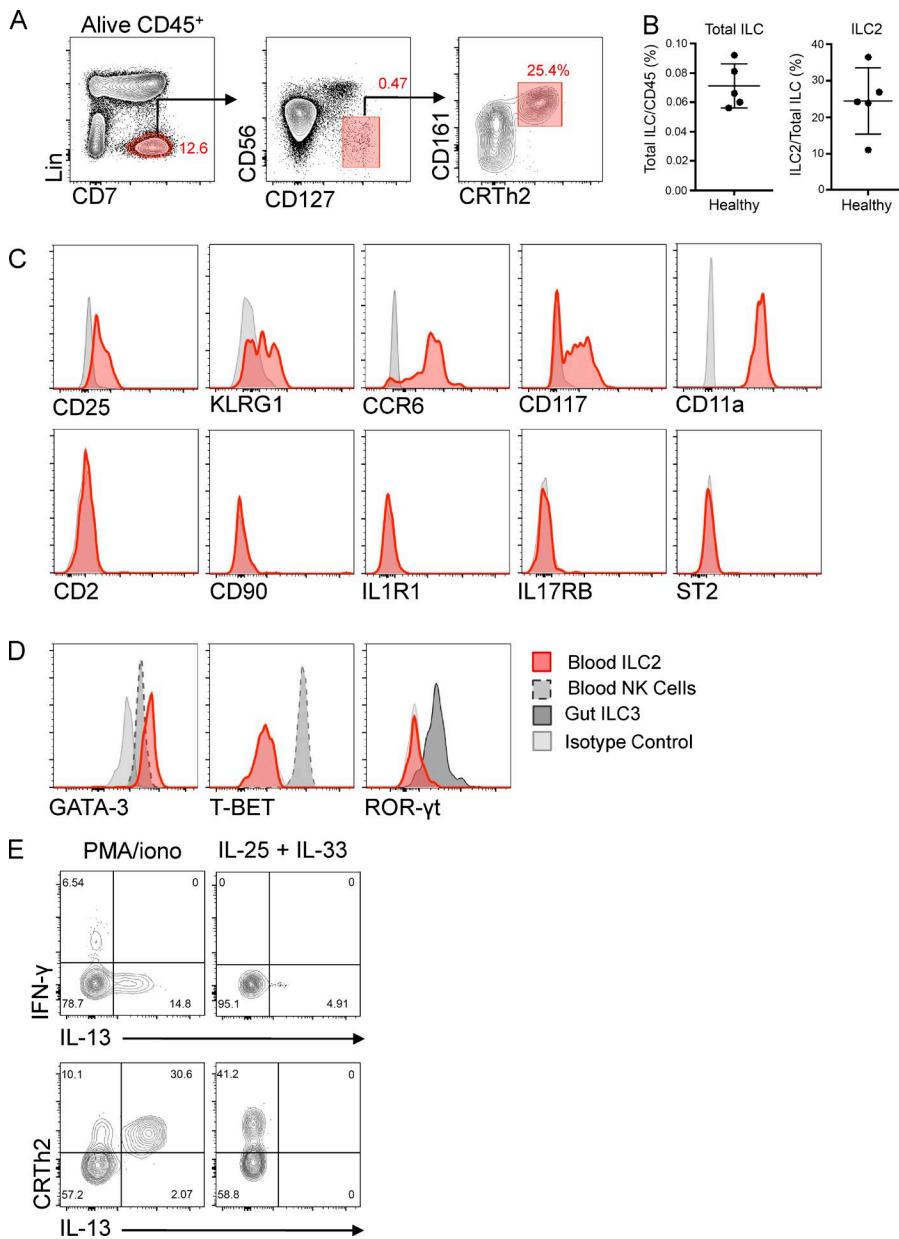


Figure 1. Phenotype and function of human peripheral blood ILC2. (A) Gating strategy for FACS analysis of human peripheral blood ILCs. ILCs were gated on viable CD45⁺ Lin⁻(CD3⁻CD5⁻TCR $\alpha\beta^+$ TCR $\gamma\delta^-$ CD14⁻CD19⁻)CD7⁺ CD127^{hi} cells. ILC2 are identified by CRTh2⁺ CD161⁺ expression. (B) Percentage of total ILCs from CD45⁺ and ILC2 from total ILCs of healthy donors. (C) Surface expression of cytokines and chemokines receptors in ILC2 (red areas) or isotype control (gray area). (D) Intracellular GATA-3, T-BET, and ROR γ t expression in ILC2 (red area), conventional CD56^{Br} NK cells (Lin⁻CD7⁺ CD56^{Br}CD127⁺; dashed gray area), or gut ILC3 (Lin⁻CD7⁺CD127⁺NKp44⁺CD117⁺; black area). (E) PBMCs were stimulated with PMA/iono for 3 h or IL-25 plus IL-33 for 12 h. IFN- γ , IL-13, and CRTh2 expression in total ILCs (Lin⁻CD7⁺CD127⁺) is shown. Data in A and C-E are representative of three independent experiments with at least three healthy donors each.

cytokine receptor expression was altered with significant induction of *IL1RL1* and *IL17RB*. Essentially, all activated ILC2 expressed either *IL1RL1*, *IL17RB*, or *TNFSFR25*, thereby allowing responses to IL-33, IL-25, or TL1A, respectively, with about half the cells concomitantly expressing all three receptors (Table 1). Concerning TF, the ILC2 signature (*GATA3*, *RORA*, *MAF*, and *ID2*) was maintained after cytokine activation with a slight induction of *ID2* and *RORA*. In contrast, Th1-associated transcriptional regulators (*TBX21* and *EOMES*) were not induced and cells failed to express *IFNG* transcripts before and after stimulation. These results demonstrate that limited cytokine signals can dramatically modify responsiveness of blood ILC2. As such, circulating ILC2 may be considered naive with requirements for fur-

ther functional competence. Our single-cell transcriptional analysis provides evidence for substantial human ILC2 clonal heterogeneity and potential molecular mechanisms to explain ILC2 responsiveness to exogenous cytokines.

In vitro culture modulates ILC2 cell surface phenotype and effector functions

The similarity in molecular profiles between CD117⁺ and CD117⁻ ILC2 subsets suggests that some ILC2 phenotypes may be flexible rather than fixed. To address this point, we sorted CRTh2⁺ ILC2 subsets and expanded them in vitro using irradiated PBMC feeders in the presence of IL-2 and IL-7, as previously described (Mjösberg et al., 2011). In some cases, we added IL-25 and IL-33 to increase the overall yield

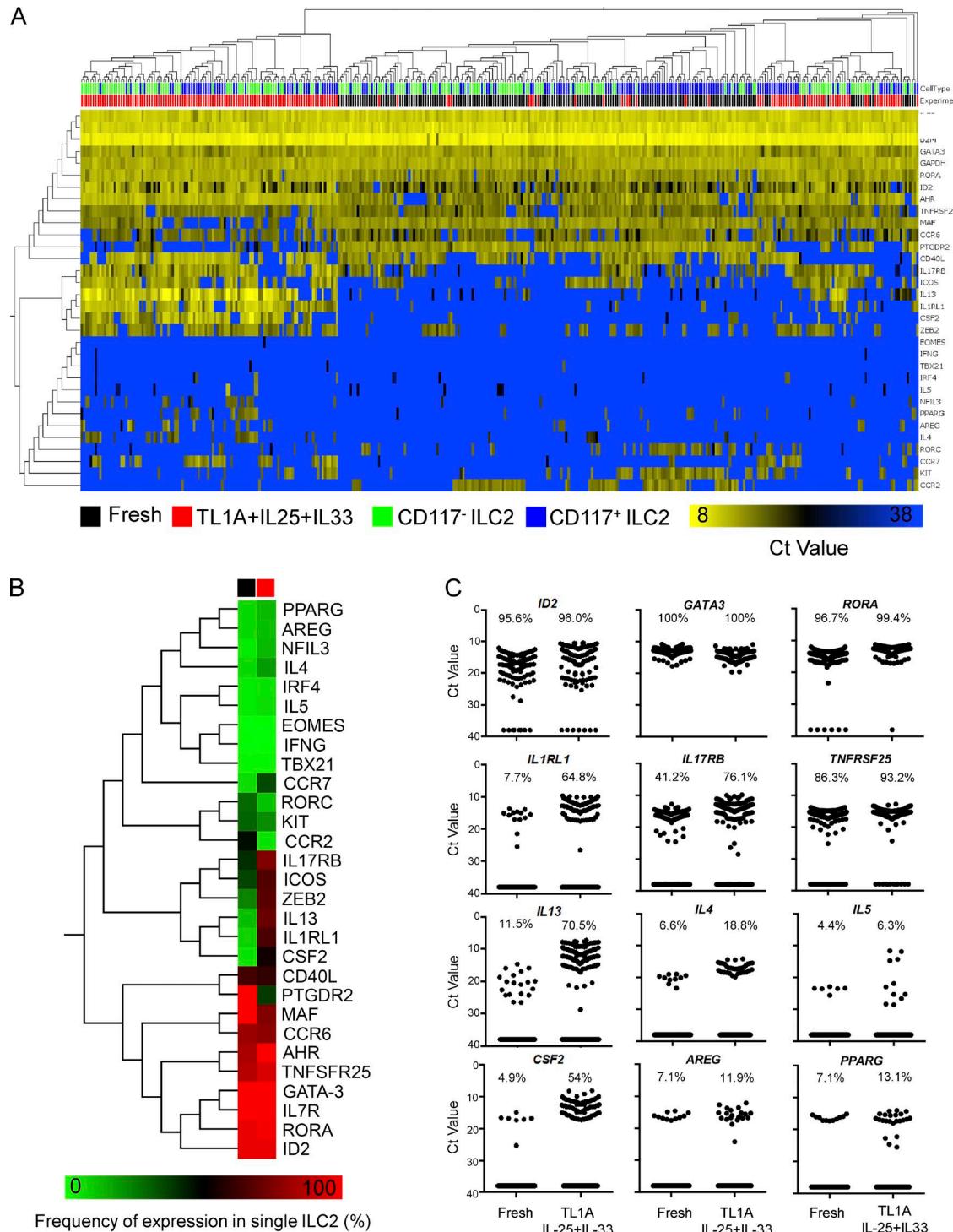


Figure 2. Single-cell gene expression profiling reveals ILC2 heterogeneity. CRTh2⁺ ILC2 were stimulated with TL1A, IL-25, and IL-33 (100 ng/ml each) or left unstimulated, and single cells were sorted into lysis buffer. Expression of a panel of mRNA transcripts was assessed by quantitative RT-PCR. For fresh ILC2, 92 CD117⁺ and 90 CD117⁻ were included in the analysis. For activated ILC2, 86 CD117⁺ and 90 CD117⁻ were included in the analysis. (A) Heat map representation of gene expression profiles using two-dimensional hierarchical clustering analysis. Each row corresponds to the expression level (Ct value) of a single gene and each column represents a single cell from fresh (black) or stimulated (red) from CD117⁺ (blue) or CD117⁻ (green) ILC2. (B) Hierarchical clustering of gene expression frequency from fresh or stimulated ILC2. Frequency of expression of each gene was calculated by number of cells with detectable signal (Ct value \leq 38) from total cells in each group. (C) Expression level and frequency of expression of selected ILC2 signature transcription factors and cytokines transcript before and after stimulation. Each dot represents one single ILC2. Data in A–C are representative of two independent experiments with one donor each.

Table 1. Expression of TNFRSF25, IL17RB, and IL1RL1 from fresh and stimulated human peripheral blood ILC2

TNFRSF25	IL17RB	IL1RL1	Fresh	TL1A + IL-25 + IL-33
			%	%
+	+	+	3.8	51.1
+	+	-	33.5	21.0
+	-	+	2.2	9.1
+	-	-	46.7	11.9
-	+	+	0.5	2.8
-	+	-	2.7	1.1
-	-	+	0.5	1.7
-	-	-	9.9	1.1

as both cytokines have been shown to expand ILC2 in both mouse and human systems (Mjösberg et al., 2011; Bartemes et al., 2014; Xue et al., 2014; Huang et al., 2015). Cultured human ILC2 formed flower-shaped clusters that were easily distinguishable from the PBMC feeder cells (Fig. 3 A). Human ILC2 could expand robustly (from 5,000- to 15,000-fold expansion) under these in vitro culture conditions and, as expected, addition of both IL-25 and -33, but not IL-12 and -18 or IL-1 β and -23, significantly increased the overall yield (Fig. 3 B). Analysis of cell surface expression showed that expanded ILC2 retained several features of the initial sorted populations: the cells uniformly expressed CD161, CD7, and CD25, but the expression of CRTh2, CD117, and CD127 was modulated (Fig. 3 C and Fig. S1 B). The latter is likely caused by the presence of IL-7 in the growth medium. Expanded ILC2 did not express other ILC markers de novo, including NKp46, NKp44, CD94 and CD16.

We next analyzed cytokine protein expression in expanded ILC2. As expected, cultured ILC2 showed enhanced expression of IL-5 and IL-13, whereas no IL-17A was detected. Curiously, a clearly distinguishable subset of IFN- γ $^+$ cells was present in both CD117 $^+$ and CD117 $^-$ expanded cells (Fig. 3 E). IFN- γ $^+$ ILC2 were found to always coexpress IL-13; these cells were already present at low frequency in steady-state cultures and could be increased after stimulation with pharmacological activators (Fig. 3 F). We further analyzed the TF profiles of the expanded ILC2 cultures. GATA-3 was, as expected, highly expressed by expanded ILC2 with levels exceeding those present in freshly isolated blood ILC2 (Fig. 3 D). Remarkably, a significant up-regulation of T-BET was observed in cultured ILC2, irrespective of the initial CD117 phenotype at the start of the culture. The increased T-BET expression may help explain the increased capacity of cultured ILC2 to produce IFN- γ under steady-state conditions and after further stimulation.

Analysis of human ILC2 clonal plasticity

Our analysis with bulk cultured human peripheral blood ILC2 suggested that these cells possess an unexpected degree of cytokine plasticity. Still, the presence of some non-ILC2 contaminants in these cultures that were able to generate the

observed plasticity could not be ruled out. To rigorously address this issue, we generated human ILC2 clones through culture of sorted single ILC2. Previous work had established conditions for human T cell cloning (Yssel and Spits, 2002); these were modified slightly to optimize growth of human ILC2, but included irradiated pooled PBMCs, phytohemagglutinin, and the cytokines IL-2, -7, -25, and -33. Similar to bulk ILC2 cultures, ILC2 clones grew as typical flower-shaped cells and could be obtained from normal healthy individuals at a frequency of 14–50% (Fig. 4 D; $n = 5$ individuals; 16–60 clones from each donor were analyzed). As was observed with bulk ILC2 cultures, a subset of cloned ILC2 clearly coexpressed IFN- γ in addition to IL-13 (Fig. 4 A). The frequency of IFN- γ -expressing cells varied from clone to clone (0–>95% IFN- γ $^+$ cells). ILC2 clones continued to express GATA-3 at high levels, whereas T-BET expression varied from low (for ILC2 clones lacking IFN- γ expression) to high (for ILC2 clones with strong IFN- γ expression) that approached levels expressed by CD56 $^+$ NK cell clones (Fig. 4 B). There was a tendency for higher CD117 expression in ILC2 clones that had lower IFN- γ expression, whereas CRTh2 expression was generally lower in ILC2 clones than that observed in freshly isolated ILC2, irrespective of the IFN- γ phenotype (Fig. 4 C).

As the frequency of IFN- γ $^+$ cells within an individual IL-13 $^+$ clone was variable, we defined plastic ILC2 clones as having >20% of IL-13 $^+$ IFN- γ $^+$ double-producing cells after stimulation (Fig. 4 E); ILC2 clones with <20% IFN- γ $^+$ cells were defined as classic. Comparing classic and plastic ILC2 clones, we found no significant differences or correlations with their expansion capacity, and the percentage of plastic ILC2 obtained was variable between donors (Fig. 4 E). Both plastic and classic ILC2 clones retained their functional properties for up to 28 d in culture (Fig. 4 F).

Roles for stromal cells and cytokines in generating human ILC2 plasticity

The results of ILC2 cloning suggested that a subset of blood ILC2 possessed the capacity to acquire novel properties, particularly cytokine plasticity, that are not normally associated with ILC2. We entertained different hypotheses to explain these observations. One possibility is that only a subset of ILC2 had the capacity to gain IFN- γ production due to expression of a unique signaling pathway. Alternatively, the variable extent of plastic ILC2 clones might depend on a pathway that was available in a limiting fashion in our culture conditions. To dissect the mechanisms behind ILC2 plasticity, we next assessed the impact of the stromal cell compartment on ILC2 phenotype at both the bulk and clonal level. ILC2 were either cultured in stromal-cell free conditions, or in the presence of murine stromal cells (OP9 expressing or not expressing the Notch ligand Delta-like 4) or under standard conditions with irradiated PBMCs. Although few ILC2 expanded in the absence of stromal cells, the resultant cultures harbored ILC2 that expressed IL-13, but not IFN- γ

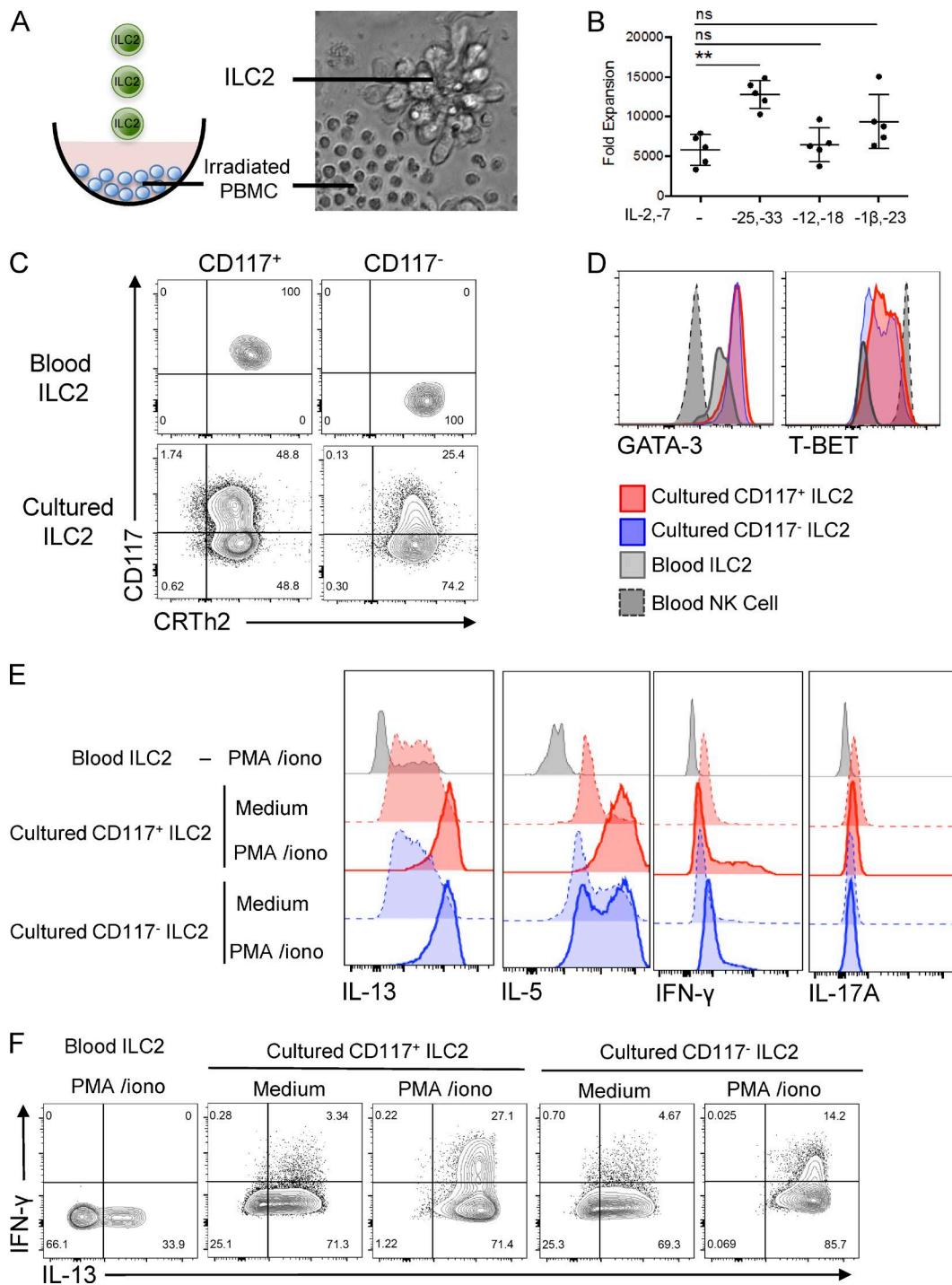


Figure 3. Phenotype and function of in vitro-expanded ILC2. (A) ILC2 morphology after bulk culture on irradiated PBMC feeder cells. (B) Fold expansion of bulk ILC2 culture for 7 d on irradiated PBMCs under different cytokine combinations. (C) Surface expression of CRTh2 and CD117 from freshly isolated blood ILC2 or 7-d ILC2 culture from CD117^{+/−} ILC2 subpopulations. (D) Intracellular expression of GATA-3 and T-BET of bulk CD117⁺ (red area) or CD117[−] (blue area) ILC2 culture for 7 d, blood ILC2 (gray area), and blood NK cell (CD56^{hi}CD127⁺, dashed gray area). (E) Intracellular cytokine staining of PMA/iono-stimulated blood ILC2 (gray area) and 7-d bulk CD117^{+/−} ILC2 culture with medium or PMA/iono for 3 h. (F) Intracellular cytokine staining for the coexpression of IL-13 and IFN- γ . Data in A and C–F are representative results from at least four experiments with one donor each cultured on irradiated PBMCs in IL-2, -7, -25, and -33 for 7 d. Data in B were obtained from five independent donors. ns, $P > 0.05$; **, $P < 0.01$ using paired Student's t test (mean \pm SD).

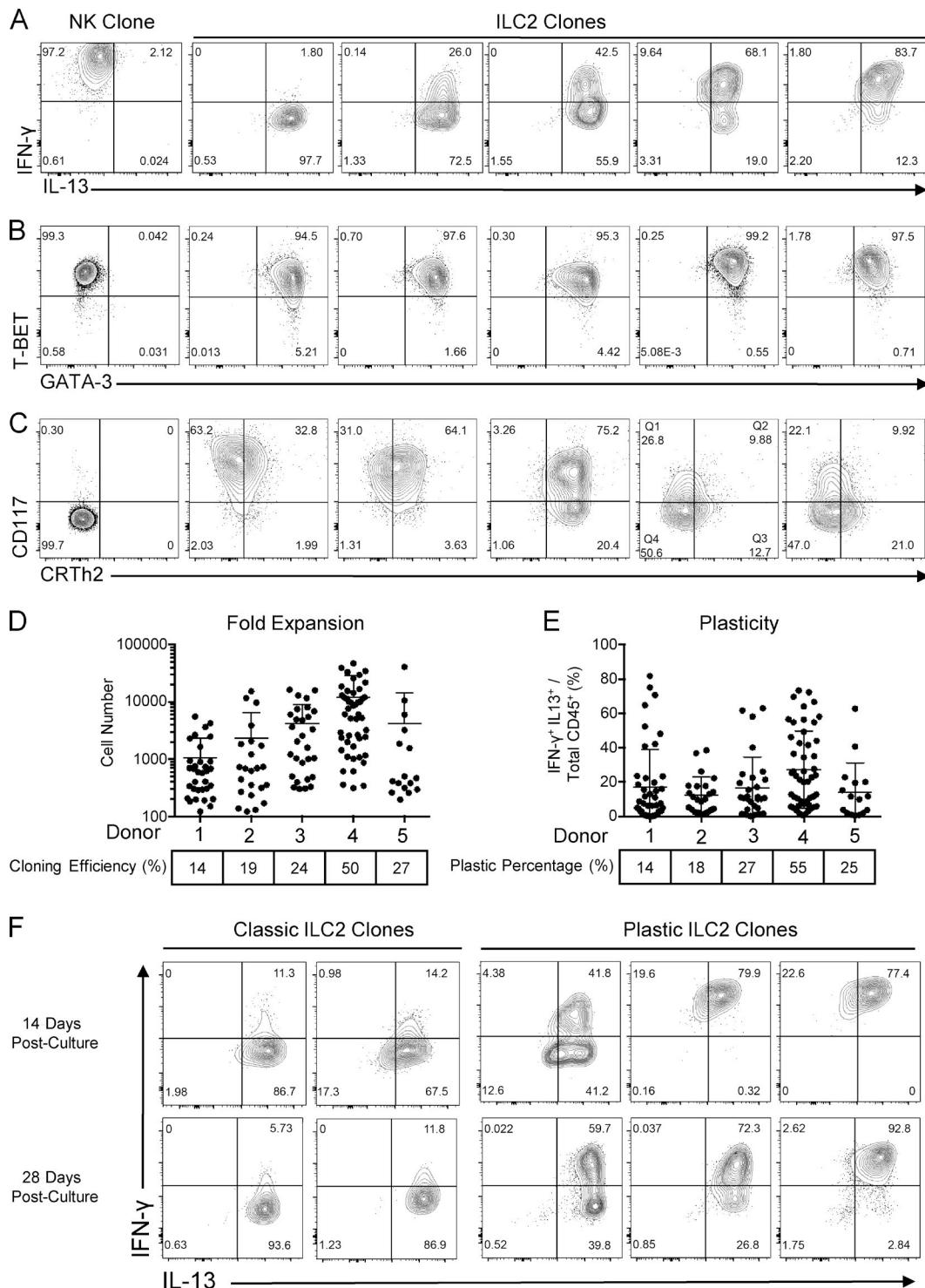


Figure 4. ILC2 cloning reveals functional plasticity. FACS-sorted single ILC2 were expanded with IL-2, -7, -25, and -33 and NK cells ($CD56^{hi}$ $CD127^+$) with expanded with IL-2, -7, -12, and -18 for 14 d on irradiated PBMCs. Clones were analyzed for intracellular IL-13 and IFN- γ production after PMA/iono stimulation (A), intracellular GATA-3 and T-BET expression (B), and surface phenotypes (C). (D) Analysis of ILC2 clonal expansion ($n = 5$ independent donors). Cloning efficiency was calculated by number of wells with recovered ILC2 against number of wells seeded. (E) Percentage of IFN- γ^+ IL13 $^+$ ILC2 in expanded cultures after PMA/iono stimulation. Plastic percentage was calculated as number of plastic clones (>20% of IFN- γ^+ IL13 $^+$ double producing ILC2) among

(Fig. 5 A), in contrast with cultures using PBMC feeders that generated plastic IFN- γ^+ ILC2. This suggested that irradiated PBMCs provided signals for the generation of ILC2 cytokine plasticity. Along these lines, OP9 stromal cell feeders could strongly support expansion of human ILC2 above levels obtained in cultures with cytokines alone, but were not able to support the generation of plastic IFN- γ^+ ILC2 at the bulk or clonal level (Figs. 5, A and C). These results strongly suggest that irradiated PBMCs provide factors that are critical for development of plastic ILC2 that are not delivered by mouse OP9 stromal cells. These experiments also indicate that ILC2 clonal expansion alone is not responsible for ILC2 functional conversion.

We next assessed a series of candidate molecules that could be involved in the generation of the IFN- γ^+ ILC2 phenotype. Cytokines such as IL-12 and -18 are important regulators of IFN- γ expression in many lymphocyte subsets (Schroder et al., 2004; Teng et al., 2015), and we next tested whether addition of these cytokines would affect the phenotype of bulk or clonal ILC2 cultures. The addition of IL-18 had no apparent effect on the percentage of IFN- γ^+ ILC2 in expanded blood ILC2 (Fig. 6, A–C). In contrast, addition of IL-12 had a dramatic effect, resulting in the conversion of the vast majority of cultured ILC2 into IFN- γ^+ -expressing cells (Fig. 6, A–C). In the bulk cultures, most IFN- γ^+ -expressing cells remained IL-13 $^+$ (Fig. 6 A); a phenotype recapitulated in ILC2 clones. Still, a fraction of bulk cultured ILC2 appeared to lose IL-13 expression and thus resembled bona fide ILC1 (IL-13 $^-$ IFN- γ^+), although these cells were never recovered as clones. Together, these results suggest that strong IL-12 signaling is sufficient to induce ILC2 plasticity and that the generation of IFN- γ^+ ILC2 is not a property of a unique subset of circulating ILC2.

To better understand how IL-12 could exert its effects in generating plastic ILC2, we studied the expression of IL-12 receptor components in freshly isolated ILC2 and after short-term culture (Fig. 6 D). The IL-12 receptor comprises two chains: IL-12R β 1 (that is shared by IL-12 and -23 receptors) and a unique IL-12R β 2; both are required for signaling in response to IL-12. Compared with circulating NK cells, blood ILC2 expressed very low levels of *IL12RB1* and *IL12RB2* at the steady state. Exposure to IL-2 and -7 led to a threefold induction of *IL12RB1*, whereas addition of IL-25 (but not IL-33) could further increase the levels of *IL12RB1* expression. The combination of IL-2, -7, -25, and -33 had a synergistic effect, increasing *IL12RB1* expression to levels observed in blood NK cells (Fig. 6 D). In contrast, *IL12RB2* expression levels were induced by IL-12 or -33 alone and strongly increased to NK cell levels in the presence of IL-2, -7, -25, -33, and -12 (Fig. 6 D). These changes in IL-12R

expression were only detected after 72 h of culture and not at earlier time points (24 h and, 48 h). Together, these data show that the expression of both *IL12RB1* and *IL12RB2* is induced in cytokine expanded ILC2 by 72 h that can help explain the kinetics of the IL-12 effect on IFN- γ production in these cells (Fig. 6 E).

The levels of intracellular IFN- γ in expanded ILC2 were significantly enhanced in the presence of IL-12, in both feeder-free and irradiated PBMC feeder systems (Fig. 6 F). Supernatants of cultured ILC2 contained detectable levels of secreted IFN- γ that could be increased by treatment with IL-12 (Fig. 6 G). These data demonstrate that plastic ILC2 effector functions (IFN- γ production) are not only observed after stimulation with pharmacological activators but can be elicited by inflammatory factors such as IL-12.

IL-12R β 1-deficient patients fail to generate plastic ILC2

We next assessed whether a functional IL-12 receptor was required for human ILC2 plasticity. Although *IL-12RB2*-deficient patients have not been described, patients with defects in *IL12RB1* suffer from a syndrome called Mendelian susceptibility to mycobacterial disease (MSMD; OMIM 209950) that includes predisposition to disease caused by virulent, weakly virulent (*Bacillus Calmette-Guerin*), and environmental mycobacteria (Bustamante et al., 2014). The phenotype and function of circulating ILC subsets in the blood of patients with inherited complete IL-12R β 1 deficiency were analyzed (Fig. 7, A and B). These patients have biallelic mutations in *IL12RB1* conferring a low production of IFN- γ by T or NK cells in response to IL-12 and -23 (Altare et al., 1998; de Beaucoudrey et al., 2008; Guia et al., 2008). Compared with healthy controls, IL-12R β 1-deficient patients demonstrated a trend toward increased percentage of Lin $^-$ CD7 $^+$ CD127 $^+$ ILCs, although this was not statistically significant (Fig. 7 B). The ILC2 subset (CRTh2 $^+$ GATA-3 $^+$) was comparable to controls and showed higher IL-13 production; in contrast, T-BET $^+$ ILC1 were significantly decreased in IL-12R β 1-deficient patients and failed to produce IFN- γ after stimulation (Fig. 7, A and B). Concerning ILC2 plasticity, cultured bulk ILC2 from IL-12R β 1-deficient patients could expand equally well as control ILC2; however, IFN- γ^+ IL-13 $^+$ ILC2 were not observed and T-BET expression was not induced (Fig. 7, C and D). All ILC2 clones obtained from IL-12R β 1-deficient patients exhibited the classic phenotype with strong IL-13 production and high GATA-3 expression. These data demonstrate the essential role for IL-12R β 1 to generate IFN- γ^+ ILC2 in humans.

ILC2 have been identified in several mucosal sites, including human lung, skin, and nasal cavity, where type 2 immune response are involved in barrier function and tissue

total number of clones obtained from each donor. (F) Analysis of intracellular IL-13 and IFN- γ production in ILC2 clones after PMA/iono stimulation. Clones were expanded using irradiated PBMCs and cytokines (IL-2, -7, -25, and 33) and analyzed on day 14 and 28. Data in A–C and F are representative of five independent experiments with one donor each. (D and E) Each dot represents one ILC2 clone from five independent donors (mean \pm SD).

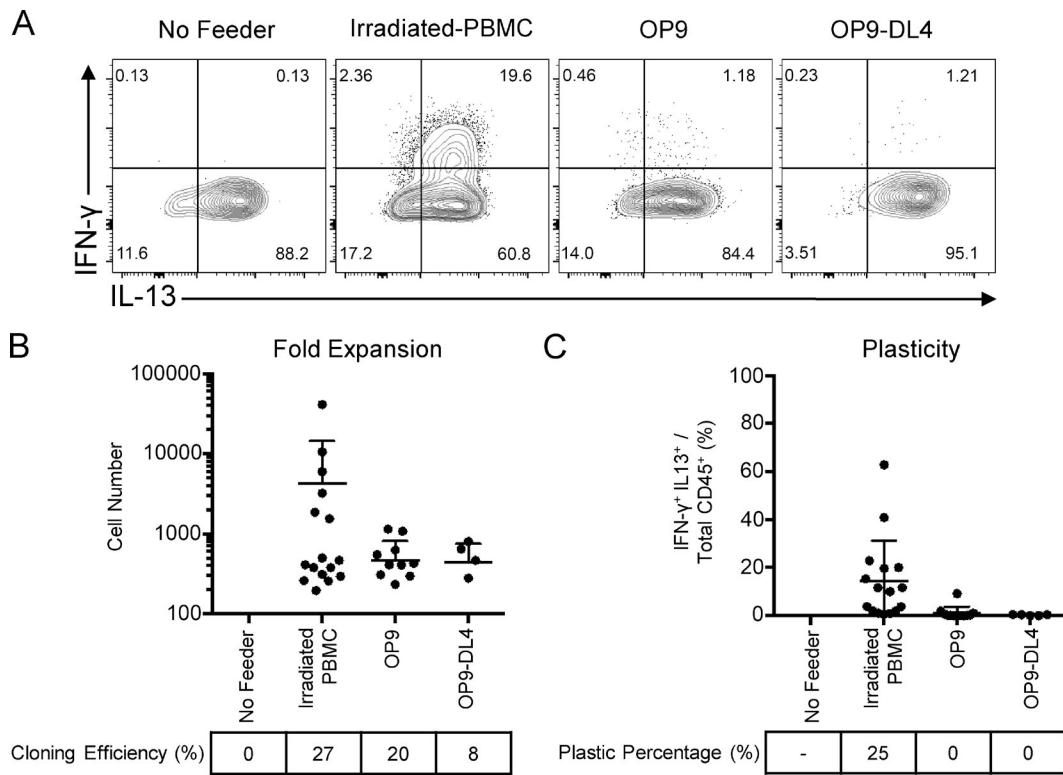


Figure 5. ILC2 expansion and plasticity requires feeder cell-derived factors. (A) FACS-sorted bulk ILC2 were cultured without feeder cells or on irradiated PBMCs, OP9, or OP9-DL4 as feeder cells in the presence of IL-2, -7, -25, and -33 for 7 d. Intracellular IFN- γ and IL-13 expression was analyzed after PMA/iono stimulation. (B and C) FACS-sorted single ILC2 clones were cultured on feeder-free or irradiated PBMCs, OP9, or OP9-DL4 in IL-2, -7, -25, and -33 for 14 d. Fold expansion rate (B), cloning efficiency (B), and plasticity (C) of ILC2 clones is shown. Each dot represents one ILC2 clones (mean \pm SD). Data in A–C are representative of three independent experiments with one donor each.

regeneration (Mjösberg et al., 2011; Monticelli et al., 2011; Salimi et al., 2013). Our results on cytokine stimulation of peripheral blood ILC2 suggest that inflammatory tissues might be a site where plastic ILC2 could be generated under the influence of soluble factors such as IL-12. We therefore examined ILC2 in intestinal samples from Crohn's disease patients who are afflicted with inflammatory bowel disease and compared them to peripheral blood ILC2. Previous studies identified ILC1 and ILC3 subsets in such patients (Geremia et al., 2011; Bernink et al., 2013; Fuchs et al., 2013). IL-13-producing ILC2s were detected in intestinal samples of Crohn's disease patients at frequencies that exceeded those found in peripheral blood (Fig. 7, E and F). Unlike circulating ILC2, a substantial percentage of intestinal IL-13-producing ILC2 from Crohn's patients could also produce IFN- γ (Fig. 7, E and F). These observations suggest that plastic ILC2 can also be generated *in vivo* in the context of inflammatory disease.

DISCUSSION

The immune system must rapidly adapt to an ever-changing set of antigenic stimuli and environmental challenges. This is perhaps best exemplified by mucosal barrier surfaces where microbial communities are dynamically changing. The im-

mune system utilizes several different strategies to confront pathogens and to maintain commensal microorganisms. First, lymphopoiesis generates a diverse and heterogeneous set of immune effector cells that can react with a large range of potential targets. Second, individual lymphocytes can further modify their effector functions (plasticity) to adapt to pathogenic or environmental changes. Although these properties have been typically associated with B and T cells of the adaptive immune system, recent evidence suggests that innate lymphocytes, including the recently identified ILCs, can also exhibit these properties (Eberl et al., 2015). In this study, we demonstrate that human ILC2 that circulate in the peripheral blood are heterogeneous and can exhibit functional plasticity in regard to cytokine production in response to external stimulation.

Previous studies have identified rare, Lin $^{-}$ CD127 $^{+}$ ILC subsets in human peripheral blood (Munneke et al., 2014). Among circulating ILCs, differential expression of CD161, CD117, and CRTh2 have been proposed to segregate ILC1 (CD161 $^{+}$ CD117 $^{-}$ CRTh2 $^{+}$), ILC2 (CD161 $^{+}$ CRTh2 $^{+}$), and ILC3 (CD161 $^{+}$ CD117 $^{+}$ CRTh2 $^{-}$) subsets using a gating strategy based on ILCs found in secondary lymphoid tissues, intestine, liver, skin, and lung (Hazenberg and Spits, 2014). Although

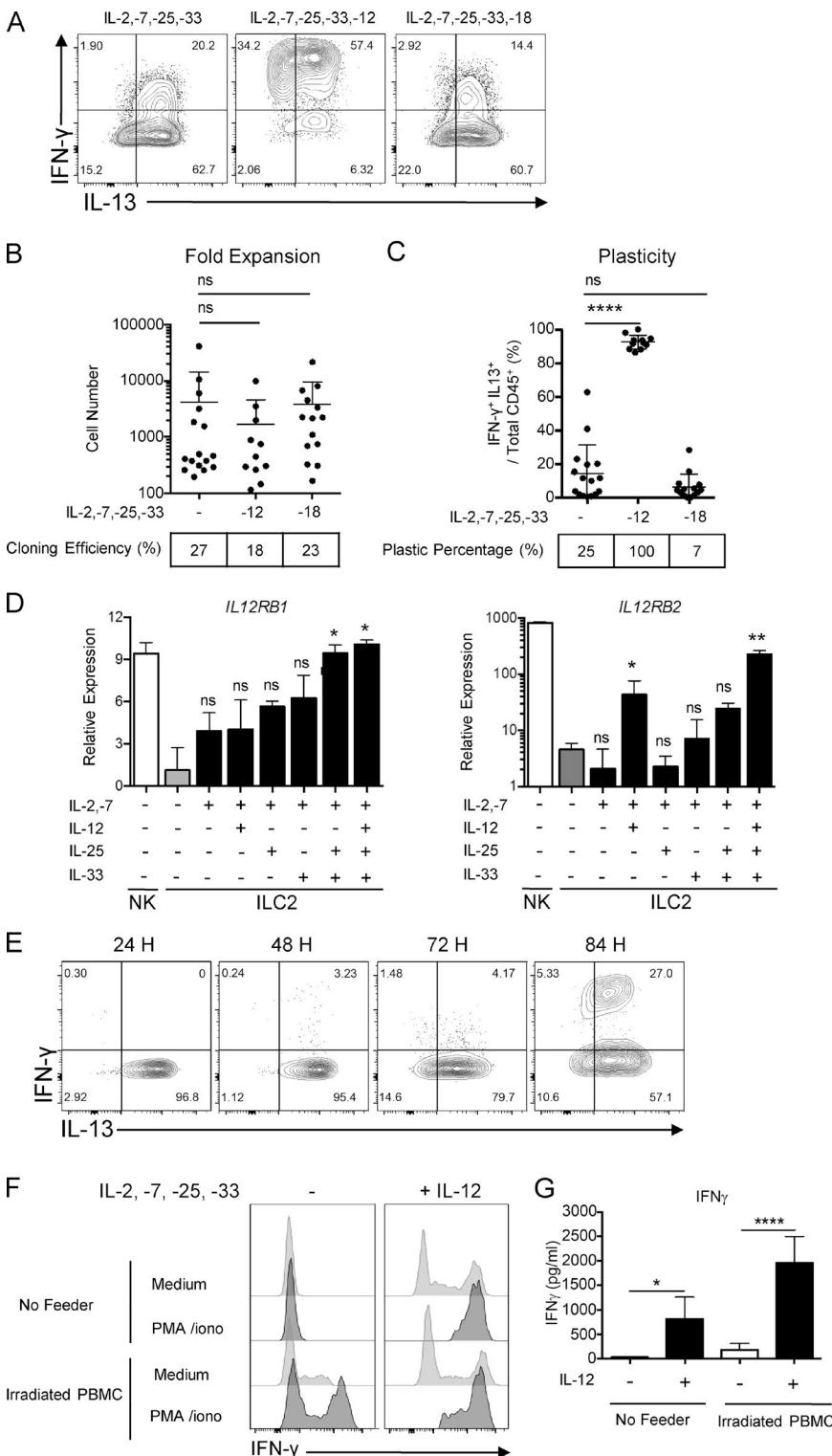


Figure 6. IL-12 induces ILC2 functional plasticity. (A) FACS-sorted bulk ILC2 were cultured on irradiated PBMCs in IL-2, -7, -25, and -33 with or without IL-12 or -18 for 7 d. Intracellular IFN- γ and IL-13 expression was analyzed after PMA/iono stimulation. (B and C) FACS-sorted single ILC2 clones were expanded on irradiated PBMCs IL-2, -7, -25, and -33 with or without IL-12 or IL-18 for 14 d. Fold expansion rate (B), cloning efficiency (B), and plasticity (C) of ILC2 clones are shown. Each dot represents an individual ILC2 clone. (D) FACS-sorted bulk ILC2 were cultured without feeder in different cytokine combinations for 72 h. Fresh NK cells ($CD56^{\text{bright}}CD127^{\text{low}}$; white bar), fresh ILC2 (gray bar), and expanded ILC2 (black bar) was analyzed for *IL12RB1* and *IL12RB2* gene expression by quantitative RT-PCR. Statistical analysis of cultured ILC2 was compared with fresh ILC2. (E) FACS-sorted bulk ILC2 were cultured without feeder cells in IL-2, -7, -25, -33, and -12 at different time-point as indicated. Intracellular IFN- γ and IL-13 expression was analyzed after PMA/iono stimulation. (F and G) FACS-sorted bulk ILC2 were cultured in feeder-free or irradiated PBMCs in IL-2, -7, -25, -33 with or without IL-12 for 4 d. (G) Culture supernatants were collected to analyze soluble IFN- γ . (F) Expanded ILC2 were analyzed for IFN- γ production after stimulation in medium or with PMA/iono. Data in A–C and E–G are representative results from three independent experiments with one donor each. Data in D and G are obtained from three independent donors. ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ using unpaired Student's *t* test (mean \pm SD).

this cell surface phenotype-based approach is easily implemented, it remains to be demonstrated that ILC subsets defined using this gating strategy do, in fact, represent bona fide functional ILCs that express the signature TFs and produce characteristic cytokines associated with the different ILC groups. We

found that IFN- γ $^{+}$ T-BET $^{+}$ ILC1 and IL-13 $^{+}$ GATA-3 $^{+}$ ILC2 are faithfully identified by this approach; in contrast, we have not observed IL-17A $^{+}$ or IL-22 $^{+}$ ROR γ t $^{+}$ ILC3 in normal human peripheral blood. As such, the nature of the circulating CD161 $^{+}$ CD117 $^{+}$ CRTH2 $^{-}$ cells remains enigmatic.

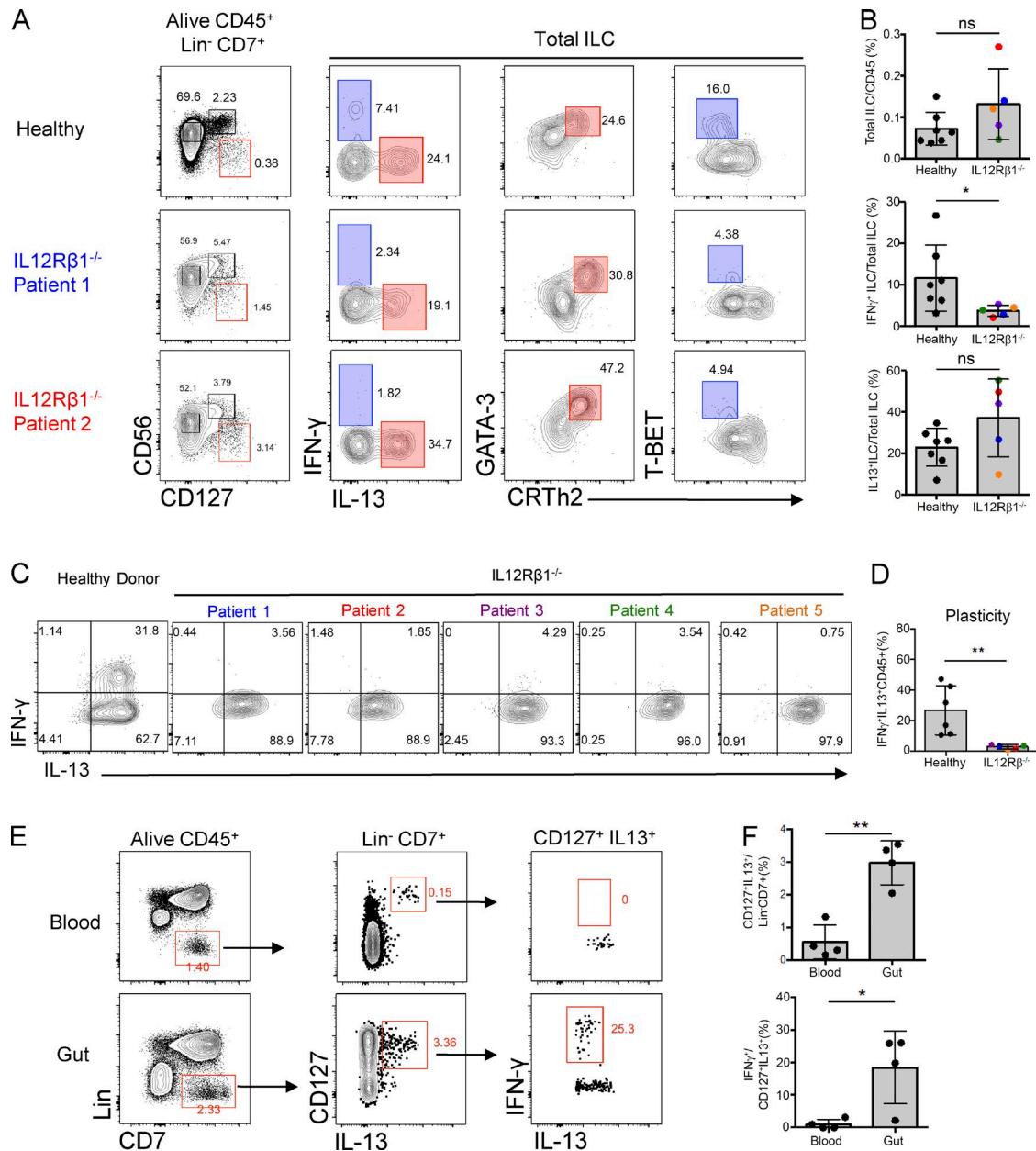


Figure 7. IL-12R β 1^{-/-} patients fail to generate plastic ILC2. (A) PBMCs from IL-12R β 1-deficient patients and healthy control were stimulated with PMA/iono. IFN- γ and IL-13 expression in total ILCs (Lin⁻CD7⁺CD127⁺) was assessed by intracellular cytokine staining. Intracellular GATA-3 and T-BET expression in total ILCs was analyzed from nonstimulated PBMCs. (B) Percentage of total ILCs in leukocytes (CD45⁺), IL13⁺ ILC2, and IFN- γ ⁺ ILC1 in total ILCs from IL-12R β 1-deficient patients and healthy controls. (C) FACS-sorted peripheral blood ILC2 from IL-12R β 1-deficient patients and healthy control were cultured on irradiated PBMCs in IL-2, -7, -25, and -33 for 7 d. Intracellular IFN- γ and IL-13 expression was analyzed in expanded ILC2 after PMA/iono stimulation. (D) Summary of analysis of ILC2 plasticity in IL-12R β 1-deficient patients. (E) Comparative analysis of ILC2 from peripheral blood of normal donors and intestinal tissues from Crohn's disease patients after PMA/iono stimulation. Percentage of IL13⁺ ILC2 in CD7⁺Lin⁻ leukocytes (CD45⁺) and percentage of IFN- γ ⁺ IL13⁺ ILC2 are shown. (F) Summary of analysis for four normal blood donors and four Crohn's disease patients. Data in A and E are representative of four independent experiments with at least one donor each. Data in B, D, and F obtained from at least four independent donors from each group: ns, P > 0.05; *, P < 0.05; **, P < 0.01 using unpaired Student's *t* test (mean \pm SD).

We focused our attention on CRTh2⁺ ILC2 to understand possible functional heterogeneity of this subset. ILC2 have multiple functional attributes (production of IL-4, -5,

-13, amphiregulin, and GM-CSF), and we wished to know if these were contributed by distinct ILC2 subsets. Surprisingly, we found that the majority of circulating ILC2 lacked

expression of many of these effector molecules, suggesting that despite an ILC2 TF signature (GATA3, RORA, and MAF), circulating ILC2 were not actively primed for effector functions. Moreover, most ILC2 lacked the critical cytokine receptors (IL-25R and -33R) that allow tissue ILC2 to respond to signals (such as IL-25 and -33) of inflammation and infection (Mjösberg et al., 2011; Bartemes et al., 2014; Xue et al., 2014) and, as such, circulating ILC2 were poorly activated by these soluble factors. Nevertheless, both ILC2 cytokine receptors and effector molecules could be rapidly up-regulated after short-term (3 h) activation, generating ILC2 that more closely resembled tissue-resident ILC2 (Monticelli et al., 2011; Barnig et al., 2013; Kim et al., 2013; Salimi et al., 2013; Brestoff et al., 2015). Together, these results suggest that circulating and tissue ILC2 may represent naive and primed/activated ILC2, respectively. T cell priming involves TCR triggering that activates multiple downstream pathways (NF- κ B, MAPK, and PLC γ); the signals that are important for ILC priming remain to be determined, but could involve a similar biochemical cascade. Direct comparison of blood and tissue ILC2 may provide the means to unravel the dominant signals that are needed to promote ILC responsiveness.

A previous study demonstrated that ILC2 in blood and in tissues express variable levels of CD117 (c-kit receptor for stem cell factor; Hazenberg and Spits, 2014). Our analysis of single ILC2 failed to identify CD117 $^+$ and CD117 $^-$ ILC2 as distinct subsets, but rather suggested that CD117 expression might be unstable; this hypothesis was confirmed after in vitro culture showing that several ILC2 markers could be modulated. Moreover, a subset of cultured ILC2 acquired novel functional attributes, including the capacity to produce IFN- γ that was associated with increased expression of the Th1 TF T-BET. We performed extensive ILC2 cloning to show that the ILC2 plasticity was not caused by cellular contaminants within the ILC2 cultures, but rather was mapped to environmental signals (such as IL-12) that could promote IFN- γ expression in these differentiated IL-13 $^+$ ILC2. As IL-12 levels are increased during inflammation, we further analyzed samples from Crohn's patients with inflammatory bowel disease. The presence of a subset of IL-13 $^+$ ILC2 that coexpressed IFN- γ further support the notion that ILC2 plasticity can be generated *in vivo* in the context of inflammatory diseases.

It is well known that the cytokine environment within secondary lymphoid organs (e.g., signal 3) and in inflamed tissues can strongly influence adaptive cellular responses. Differentiation of IFN- γ -producing T cells is regulated by several cytokines, including IL-2, -12, -15, -18, and -27, among others (Teng et al., 2015). In our system, cytokines produced by irradiated PBMCs played a role in generating ILC2 plasticity, and their effect could be substituted by exogenous IL-12 that strongly promoted IFN- γ $^+$ ILC2. Moreover, our analysis of Mendelian susceptibility to mycobacterial disease patients revealed that differentiation of plastic ILC2 required expression of *IL12RB1*. Together, these results strongly suggest a role for IL-12 in promoting IFN- γ -producing ILC2

in humans. As *IL12RB1* mutations inactivate both IL-12 and -23 signaling pathways (Bustamante et al., 2014), it remains possible that IL-23 signals are also involved. We think that this is unlikely, given that ILC2 fail to express IL-23R. Future studies of patients harboring *IL12RB2* or *IL23R* mutations will be useful to address the role for IL-12 versus IL-23 in promoting IFN- γ production in plastic ILC2.

Previous studies identified a peculiar subset of IL-22-producing ILC2 (Mjösberg et al., 2011). Regulation of IL-22 expression is poorly understood, and several TFs (RORC and AHR) and soluble factors (IL-1 β , -6, -23, and TGF β) have been proposed to promote IL-22 expression in differentiated Th cells and in ILC3 (Rutz et al., 2014). As for IFN- γ $^+$ ILC2, IL-22 production in ILC2 may represent another example of cytokine plasticity (ILC2 \rightarrow ILC3?) that can be induced by environmental signals. Future studies in rare human patients that manifest defects in the TFs (RORC; Okada et al., 2015) or in the aforementioned cytokine signaling pathways may help to elucidate the mechanisms behind ILC2 functional plasticity.

Analysis of IL-12R β 1-deficient patients suggests an essential role of IL-12–IL-23 signaling in the homeostasis of ILC1. We found few, if any, IFN- γ $^+$ ILC1 in patients lacking *IL12RB1*, whereas IFN- γ $^+$ NK cells (although clearly reduced) were detected as previously reported (Altare et al., 1998; Guia et al., 2008). These results are in line with the unique developmental origins of NK cells and ILC1 (Serafini et al., 2015).

What could be the biological implications for ILC2 cytokine plasticity? IFN- γ plays myriad roles in host immunity and it is critical for defense against several pathogens, including intra- and extracellular bacteria, and fungi (de Beaucoudrey et al., 2010). IFN- γ can also play roles in tissue homeostasis and remodeling during physiological processes (i.e., pregnancy; Ashkar et al., 2000) and after inflammation. As ILC2 are systemically distributed, the ability to elicit IFN- γ production in these cells could afford the organism with an additional rapid source of this important cytokine that participates during diverse disease contexts. Fine-tuning of ILC2 plasticity may be important to optimize immune benefit and to avoid pathological exacerbations.

MATERIALS AND METHODS

Human samples and cell isolation. Blood samples from healthy donors were obtained from *Establishement Français du Sang* (Paris, France) in an agreement signed with the *Institut Pasteur* (Paris, France). Blood samples from patients with complete IL-12R β 1 deficiency (P1, c.1791+2T>G/1791+2T>G; P2, c.1791+2T>G/p.R486X; P3, c.C1207T/C1207T; P4, p>R173W/R173W; P5, p.C186Y/C186Y) have been previously reported (Ouederni et al., 2014). Intestinal samples were obtained from Crohn's patients with refractory disease that underwent ileocecal resection for medical emergencies (for patient details, see Table S2). Informed consent was obtained from each patient as requested and approved by the institutional review boards of Necker Medical School, Paris

Descartes University, and the Hôpital Saint Louis, Assistance Publique - Hôpitaux de Paris. Human PBMCs were isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation. Mucosal samples were obtained from the intestinal resection, and cell suspensions were isolated by collagenase digestion, followed by Percoll centrifugation as previously described (Serafini et al., 2014).

FACS analysis and cell sorting. Antibodies used for analysis of cell surface phenotype, TFs, and cytokines are listed in Table S3. Cell surface molecules were analyzed using Brilliant Stained Buffer (BD), and intracellular TFs were analyzed using Foxp3/TF Staining Buffer Set (eBioscience). For intracellular cytokine staining, cells were stimulated with PMA (10 ng/ml; Sigma-Aldrich) plus iono (1 µg/ml; Sigma-Aldrich) in the presence of Golgi Plug (BD) for 3 h. Cells were fixed, permeabilized, and stained by Cytofix/Cytoperm kit (BD). Samples were acquired on LSRFortessa (BD) and analyzed by FlowJo software (Tree Star). For cell sorting, PBMCs were depleted of T cells, B cells, pDCs, and monocytes by labeling with biotin-conjugated anti-CD3, anti-CD4, anti-CD19, anti-CD14, and anti-CD123, followed by antibiotin microbeads (Miltenyi Biotec) according to manufacturer's instructions. Bulk populations were sorted to a purity \geq 99% or as single-cell index sorting (both using FACSAria III; BD).

Bulk and single ILC2 cell culture. Sorted bulk ILC2 and NK cells were plated in 96-well round-bottom plates with irradiated allogeneic PBMCs (45 Gy; combined from 6 healthy donors) and purified phytohemagglutinin (0.2 µg/ml; Oxoid) in Yssel's medium (Yssel and Spits, 2002) supplemented with 2% human AB serum (Establishment Français du Sang). ILC2 and NK clones were obtained after sorting single cells directly into 96-well plates preseeded with irradiated PBMCs or stromal cells lines, OP9 or OP9-DL4. Cytokines IL-2, -7 (5 ng/ml each; Miltenyi Biotec), -12, -18, -25, -33, -1β, -23 (10 ng/ml each; R&D Systems) are provided in various combinations, as indicated. Fresh cytokines and medium were replenished every 4 d. Unless otherwise indicated, bulk cultures were analyzed after 7 d and clones after 14 d.

Single-cell gene expression analysis and quantitative RT-PCR. Single cells were directly sorted into 96-well PCR plates containing lysis buffer. Quantitative RT-PCR was performed by using Two-Step Protocol with SuperScript VILO cDNA Synthesis kit (Invitrogen) for reverse transcription reaction and TaqMan PreAmp Master Mix (Applied Biosystems) for specific target amplification. Gene expression profiling was assessed using a Biomark (Fluidigm) with EvaGreen Supermix (Bio-Rad Laboratories) according to the manufacturer's protocols. Primers (Fluidigm) used are listed in Table S1. Mean and SD of Ct values from housekeeping genes (*B2M*, *ACTB*, and *GAPDH*) for all cells were calculated. Cells with Ct values of housekeeping genes exceeding the SD by more than twofold were eliminated from the analysis. 182 freshly isolated single ILC2 and 176 single ILC2 after 3-h culture

in TL1A, IL-25, and IL-33 (R&D Systems; 100 ng/ml each) were included in the dataset shown in Fig. 2. Frequency of expression of each gene was calculated by number of cells with detectable signal (Ct value \leq 38) from total cells in each group. Heat maps of two-dimensional hierarchical clustering analysis were performed by Qlucore Omics Explorer software. Dot plots were obtained by analyzing Ct values in Prism software (GraphPad Software).

Quantitative RT-PCR analysis of IL-12R subunits from cultured cells was performed using Power SYBR Green Cells-to-Ct kit (Thermo Fisher Scientific) according to manufacturer's instructions. Primer 5'-GCCATATCC GGATGCAGAC-3' and 5'-AGGAGCACTCGTAACGAT CA-3' for *IL12RB1* and 5'-GGAGTGAATCATTGAGAG CACAA-3' and 5'-TGCCGTTTCATGTACCAGAC-3' for *IL12RB2* were used. Expression levels of genes of interest were normalized to GAPDH.

ELISA. Human IFN-γ in culture supernatants was quantitated by ELISA (Thermo Fisher Scientific).

Statistical analysis. Experimental results are reported as mean \pm SD unless otherwise specified. Statistical differences between groups were determined using unpaired Student's *t* test. P-values $<$ 0.05 were considered significant. All data were analyzed with Prism v6 software.

Online supplemental material. Fig. S1 describes the gating strategy for ILC2 analysis from peripheral blood, cultured cells, and tissue samples. Table S1 describes PCR primers. Table S2 lists Crohn's patient characteristics. Table S3 lists antibodies used in FACS analysis. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20151750/DC1>.

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J.P. Di Santo is a founder and stakeholder in AXENIS (Paris, France). The other authors declare no additional competing financial interests.

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