Crohn’s disease: Mucosal immunology and immune modulating therapy

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Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues
Abstract

Innate lymphoid cells (ILCs) are effectors of innate immunity and regulators of tissue modeling. Recently identified ILC populations show a cytokine expression pattern that resembles that of Th2, Th17, and Th22 cells. Here we describe a distinct ILC subset similar to Th1 cells, which we call ILC1. ILC1 expressed the transcription factor T-bet and responded to IL-12 by producing interferon-γ (IFN-γ). ILC1 were distinct from natural killer (NK) cells since they lacked perforin, granzyme B and the NK markers CD56, CD16, and CD94 and could develop from RORγt+ ILC3 cells under the influence of IL-12. The frequency of the ILC1 subset was significantly higher in inflamed intestine of Crohn's disease patients, pointing towards a role for these IFN-γ producing ILC1 in the pathogenesis of gut mucosal inflammation.
**Introduction**

Innate lymphoid cells (ILCs) are emerging as critical effectors of innate immunity and tissue remodeling. ILCs lack rearranged receptors, are dependent on the transcriptional repressor Id2, share a lymphoid morphology, and express and are dependent on the common γ-chain of the interleukin 2 receptor (IL-2R). Several subpopulations of ILCs have now been found that differ from each other in their ability to produce cytokines and their dependency on transcription factors. Interestingly, the cytokine production profiles of ILC subpopulations are very similar to those of Th cell subsets. ILCs have been identified that produce interleukin (IL)-17 and/or IL-22 and are dependent on the transcription factor RORγt, similar to Th17 cells, whereas other ILC subsets, called ILC2, natural helper (NH) cells, nuocytes, and innate helper cells type 2 produce the type 2 cytokines IL-5 and IL-13 and, like Th2 cells, depend on GATA3 for their development and function. These various ILC subsets have now been classified into three groups; group 1 ILCs include NK cells and ILC1 (which are described here), GATA3-dependent group 2 ILCs and RORγt-dependent group 3 ILCs. ILCs are instrumental in immunity against invading microbes. ILC2 are critical in controlling helminth and other parasite infections and IL-22-producing ILC3 which are instructed by IL-23 provide protection during the acute phase of *Citrobacter rodentium*-induced colitis. ILCs also play important roles in tissue remodeling and repair. LTi cells, which belong to the ILC3 subset, are involved in repair of tissue damage inflicted by lymphocytic choriomeningitis virus following clearance of infection, whereas ILC2 mediate tissue repair in the lung following infection with influenza virus. Notwithstanding the importance of ILCs in maintaining the epithelial integrity at mucosal tissues, these cells have also been associated with pathophysiological conditions. ILC2 numbers are elevated in nose polyps that emerge in chronic rhinosinusitis, a disease characterized by eosinophilia and high amounts of IgE. Furthermore, ILC2 drive airway hyper-reactivity in mouse models of allergic asthma. IL-23 has been shown to be the main driver of innate gut inflammation by instructing ILCs to produce IL-17A, IL-17F and IFN-γ in a *Helicobacter hepaticus*-induced model of innate colitis. Moreover, numbers of IL-17-producing ILC3 are significantly increased in patients with Crohn’s disease.

Whereas the ILC equivalents of Th2, Th17 and Th22 cells have now been identified, a Th1-like ILC population has not yet been well characterized, although some reports have described ILCs that produce substantial amounts of IFN-γ, either alone or in combination with IL-17. Several studies in mice that lack a functional adaptive immune system, demonstrated that IFN-γ produced by ILCs is a potent inducer of gut inflammation and that neutralization of IFN-γ was sufficient to ameliorate disease progression. It has been documented that IL-23-responsive ILC3 that produce IL-22, partly shift towards an IFN-γ producing subset when cultured with IL-12 plus IL-18. Moreover, the conversion from IL-22 towards IFN-γ production was described to be accompanied by the down-regulation of the transcription factor RORγt, probably as a consequence of changes in the local cytokine environment.
Here we identified a Lin^−CD127^+c-Kit^−NKp44^− ILC population in humans that produced the pro-inflammatory cytokine IFN-γ, but is distinct from NK cells. Interestingly, we found that the IFN-γ–producing ILCs accumulated in inflamed intestine from patients that suffer from Crohn’s disease.

### Methods

#### Tissue collection

All tissues were collected after patient’s informed consent with approval of tissue specific protocols by the Medical Ethical Committee of the Academic Medical Centre, Amsterdam, The Netherlands. Tonsils were obtained from pediatric tonsillectomies. Inflamed intestinal mucosa of patients with Crohn’s disease was freshly obtained and processed after intestinal resection of inflamed ileum. Non-inflamed ileum referred to as “non-inflamed control”, was collected after intestinal resection of colon cancer, where the ileum was considered unaffected. Patients were excluded when undergoing chemo- or radiotherapy before resection. Human fetal tissues were obtained from elective abortions at the Stichting Bloemenhove clinic in Heemstede, the Netherlands, upon on the receipt of informed consents. 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 14-17 weeks.

#### Isolation of cells

Tonsil tissue was cut in small pieces and mechanically disrupted using the Stomacher 80 Biomaster (Seward). Cell suspension was passed through a 70 mm cell strainer and mono-nuclear cells were isolated by Ficoll-Paque PLUS (GE Healthcare).

Intestinal lamina propria was incubated for 15 min with HBSS (Gibco) containing DTT (154 μg/ml), 0.1% β-mercaptoethanol and 5 mM EDTA at 37 °C to eliminate mucus and epithelial cells. Thereafter lamina propria was cut into small pieces and digested for 30 min at 37 °C with HBSS (Gibco) containing Liberase TM (125 μg/ml) and DNaseI (200 μg/ml) (Roche). Cell suspensions were filtered through a 70-μm nylon mesh and lamina propria mononuclear cells (LPMC) were isolated by Ficoll-Paque PLUS (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-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-NKp46 (9E2), anti-NKp44 (P44-8), anti–granzyme B (GB11), allophycocyanin (APC)-conjugated anti-NKG2D (1D11), anti-T-bet (ebio4B10), Alexa Fluor 700–conjugated anti-CD56 (HCD56; all from BioLegend); FITC-conjugated anti-CD4 (RPA-T4), anti-CD14
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(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-CXCR3 (IC6/CXCR3), Alexa Fluor 647–conjugated anti-CRTH2 (CD294; BM16), APC-indotricarbocyanine (Cy7)–conjugated anti-CD45 (2D1); APC-conjugated anti-CD4 (S3.5; Invitrogen); PE-conjugated; PE-Cy7–conjugated anti-CD127 (R34.34; Beckman Coulter); PE-conjugated anti-ROrγt (AFKJS-9; eBioscience); and FITC-conjugated anti-BDCA2 (CD303; AC144; Milenyi), cell-trace violet cell proliferation dye (c34557, invitrogen). Phenotypical analyses were performed by flow cytometry in LSR Fortessa (BD), and analyzed with FlowJo software (TreeStar, Inc.). For sorting by flow cytometry, tonsil mononuclear cell samples were depleted of T cells, B cells, and NK cells by labeling with FITC-conjugated anti-CD3, anti-CD16 and anti-CD19 (described above) plus anti-FITC microbeads (Milenyi).

Establishment of cell lines and analysis of cytokine production

Lin−CD127+ ILCs were expanded with irradiated allogeneic peripheral blood mononuclear cells (25 Gy), irradiated Epstein-Barr virus–transformed JY human B cells (50 Gy), phytohemagglutinin (1 μg/ml; Oxoid) and IL-2 (100 U/ml; Novartis) in Yssel’s medium (AMC; made ‘in-house’) supplemented with 1% (vol/vol) human AB serum. Cells were cultured up to 6 weeks and resorted before further analysis. Fresh Lin−CD127+c-KitNKp44−ILCs were stimulated for 3–4 d with either or combinations of IL-2 (10 U/ml; Novartis), IL-23 (50 ng/ml; R&D Systems), IL-1β (50 ng/ml (R&D Systems), or IL-12 (50 ng/ml; R&D Systems) and IL-18 (50 ng/ml; R&D Systems). IFN-γ was measured in supernatants by enzyme-linked immunosorbent assay (eBioscience).

Intracellular cytokine staining

Cell lines expanded ex vivo were stimulated for 6 hours with PMA (10 ng/ml; Sigma) and ionomycin (500 nM; Merck) in the presence of GolgiPlug (BD) for the final 4 h of culture. A Cytofix/Cytoperm kit (BD) was used for cell permeabilization, staining and subsequent washing. The following antibodies were used: APC-conjugated anti-IL-13 (JES10-5A2; BioLegend), APC-conjugated IL-17 (BL168; BioLegend) and PE-conjugated anti-IL-22 (142928; R&D Systems), anti-IFN-γ (B27; BD Bioscience). Data were acquired on an LSRFortessa (BD) and were analyzed with FlowJo software (TreeStar).

Quantitative real-time PCR

RNA was isolated by either the RNeasy micro kit (Qiagen) or the NucleoSpin RNA XS kit (Macherey-Nagel) according to the manufacturer’s protocol. Synthesis of complementary DNA was established with the high-capacity cDNA archive kit (Applied Biosystems). PCR’s were performed on a LightCycler 480 Instrument II (Roche) with SYBR Green I master mix (Roche). Used primers are listed in Supplementary Table 3 and primers for 18S, IFNG, IL22, IL17, IL13, and RORC were used as previously published.4 LinRegPCR Software was used to quantify expression.4
Human immune system mice
CD34<sup>+</sup> CD38<sup>-</sup> hematopoietic stem cells (HSC) isolated from human fetal liver (0.2-2 x 10<sup>6</sup> cells) were transplanted intrahepatically into sublethally irradiated (3.50 Gy) newborn NOD SCID common gamma chain (γ<sub>c</sub>)<sup>-/-</sup> (NSG) mice (younger than 1 week of age). Peripheral blood (PB) was collected from a facial vein every 3 to 4 weeks after transplantation to determine the kinetics of human cell engraftment. Mice of 8-12 weeks were treated with either water or DSS (3% (w/v)) for a period of 7 days.

Histological scoring
The longitudinally divided colons were rolled, fixed in 4% formalin and embedded in paraffin for routine histology. An experienced pathologist evaluated formalin-fixed haematoxylin tissue sections microscopically, in a blinded fashion. Colons were evaluated, and graded from 0 to 4 as an indication of incidence and severity of inflammatory lesions based on the extent of the area involved, the number of follicle aggregates, oedema, fibrosis, hyperplasia, erosion/ulceration, crypt loss and infiltration of granulocytes and mononuclear cells as indicated in Supplementary Table 2. The total inflammation score was calculated as the sum score of the above.

Statistical analysis
Statistical significance was determined with the ANOVA or Students t-test. Prism graphpad software was used.
Results

Phenotype and gene expression profile of ILC populations

Human innate lymphoid cell (ILC) subsets have been described\(^ {24} \) that can be considered as the innate equivalents of the Th22, Th17, and Th2 subsets. As these ILC populations are readily found in human tonsils, we searched this organ for an ILC population with Th1 cell characteristics. By gating on CD127\(^ + \) hematopoietic cells (CD45\(^ + \)) with a lymphoid morphology that lacked markers for hematopoietic precursors (CD34), B, T, NK cells, or myeloid cells, we identified a CRTH2\(^ + \) ILC2 population (Figure 1a, middle) that expressed high amounts of *IL13* mRNA, and the transcription factors *GATA3* and *ROR\(\alpha\)* (Figure 1b). Furthermore, we observed a c-Kit\(^ +\)NKp44\(^ +\) ILC (which we call here NKp44\(^ +\) ILC3), (Figure 1a, right), expressing *IL22*, *IL23R*, the aryl hydrocarbon receptor (AHR) and retinoic acid related orphan receptor (RORC) (Figure 1c). We did not detect any *IL17* transcripts in any of the ILC populations from human tonsils (Figure 1c), however, stimulation with phorbol 12-myristate 13-acetate (PMA) plus ionomycin for 2 and 4 hours induced *IL17* expression in the NKp44\(^ +\) ILC3, indicating that these cells have the capacity to produce IL-17. In addition to the ILC2 and NKp44\(^ +\) ILC3, we identified two additional populations, c-Kit\(^ +\)NKp44\(^ –\) and c-Kit\(^ –\)NKp44\(^ –\) (Figure 1a, right). The c-Kit\(^ –\)NKp44\(^ –\) cells were clearly distinct from ILC2 and NKp44\(^ +\) ILC3, as they did not express transcripts for IL-22 nor IL-13 or IL-17, and expressed no or relatively low amounts of *GATA3*, *RORA* and *RORC* transcripts (Figure 1b,c). The c-Kit\(^ +\)NKp44\(^ –\) cells also expressed low amounts of *GATA3* and *RORA* and expressed *RORC*. Furthermore, these ILCs did not contain CD34\(^ +\) common lymphoid progenitors (CLPs).\(^ {25} \) Thus, in tonsil we identified two CD127\(^ +\) ILC populations that are distinct from ILC2 and ILC3.

**c-Kit\(^ +\)NKp44\(^ –\) ILCs show characteristics of Th1 cells**

Given the diversity in cytokine production profiles of ILCs we examined the cytokine expression profile in the NKp44\(^ –\) ILC subsets. *Ex vivo* isolated c-Kit\(^ +\)NKp44\(^ –\), but not c-Kit\(^ +\)NKp44\(^ +\) ILCs, expressed transcripts for IFN-\(\gamma\) (Figure 2a), suggesting that Th1-like ILCs were contained within the former population. Consistent with this notion, we observed that c-Kit\(^ +\)NKp44\(^ +\) ILCs expressed much higher amounts of transcripts of T-bet-encoding *TBX21* and T-bet protein (Figure 2a,b) compared to c-Kit\(^ +\)NKp44\(^ –\) and NKp44\(^ +\) ILC3. Furthermore, the c-Kit\(^ +\)NKp44\(^ –\) ILC subset expressed very low amounts of *RORC* transcripts (Figure 1c) and ROR\(\gamma\)t protein (Figure 2b).

T-bet controls expression of many genes including the chemokine CCL3, and the chemokine receptor CXCR3, \(^ {26,27} \) which is also expressed on Th1 cells that migrate to inflammatory sites.\(^ {28} \) Indeed, the c-Kit\(^ +\)NKp44\(^ –\) ILC subset expressed significantly more CCL3 and CXCR3 as compared to the other ILC subsets (Figure 2a). Flow cytometry confirmed that a substantial part of the c-Kit\(^ +\)NKp44\(^ –\) subset expressed CXCR3 and T-bet protein (Figure 2b). The bimodal distribution of CXCR3 expression by the c-Kit\(^ +\)NKp44\(^ –\) cells might be due to the internalization of the receptors as a consequence of the processing procedure, or due to differences in their activation status. We cannot, however, completely rule out that the c-Kit\(^ +\)NKp44\(^ –\) ILC subset is heterogeneous.
**Figure 1 | Phenotype and gene expression profile of ILC populations in human tonsil.**

**a.** Flow cytometry analysis of ILC populations based on the expression of CRTH2, c-Kit, and NKp44. Tonsil mononuclear cells were depleted from T cells (CD3) and B cells (CD19) through magnetic bead-based separation, followed by gating on Lin- cells (CD1a-, CD3-, CD11c-, CD14-, CD19-, CD94-, CD34-, CD123-, TCRαβ-, TCRγδ-, BDCA2-, FcεR1-) and CD127+ cells. Numbers in gates or quadrants indicate percent of cells.

**b.** Gene expression of IL13, GATA3, and RORA relative to β-actin in ILC populations as sorted in (a): c-Kit+NKp44, NKp44+ILC3 (c-Kit+NKp44+), c-Kit NKp44-, ILC2 (CRTH2+). c. Gene expression of IL22, AHR, RORC, IL23R, and IL17 relative to β-actin in ILC populations as sorted in (a): c-Kit+NKp44-, NKp44+ ILC3, c-Kit NKp44-, NK cells (CD45+, CD127 CD94+). IL17 transcripts in NKp44+ ILC3 have been stimulated with P/I for 2 and 4 h. Bar graphs in (b and c) represent at least 3 independent experiments with 1 to 3 donors each. Error bars show SEM and horizontal bars show means, *P < 0.05; **P < 0.01; ***P < 0.001; ND, not detectable.
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Experiments were performed with 2000 cells/well in a 96-wells plate in 200 µl, NKp44−/NKp44+ NK cells, sorted by flow cytometry as indicated (1a), and cultured for 4 days, either alone or with combinations of IL-2 (10 U/ml), IL-12, IL-1β and/or IL-18 (all 50 ng/ml).

Figure 2 | C-Kit NKp44+ ILCs have characteristics of Th1 cells. a. Gene expression of IFNG, TBX21, CCL3, CXCR3, IL12RB1, IL12RB2, LTA, and LTB relative to β-actin in tonsil ILC populations as sorted in (1a): c-Kit+ NKp44−, NKp44+ ILC3, c-Kit NKp44+, NK cells. Bar graphs represent at least 3 independent experiments with one to three donors each. b. Flow cytometry characterization of Lin−CD127− c-Kit NKp44− cells, Lin−CD127+ c-Kit+ NKp44+, NKp44− ILC3, and cNK cells. Data are representative of at least 4 experiments with 1 donor each. c. IFN-γ production by tonsil CD45+lin−CD127+ c-Kit NKp44+ ILCs, sorted by flow cytometry as in (1a), and cultured for 4 days, either alone or with combinations of IL-2 (10 U/ml), IL-23, IL-12, IL-1β and/or IL-18 (all 50 ng/ml). d. IFN-γ production by tonsil CD45+lin−CD127+ c-Kit NKp44+, c-Kit+ NKp44−, NKp44− ILC3 (c-Kit+ NKp44+) and NK cells, sorted by flow cytometry as indicated in the legends of figure 1a, and cultured for 4 days in the presence of IL-2 (10 U/ml), IL-12 and IL-18 (50 ng/ml). (c,d) Experiments were performed with 2000 cells/well in a 96-wells plate in 200 µl. Graphs represent 3 independent experiments with 1 to 3 donors each. Error bars show SEM and horizontal bars show means, * P < 0.05; ** P < 0.01; *** P < 0.001.
IFN-γ expression is under direct control of T-bet, and T-bet expression is amplified through TCR-independent IL-12 signaling. Analysis of the c-Kit^−NKp44^− subset revealed significantly more IL12RB2 transcripts as compared to the c-Kit^+NKp44^− cells, NK cells, or the NKp44^+ ILC3 subset (Figure 2a). In contrast, transcripts encoding the IL-12 receptor subunit β1 (IL-12Rβ1) were uniformly distributed between the ILC subsets (Figure 2a). We also assessed the expression of LTA, as LTα is expressed on Th1 but not Th2 cells. The amounts of LTA transcripts were significantly more abundant in the c-Kit^−NKp44^− subset as compared to the other ILC subsets, whereas LTB was expressed on all ILC populations at comparable amounts (Figure 2a).

Next we wanted to assess which physiological factors are responsible for regulating these c-Kit^−NKp44^− cells. IL-23, which is a potent activator of RORγt dependent ILC3 subsets in mice, was shown to induce IFN-γ production in a subset of mouse ILCs. However, IL-23 was unable to induce IFN-γ production by freshly isolated tonsil c-Kit^−NKp44^− ILCs (Figure 2c). Similarly, the pro-inflammatory cytokine IL-1β did not upregulate IFN-γ (Figure 2c). In contrast, activation with IL-12 triggered a five-fold increase of the IFN-γ production, which was synergistically enhanced by IL-18 (Figure 2c). The amounts of IFN-γ protein induced by IL-12 plus IL-18 by c-Kit^−NKp44^− ILCs were comparable to those in NK cells (Figure 2d). Together, these data clearly identified a distinct CD127^+ c-Kit^−NKp44^− ILC population with many functional and phenotypical characteristics in common with Th1 cells. Hence, we designate this subset ILC1.

ILC1 are distinct from NK cells

Having identified an ILC population that expresses IFN-γ and T-bet it was important to determine whether these cells are distinct from conventional natural killer (cNK) cells or cNK cell precursors, as cNK cells also express IFN-γ and T-bet. ILC1 did not express any of the NK cell signature cytotoxic molecules, perforin or granzyme B (Figure 3a). Neither did they express the killer immunoglobulin-like receptor KIR3DL1, nor the IL-15α receptor component of the IL-15R complex, which is essential for cNK cell development. ILC1 lacked the NK-associated marker CD56, in contrast to NKp44^+ ILC3, which were heterogeneous in their expression of CD56 (Figure 3a), as we documented previously. ILC1 and NK cells also differed in expression of the receptor for IL-1β (IL1R1) (Figure 3b). IL1R1 was most abundant on NKp44^+ ILC3, consistent with the important role of IL-1β in development and maintenance of these cells. The presence of high amounts of CD127 and the absence of c-Kit and CD34 on ILC1 strongly suggest that these cells were also distinct from committed NK precursors or immature NK (iNK) cells, as these cells are defined by expression of CD34 and c-Kit, respectively. The ILC1 subset expressed CD161, which is a marker commonly expressed on RORγt^+ ILC3 and ILC2 subsets. Furthermore, approximately 50% of the ILC1 subset expressed CD69, whereas only few of the cNK cells expressed this antigen, indicating that the ILC1 were activated in situ, whereas the majority of cNK cells were not. Thus although ILC1 and NK cells share the capacity to produce high amounts of IFNγ, they represent distinct cell types.
Figure 3 | ILC1 are distinct from mature NK cells. a. Flow cytometry characterization of tonsil ILC1, NKp44+ ILC3, and cNK cells. Histograms are representative of at least 3 experiments with 1 donor each. b. Gene expression of IL1R1 in ILC populations as sorted in figure 1a. Black: c-Kit+ NKp44−; light grey: NKp44+ ILC3 (c-Kit+ NKp44+); dark grey: ILC1; white: cNK cells (CD45+, CD127 CD94+). Graphs represent 3 independent experiments with 1 to 3 donors each. Error bars show SEM and horizontal bars show means, * P < 0.05.
The ILC1 subset represents a stable cell type

To further validate that ILC1 is a stable cell type, we generated cell lines from the freshly isolated tonsil ILC1, using a feeder cell mixture that we previously successfully used to generate cell lines of NK cells, ILC2 and ILC3. After 4-6 weeks culture in a feeder cell mixture that contained IL-12, the ILC1 cells expressed CD127, CD161 and CXCR3, but lacked c-Kit and NKp44 (Figure 4a). In contrast to freshly isolated ILC1, in vitro cultured ILC1 did not express the activation marker CD69, whereas cultured ILC3 expressed low amounts (Figure 4a). Furthermore, these cell lines lacked expression for CD3, CD94 and CD56, whereas the NKp44+ ILC3 were heterogeneous for CD56 expression (Figure 4a). To determine whether the ILC1 cell lines also maintained its functionality upon culture, we stimulated cells with PMA plus ionomycin, and assessed the cytokine expression profile. Similar to the freshly isolated ILC1, ILC1 cell lines produced high amounts of IFN-γ, but no IL-17 or IL-22 (Figure 4b). A minor proportion of the ILC1 subset also produced IL-13, similar to cultured NKp44+ ILC3. Cultured ILC1 expressed abundant TBX21 transcripts, similar to those in freshly isolated ILC1, whereas cultured ILC1 expressed less RORC transcripts as compared to NKp44+ ILC3 (Figure 4c). IL-12 plus IL-18, but not IL-23, was able to induce IFN-γ production by cultured ILC1 (Figure 4d). Together, these data show ILC1 cultured in the presence of IL-12 maintain their phenotype, transcription factor expression pattern, and capacity to produce IFN-γ, indicating that the ILC1 subset is stable.

Accumulation of ILC1 in Crohn's disease intestine

Patients with inflammatory bowel disease have chronic inflammation of their intestines. Two major phenotypes can be distinguished: ulcerative colitis and Crohn’s disease. Crohn’s disease is a type 1-mediated inflammatory disease since patients have enhanced IFN-γ production in their intestinal lamina propria and show elevated amounts of the pro-inflammatory cytokines IL-12 and IL-18. Therefore we hypothesized that Crohn’s disease patients might have an altered ILC composition that is more polarized towards ILC1 compared to non-inflamed control patients. To examine this, we first analyzed the composition of ILCs in fetal gut, which have not been colonized with microbes. As documented before, we observed that all ILCs in fetal gut express c-Kit, and the majority were positive for NKp44, whereas no ILC1 were found in fetal gut (Figure 5a). Next we compared the ILC composition in inflamed and non-inflamed intestinal lamina propria of Crohn’s disease patients with non-inflamed non-affected intestine from patients undergoing intestinal resection for colorectal cancer. The frequencies of ILC1 were significant higher in inflamed tissues from Crohn’s disease patients as compared to controls without inflammatory bowel disease (Figure 5a), whereas the percentages of NKp44+ ILC3 were significantly reduced compared to the non-inflamed controls (Figure 5a). Relative to other leukocytes, the overall frequencies of ILCs in inflamed and non-inflamed tissues were similar (Figure 5a). Like tonsil ILCs, the gut ILC1 subset expressed IFNG and CXCR3, but lacked CD94 and the cytotoxicity molecules perforin and granzyme B (Figure 5b,c). Taken together, these data indicate that in Crohn’s disease patients, the ILC1 represent the most prominent ILC subset, expressing transcripts for the pro-inflammatory cytokine IFN-γ.
Figure 4 | Stable cell lines can be generated from ILC1 subset. a. Flow cytometry analysis of expanded tonsil ILC1 (black line) and NKp44+ ILC3 (grey line) subsets. Data are representative of at least 8 experiments with 1 donor each. b. Flow cytometry analysis of ILC1, and NKp44+ ILC3 subsets, stimulated with PMA and ionomycin and stained for intracellular IL-17 and IL-22, or IFN-γ and IL-13. Numbers in quadrants indicate percent of cells in each, and are representative of 5 experiments with 1 donor each. Data are summarized in bar graphs (b, right). c. Gene expression of TBX21 and RORC in expanded ILC1 (black) and NKp44+ ILC3 (grey). Graphs represent 2 independent experiments with 1 to 2 donor each. d. IFNγ production by expanded ILC1; cultured for 4 days, either with IL-2 (100 U/ml) and IL-23 (50 ng/ml), or IL-2 (100 U/ml), IL-12 and IL-18 (both 50 ng/ml). Experiments were performed with 2000 cells/well in a 96-wells plate in 200 μl. Graphs represent 3 independent experiments with 1 donor each. Error bars show SEM and horizontal bars show means, P < 0.05; ** P < 0.01; *** P < 0.001.
Figure 5 | Accumulation of ILC1 in inflamed intestine of Crohn’s disease patients. a. Flow cytometry analysis of ILC populations on freshly isolated lamina propria mononuclear cells from fetal gut, controls, and inflamed ileum from Crohn’s disease patients. Dotplots are representative of at least 7 independent experiments with 1 donor each. Numbers in gate or quadrant indicate percent of cells. Bar diagrams represent quantification of total ILCs (defined as CD45<sup>+</sup>lin<sup>-</sup>CD127<sup>+</sup>), the NKp44<sup>+</sup> ILC3, ILC1, and c-Kit<sup>+</sup>NKp44<sup>-</sup> subsets in fetal gut, non-inflamed control, Crohn’s disease non-inflamed, and Crohn’s disease inflamed in at least 7 individuals. Error bars show SEM and horizontal bars show means, **P < 0.01; ***P < 0.001. b. Flow cytometry characterization of Crohn’s disease-derived-ILC1, c-Kit<sup>+</sup>NKp44<sup>-</sup>, NKp44<sup>+</sup> ILC3, and cNK cells. Histograms are representative for at least 7 independent experiments with 1 donor each. c. Gene expression of IFNG, IL22, RORC, and IL17 relative to 18S in ILC populations as sorted in (1a): c-Kit<sup>+</sup>NKp44<sup>-</sup>, NKp44<sup>+</sup> ILC3, ILC1. Bar graphs represent 3 independent experiments with 1 donor each. Error bars show SEM and horizontal bars show means; NS, not significant.
Figure 6 | Expansion of the ILC1 subset during gut inflammation in NOD SCID γc−/− mice reconstituted with fetal human hematopoietic stem cells (CD34+CD38−). a. Flow cytometry analysis of human ILC populations based on the expression of CRTH2, c-Kit, and NKp44 in HIS-mice. Gating was on human CD45+ lamina propria mononuclear cells, followed by gating strategy as indicated in (1a). Numbers in gates or quadrants indicate percent of cells and are quantified in right bar graph. b. Characteristics of inflammation on day 7 of DSS treatment (3%). Bar graphs (top) indicate the quantifications of total human hematopoietic (CD45+) cells in the lymphocyte-gate, and colon length in water- vs. DSS-treated mice. The curve indicates the weight loss of water- vs. DSS-treated mice. Scatter plot indicates total histological score, which is the sum of inflammatory parameters.
Figure 7 | IL-12 induces differentiation of ILC1. (a, dotplots) Purified tonsil c-Kit⁺NKp44⁻ ILCs can differentiate into the NKp44⁺ IL3 and ILC1 subsets. Stimulation of highly purified c-Kit⁺NKp44⁻ ILCs for 8 days either with IL-2 (100 U/ml) or with combinations of IL2 (100 U/ml), IL-23 and IL-1β (50 ng/ml) or IL-2 (100 U/ml) and IL-12 (50 ng/ml), followed by flow cytometry analysis of c-Kit and NKp44. Numbers in quadrants indicate percent cells in representative plots of at least 5 independent experiments, and are quantified in Supplementary Figure 2a. (a, bar graphs) Gene expression of IFNG, TBX21, CXCR3, and RORC relative to β-actin of the differentiated c-Kit⁺NKp44⁻ derived-ILC populations (only IL-2) as indicated in the figure: c-Kit⁺NKp44⁻, NKp44⁺ ILC3, ILC1. Bar graphs represent at least 3 independent experiments with 1 to 3 donors each. b. Representative histogram of either c-Kit⁺NKp44⁻ derived NKp44⁺
ILC3 or ILC1 subsets, stimulated with PMA and ionomycin and stained for intracellular IL-22 or IFN-γ. Bar graphs indicate percent of cytokine-producing cells of at least 6 independent experiments. c. Stimulation of highly purified c-Kit⁺NKp44⁻ ILCs for 3 days with combinations of IL2 (100 U/ml), IL-23 and IL-1β (both 50 ng/ml) or IL-2 (100 U/ml) and IL-12 (50 ng/ml). Cells were stained with a proliferation dye and followed over time by flow cytometry. Histograms are representative of 4 independent experiments. Filled histogram represents non-proliferation control. Numbers in histograms indicate percent of non-proliferating cells, which are quantified in the bar graph. Error bars show SEM and horizontal bars show means, * P<0.05; ** P<0.01.

Accumulation of human ILC1 in inflamed gut of HIS-mice

To investigate whether the expansion of the ILC1 subset seen in IBD patients was the consequence of an ongoing chronic inflammation or emerges at the onset of the mucosal inflammation, we treated human immune system (HIS) mice with dextran sodium sulfate (DSS) to induce innate gut inflammation. NOD-SCID common gamma chain (γc⁻/⁻) (NSG) mice, which lack T, B, NK cells, and ILCs were used as recipient mice. Newborn NSG mice were sub-lethally irradiated and injected with human CD34⁺CD38⁻ hematopoietic stem cells (HSCs) isolated from human fetal liver, which resulted in appearance of human immune cells in these mice, as described previously. Evaluation of lamina propria mononuclear cells (LPMCs) of HIS-mice 2 months after injection with HSCs revealed a reconstitution of human CD45⁺ cells in the colon that varied from 15 to 30% of the total colonic mononuclear leukocytes (Supplementary Table 1). By gating on human CD45⁺ lymphocytes that lacked lineage markers and expressed CD127, we detected human ILCs. Almost all of these cells expressed c-Kit and the majority of these cells expressed NKp44 (Figure 6a). Thus the composition of the ILC pool in HIS mice is similar to that in non-inflamed human gut (Figure 5a, 6a). To examine the consequences of gut inflammation on the composition of human ILCs in the gut of HIS mice we challenged 2 months old HIS mice with DSS for 7 days. This treatment triggered an acute inflammatory response, as demonstrated by shortening of the colon and wasting-disease which was accompanied by expansion of the total pool of human CD45 cells as compared to control HIS mice (Figure 6b). Moreover substantial inflammation was observed upon inspection of the intestine of DSS-treated but not in control HIS mice as reflected by the histology score (Figure 6b, lower left). These data indicated that the human immune cells responded to the mucosal barrier damage and bacterial influx in the intestine evoked by DSS. However the human leukocytes were not responsible for the inflammation because similar pathology scores were observed in mice, which were not reconstituted with human HSCs. Nonetheless the inflammatory environment induced by DSS resulted in substantially higher frequencies of ILC1 cells in the gut as compared to control mice (Figure 6c). The gut-residing ILC1 expressed IFNG transcripts similar to those in T cells, but the amounts were lower than in NK cells isolated from the gut of the DSS-treated HIS mice (Figure 6b). Collectively these data indicate that ILC1 are accumulating as a consequence of acute inflammation in the gut.
IL-12 induces differentiation of c-Kit+ ILCs into ILC1

In addition to ILC1, ILC2 and NKp44+ ILC3 we identified a c-Kit+NKp44- population, which showed low expression of IL23R and IL12RB2 and lacked IL13, IL22, IL17 and IFNG raising the possibility that this subset may represent an immature ILC subset capable of acquiring features of mature ILC subpopulations. To test this idea, we purified the c-Kit+NKp44- subset and stimulated these cells ex vivo with IL-2, a cytokine that acts as a growth and maintenance factor for ILCs. Incubation of c-Kit+NKp44- cells with IL-2 resulted in appearance of cells with a phenotype similar to ILC1 and NKp44+ ILC3. Expression analysis confirmed that these cells indeed were ILC1 and NKp44+ ILC3, as they expressed high amounts of TBX21, IFNG and CXCR3, or RORC, respectively (Figure 7a).

Figure 8 | RORγt+ ILCs of fetal gut can differentiate into ILC1. a. Flow cytometry analysis of ILC populations based on the expression of CRTH2, CD117 and NKp44. Gating on CD45+ lamina propria mononuclear cells, followed by gating on Lin- CD127+ ILCs. Plots are representative of 7 independent experiments with 1 to 3 donors each. Numbers in gate or quadrant indicate percent of cells. b. Highly purified c-Kit+NKp44+ ILCs or NKp44+ ILC3 were cultured (8 days) either with IL-2 (100 U/ml) or with combinations of either IL-2 (100 U/ml), IL-23, and IL-1β (both 50 ng/ml) or IL-2 (10 U/ml) and IL-12 (50 ng/ml) and analyzed by flow cytometry. Numbers in quadrants indicate percent cells in representative plots of at least 3 independent experiments with 1 to 3 donors each, and are quantified in Supplementary Figure 2b,c.
**Figure 8 (continued)**  

**c.** Purified NKp44+ ILC3 were stained with a proliferation dye and then cultured for 3 days as described in b. Histograms are representative of 3 independent experiments. Filled histogram represents non-proliferation control. Numbers in histograms indicate the percentage of non-proliferating cells, and in quadrants indicate the percentage of non-proliferating cells and is quantified in Supplementary Figure 3.  

**d.** Gene expression of RORC, TBX21, IFNG in freshly isolated tonsil NKp44+ ILC3 and ILC1 populations or stimulated with either IL-2 (100 U/ml) and IL-12 (50 ng/ml) or IL-2 (100 U/ml), IL-23 and IL-1β (both 50 ng/ml). Data represent 1 experiment with 2 donors. Error bars show SEM and horizontal bars show means, *P < 0.05.
Next, we assessed whether c-Kit\(^{+}\)NKp44\(^{-}\) ILCs could develop into mature ILC1 or ILC3. Indeed, culturing purified c-Kit\(^{+}\)NKp44\(^{-}\) cells for 8 days with IL-2, IL-1\(\beta\), and IL-23 resulted in a clear shift towards NKp44\(^{+}\) ILC3, whereas culturing with IL-2, and IL-12 induced a shift towards ILC1 (Figure 7a and Supplementary Figure 2a). Under the latter culture conditions, we found, in addition to cells with an ILC1 phenotype, cells with a c-Kit NKp44\(^{+}\) phenotype. These cells might be similar to a very minor population of cells with a same phenotype found in freshly isolated tonsils (Figure 1a). Stimulation of the differentiated c-Kit\(^{+}\)NKp44\(^{-}\)-derived NKp44\(^{+}\) ILC3 and ILC1 with PMA plus ionomycin resulted in production of IL-22 and IFN-\(\gamma\), respectively, indicating a functional differentiation of c-Kit\(^{+}\)NKp44\(^{-}\) cells into IL-22\(^{+}\) ILC3 and ILC1 (Figure 7b). To rule out the possibility that the observed divergence in phenotype, induced by IL-23 and IL-1\(\beta\) or IL-12 was a result of preferential outgrowth of contaminating, already differentiated cells in the c-Kit\(^{+}\)NKp44\(^{-}\) subset, we stained c-Kit\(^{+}\)NKp44\(^{-}\) cells with a cell tracking dye, and cultured them under ILC1 or NKp44\(^{+}\)ILC3 favoring conditions without feeder cells, and followed these cells over time. After three days of culture cells with an ILC1 and NKp44\(^{+}\) ILC3 phenotype appeared within the non-proliferating fractions, indicating that the appearance of ILC1 or NKp44\(^{+}\) ILC3 was not due to preferential outgrowth of contaminating cells, but was the result of differentiation of the c-Kit\(^{+}\)NKp44\(^{-}\) population (Figure 7c).

**ROR\(\gamma\)t\(^{+}\) ILCs can differentiate into ILC1**

A fraction of the c-Kit\(^{+}\)NKp44\(^{-}\) ILCs in tonsils expressed ROR\(\gamma\)t, raising the question whether the ILC1 subset that differentiated under influence of IL-12 could be derived from ROR\(\gamma\)t ILCs. As all CRTH2\(^{-}\) ILCs in fetal gut express ROR\(\gamma\)t\(^{+}\), we purified c-Kit\(^{+}\)NKp44\(^{-}\) cells from fetal intestine, according to the strategy that we also employed for tonsils (Figure 8a), and cultured these cells in IL-2 and IL-12 for 8 days. Similar to tonsil cells, we observed that c-Kit\(^{+}\)NKp44\(^{-}\) ILCs acquired the ILC1 phenotype when cultured with IL-2 and IL-12, whereas when cultured with IL-2, IL-23 and IL-1\(\beta\), these cells differentiated into NKp44\(^{+}\) ILC3 (Figure 8b, top and Supplementary Figure 2b).

To further explore the plasticity of ILCs, we examined whether the ROR\(\gamma\)t\(^{+}\) fetal gut NKp44\(^{+}\) ILC3 could differentiate into the ILC1 subset when cultured with IL-2 and IL-12. Indeed, a highly purified fetal gut NKp44\(^{+}\) ILC3 rapidly lost NKp44 and c-Kit expression, indicating that they differentiated into ILC1 when cultured with IL-2 and IL-12 (Figure 8b, bottom and Supplementary Figure 2c). To exclude that appearance of ILC1 in these cultures was the result of proliferating contaminating cells, we labeled purified fetal NKp44\(^{+}\) ILC3 with a cell tracking dye, and followed the expression in time when cultured with IL-2 and IL-12. The non-proliferating fraction of cells did change their phenotype (Figure 8c and Supplementary Figure 3), confirming that ILCs have the capacity to change their phenotype upon exposure to changes in their local cytokine environment. We obtained similar data when culturing NKp44\(^{+}\) ILC3 from tonsil with IL-2 and IL-12 (Supplementary Figure 4).

The finding that ROR\(\gamma\)t\(^{+}\) ILC3 cultured in IL-2 plus IL-12 could differentiate into ILC1 raised the question whether freshly isolated ILC1 can also differentiate into ROR\(\gamma\)t-expressing ILC3. ILC1 survive poorly when cultured for prolonged periods of time without IL-12, which complicates in vitro culturing assays. However, when we cultured ILC1 cells for 3 days in IL-2...
plus IL-23 we observed that expression of $TBX21$ was severely reduced but that expression of RORC remained low (Figure 8d).

Taken together these data indicate that the appearance of ILC1 in IL-2 plus IL-12 cultures is a result of differentiation rather than proliferation of contaminating cells and indicate that IL-12 can drive differentiation of IL-22-producing RORγT$^{\text{dim}}$ ILCs into IFN-γ-producing RORγT$^{\text{lo}}$/T-bet$^{\text{lo}}$ ILC subset. Our data with ILC1 in a short-term culture with IL-2 plus IL-23 seem to suggest that ILC1 cells also have the potential to change its transcription factor expression profile.
Discussion

Here we described a distinct CD127^+ c-Kit^NKp44^- innate lymphoid cell type, which differs from the currently known RORγt-dependent (ILC3) and GATA3- and RORα-dependent (ILC2) ILCs. The ex vivo isolated c-Kit^NKp44^- ILC expressed IFNG but low amounts of IL13, and no IL22, nor IL17. In addition, these ILCs expressed high amounts of the transcription factor T-bet and its downstream targets, CXCR3 and CCL3, and expressed very low amounts of RORγt. The c-Kit^NKp44^- ILCs were distinct from NK cells as they lacked IL-15Rα and were devoid of CD16, CD94, NKp46, Killer Immunglobulin-like receptors, granzyme B and perforin. C-Kit^-NKp44^- cells were also distinct from c-Kit^+ immature NK (iNK) cells, because they expressed high levels of IL-7Rα, which is not present on iNK cells and lacked c-Kit. In addition, the c-Kit^NKp44^- ILCs were different from the recently described thymus-dependent IL-7Rα^+ NK cells since these latter cells expressed CD56, which is in contrast to c-Kit^NKp44^- ILCs. Thus IFN-γ-producing c-Kit^NKp44^- ILCs were distinct from NK cells and can be considered as the innate equivalent of the Th1 subset, and as such we designated these cells ILC1.

Strikingly, we observed that inflamed intestinal lamina propria of patients suffering Crohn’s disease had elevated proportions of ILC1, which is in agreement with a previous report indicating that CD56^- ILCs that include IFN-γ producing cells is overrepresented in inflamed intestine of Crohn’s disease patients. Consistent with another report we also detected CD56^-IL-17A-expressing ILCs in inflamed tissues of a few Crohn’s disease patients. However, in contrast to ILC1, these IL17A-expressing cells were c-Kit^+. The elevated proportions of ILC1 may be a direct consequence of acute inflammation rather than a bystander effect of a long-term ongoing inflammation as suggested by our experiments with DSS-treated HIS mice.

IL-17 and IFN-γ producing IL-23-responsive ILCs were described to be potent inducers of gut inflammation in an innate mouse model of colitis and neutralization of IFN-γ was sufficient to ameliorate disease progression. Elevated amounts of IFN-γ are also present in the lamina propria of patients with Crohn’s disease. In line with these findings, clinical trials with antagonistic antibodies against IFN-γ (fontolizumab) led to decreased amounts of C-reactive protein, a marker of inflammation, although in this small trial no significant improvement in clinical response, as determined by the subjective Crohn’s disease activity index (CDAI) was found for fontolizumab versus placebo.

Also, antibodies against the p40 component of IFN-γ-inducing cytokine IL-12 failed to demonstrate clear therapeutic efficacy of neutralizing IL-12\textsuperscript{p40} arguing against a prominent role of the IL-12/IFN-γ axis in Crohn’s disease. However, interpretation of the results of this latter trial is confounded by the fact that antibodies against p40 also target IL-23, which shares p40 with IL-12. IL-23 induces IL-17, which might be protective in Crohn’s disease since in these patients anti-IL-17 therapies appear to slightly exacerbate disease activity. Antibodies against the p35 chain that exclusively target IL-12 might be tested to further examine the role of the IL-12 and IFN-γ in Crohn’s disease.

We also identified in various tissues CD127^+ c-Kit^+ NKp44^- ILCs, which did not express IL-13, IL-17, IL-22 nor IFN-γ ex vivo. The c-Kit^+ NKp44^- population contained precursors for both
ILC3 and ILC1, as c-Kit+NKp44 ILCs differentiated to IL-22-producing ILC3 in the presence of IL-2 and IL-23, and to IFN-γ-producing ILC1 in the presence of IL-2 and IL-12. Differentiation occurred before proliferation was manifest, indicating that the observed changes in phenotype and cytokine expression profile was not a consequence of outgrowth of a small contaminating, already differentiated, cell population. We also observed that highly purified NKp44+ ILC3 from both fetal gut and tonsil were able to differentiate into ILC1 in response to IL-2 and IL-12 by first losing c-Kit and then NKp44. This data indicated that at least a proportion of ILC1 are derived from RORγt+ ILC3, which is consistent with observations that ILC3 in RORγt-fate mapped mice eventually lose RORγt and converted into IFN-γ-producing cells without losing the fate marker.21 A phenotypic shift from RORγt+ IL-17 and IL-22 producing cells to RORγt- IFN-γ-producing cells is not without precedent, as also with T cells such shifts have been observed.46,47 Conversely, freshly isolated ILC1 rapidly downregulated TBX21 upon short time incubation in IL-2 and IL-23, although expression of RORγt remained low when compared to NKp44+ ILC3 incubated with IL-2 plus IL-23. Whether a conversion of ILC1 into T-bet negative ILCs occurs in vivo remains to be determined.

Our data indicate a high degree of plasticity among the ILC populations as was already suggested by other recent studies.21,34 How the plasticity amongst ILCs is regulated remains to be established but T-bet might be a central regulator. Recently it was reported that IL-17-producing ILCs were involved in pathogenesis in mice deficient for Tbx21 and Rag1 that develop colitis resembling human ulcerative colitis.48 In the latter study it was also found that T-bet positively regulated IFN-γ, and strongly reduced the capacity of ILCs to produce IL-17. In another recent study T-bet was found to be essential for differentiation of NKp46+ ILC3.49 It was also shown that IL-12 but not IL-23 induced NKp46+ ILC3 to produce IFN-γ. Our results extend these results by showing that IL-12 results in a rapid downregulation of RORγt and upregulation of T-bet combined with loss of c-Kit and NKp44. Thus tunable expression of T-bet emerges as an important regulator of ILC effector functions.

In summary we demonstrated here the existence of ILC1 capable of producing high amounts of IFN-γ, which were distinct from NK cells. At least part of these cells differentiated from ILC3 under the influence of IL-12. We hypothesize that there is also an ILC1 population that develops independent from the transcription factor RORγt. Cell fate mapping experiments in mice would be needed to verify this hypothesis. Because no ILC1 were present in the fetal gut we speculate that ILC1 develop following colonization of the gut with commensals and that ILC1 may be involved in the early innate immune response against certain bacteria such as Salmonella.49 Since ILC1 accumulated in inflamed tissues in Crohn’s disease these cells may contribute to the pathogenesis of this disease.

Acknowledgements
We thank B. Hooibrink for help with flow cytometry; staff of the Bloemenhove clinic in Heemstede, the Netherlands, for fetal tissues; A. Voordouw for proving fetal material. We thank W. Fokkens and C. van Drunen of the department of Otorhinolaringology for providing us with human tonsils.
Supplementary tables and figures

Supplemental table 1 | % of human CD45+ cells in colon of HIS mice

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Percentage of human (h)CD45+ cells, hCD3cells, hCD14cells, and hILCs in colon of HIS mice after a ficoll density gradient purification step. Table represents percentage of total CD45+ cells = (human + mouse CD45+cells) in colon of NSG mice that are reconstituted with human hematopoietic stem cells. Data are from 6 experiments with 2 - 4 pooled donors, each.

Supplemental table 2 | Histological scoring

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Histology score parameters The total inflammation score was determined by the average score of the following criteria: area involved, the number of follicle aggregates, oedema, fibrosis, hyperplasia, erosion/ulceration, crypt loss and infiltration of granulocytes and mononuclear cells.


**Supplemental table 3 | Sequences of real-time PCR primers designed in house**

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CHAPTER 2

Supplemental figure 1 | Differentiation of ILC1 and NKp44+ ILC3. 

**a.** Differentiation of tonsil ckit+NKP44- derived ILCs towards the ILC1 subset and NKp44+ ILC3 subset when cultured in IL-2 and IL-12 or IL-2, IL-23 and IL-1β, respectively (N = 5; **P < 0.01).

**b.** Differentiation of fetal gut ckit+NKp44- derived ILCs towards the ILC1 subset and NKp44+ ILC3 subset when cultured in IL-2 and IL-12 or IL-2, IL-23 and IL-1β, respectively (N = 3; **P < 0.01).

**c.** Differentiation of fetal gut NKp44+ ILC3 derived ILCs towards the ILC1 subset and NKp44+ ILC3 subset when cultured in IL-2 and IL-12 or IL-2, IL-23 and IL-1β, respectively (N = 3; **P < 0.01).
Supplemental figure 2 | Histological Scoring. Histological scoring of: area involved, the number of follicle aggregates, oedema, fibrosis, hyperplasia, erosion/ulceration, crypt loss and infiltration of granulocytes and mononuclear cells in huminzed immune system (his) mice vs. non-reconstituted control mice. Mice are treated for 7 days with 3% DSS. Each dot represents 1 mouse.
Supplemental figure 3 | Differentiation of non-proliferating fraction of NKp44+ ILC3 from fetal gut differentiate into ILC1. Quantification of the fetal gut non-proliferating fraction of NKp44+ ILC3 derived ILCs towards the ILC1 subset when cultured in IL-2 and IL-12 or IL-2, IL-23 and IL-1β, respectively (N = 3; ** P<0.01)

Supplemental figure 4 | NKp44+ ILC3 from tonsil differentiate into ILC1 under influence of IL-12. ILC3 from tonsil differentiate into ILC1 under influence of IL-2 and IL-12. Freshly purified ILC3 from tonsil are cultured for 8 days either alone or with combinations of IL-2 and IL-12, or IL-23 and IL-1β, followed by flow cytometry analysis of ckit and NKp44. Numbers in quadrants indicate percent cells in each. Data shown is representative of 3 experiments.
Human ILC1 accumulate in inflamed tissues

References


