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CHAPTER 3

Interleukin-12 and -23 Control Plasticity of CD127+ Group 1 and Group 3 Innate Lymphoid Cells in the Intestinal Lamina Propria

INTRODUCTION

The intestinal immune system is tolerant towards commensal bacteria but adroitly poised to fight invading pathogens. Homeostatic interactions between commensal bacteria and the intestinal immune cells contribute to the surveillance of the mucosal surface and maintenance of the epithelial barrier. Upon a pathogenic assault, the innate immune system responds promptly by tailoring an immune response accordingly to eliminate the pathogen or to instruct the adaptive arm of the immune system. Following the mounted immune response, a phase of resolution precedes the recovery of intestinal homeostasis and restoration of the intestinal architecture.

Innate lymphoid cells (ILCs) are a family of innate effector cells that are important not only for the integrity and maintenance of the intestinal tract but also in the protection against infiltrating pathogens at the acute phase of infection (Artis and Spits, 2015; Spits and Cupedo, 2012). Distinct ILC subsets respond to and protect against different pathogens by secreting cytokines that are similar to the cytokine and transcription factor expression pattern of the T helper cell family (Spits et al., 2012). RORγt-expressing CD127+ group 3 ILCs are involved in organizing tertiary lymphoid structures (van de Pavert and Mebius, 2010), are critical in controlling containment of commensals (Sonnenberg et al., 2012), and also promote antimicrobial peptide production and the proliferation of epithelial cells (Cella et al., 2009; Crellin et al., 2010). Furthermore, IL-22 producing group 3 ILCs protect against certain bacterial pathogens such as Citrobacter rodentium, which is used as a model in mice for the attaching and effacing enteropathogenic Escherichia coli (EPEC) and enterohaemorrhagic E. coli (EHEC) (Satoh-Takayama et al., 2008; Sonnenberg et al., 2011). T-bet-expressing group 1 ILCs protect against bacteria such as Helicobacter typhlonius (Powell et al., 2012) and intracellular pathogens such as Salmonella enterica in an IFNγ-dependent manner (Klose et al., 2013).

Recently we identified a human ILC1 subset that like ILC2 and ILC3 expressed high amounts of the IL-7 receptor α-subunit (CD127), which promoted polarization from ILC3 to CD127+ ILC1. In contrast, CD14+ DCs, promoted differentiation from CD127+ ILC1 towards ILC3. These observations suggest that environmental cues determine the composition, function and phenotype of CD127+ ILC1 and ILC3.
Here we addressed the question whether IFNγ-producing group 1 ILCs have the potential to differentiate towards IL-22-producing ILC3. We demonstrated both in vitro and in vivo that human CD127+ group 1 - but not CD103+ ILC1 or cNK cells - can differentiate into ILC3, depending on the cytokines they are exposed to, a process which is dependent on the transcription factor RORγt. Furthermore, we demonstrated that the gut metabolite retinoic acid (RA) accelerates transition of CD127+ ILC1 to IL-22-producing ILC3, and we identified RA-producing CD103+ dendritic cells (DCs) as a physiological source that could drive ILC differentiation and maturation of ILC3.

RESULTS
CD127 and CD103 define distinct ILC1 subsets
Human group 1 ILCs comprise a heterogeneous population of IFNγ-producing effector cells, including conventional natural killer (cNK) cells and two populations of ILC1. The first ILC1 subset described by our group (Bernink et al., 2013) expresses high levels of CD127 (the IL-7Rα-chain), and is referred to as CD127+ ILC1. The ILC1 subset described by Fuchs et al. (Fuchs et al., 2013) expresses CD103 (an integrin αE subunit), which in association with β7 may play a role in homing of intraepithelial lymphocytes. This ILC1 subset is therefore referred to as CD103+ ILC1. In order to obtain more insight into the functional differences between CD127+ and CD103+ ILC1, we first compared the phenotype and tissue distribution of the two ILC1 subsets in human palatine tonsils, ileum and mesenteric lymph node. CD103+ ILC1, which could be distinguished from CD127+ ILC1 by the expression of CD56, Nkp44, and CD103 were detected in tonsil and ileum, but not in mesenteric lymph node (Figure 1A). The frequencies of CD127+ and CD103+ ILC1 subsets in tonsils were similar, but were less compared to Nkp44+ and Nkp44+ ILC3 (Figure 1A). In the ileum the CD103+ ILC1 were found in the epithelium as documented before (Fuchs et al, 2013), whereas the CD127+ ILC1 were present in the lamina propria (Figure 1A). Compared to CD127+ ILC1, CD103+ ILC1 expressed very little CD127 if at all (Figure 1B). CD103+ ILC1 from tonsils expressed CD94 and are heterogeneous for CD160, whereas CD127+ ILC1 were negative for both markers (Figure 1B). Both ILC1 subsets expressed CD161, but CD103+ ILC1 showed much lower expression compared to CD127+ ILC1 (Figure 1B). Like CD103+ ILC1, conventional cNK cells expressed CD56 and CD94 and were heterogeneous for CD160, but lacked the expression of CD103, and CD161 (Figure 1B).

We next analyzed the expression of transcription factors in freshly isolated ILC subsets. Transcripts of the transcription factor RORγt (RORC) were undetectable in ex vivo isolated CD103+ ILC1, and low -but not absent- in CD127+ ILC1 compared to Nkp44+ and Nkp44+ ILC3 subsets (Figure 1C). TBX21, which encodes for the transcription factor T-bet was expressed at highest levels in CD103+ ILC1 and CD127+ ILC1 as compared to both ILC3 subsets. The transcription factor Eomesodermin (Eomes), which has been associated with cNK cells in mice (Daussy et al., 2014; Gordon et al., 2012; Kloé et al., 2014), was highly expressed in CD103+ ILC1, but absent in all CD127+ ILC1 and ILC3 subsets (Figure 1C). As expected, both ILC1 subsets but not ILC3, expressed transcripts for IFNG, which was most pronounced in CD103+ ILC1. IL22 transcripts were highly expressed in the Nkp44+ ILC3 subset.

The transcription factor arylhydrocarbon receptor (AHR) was expressed in all ILC subsets, but highest in ILC3. Freshly isolated intestinal ILC subsets showed a similar transcription factor expression profile compared to their tonsil counterparts (Supplemental Figure 1A).

In contrast to ex vivo isolated cNK cells, CD103+ and CD127+ ILC1 did not produce IFNγ in response to IL-15 alone as measured by enzyme-linked immnosorbant assay (ELISA) (Figure 1D). Combined stimulation with IL-15 and IL-12 resulted in a robust IFNγ response in all ILC1 subsets, which was most pronounced in cNK cells. Stimulation with the pro-inflammatory cytokines IL-12 plus IL-18 induced a similar response in all group 1 ILC subsets, and was higher compared to stimulation with IL-12 plus IL-15.

Together, these data demonstrate that in addition to cNK cells, two distinct human IFNγ producing group 1 ILC subsets are present in the tonsil and ileum, which have the ability to respond to the pro-inflammatory cytokines IL-12, IL-15 and IL-18 ex vivo.

When comparing the ILC composition of fetal intestine to that of non-infamed and inflamed resection specimens from patients that suffer from Crohn’s disease, we observed that CD127+ ILC1 in fetal intestines, which were not yet colonized with commensal bacteria, were very low in frequency, and we did not observe any CD103+ ILCs (Figure 1E and Supplemental figure 1B). Furthermore, we observed that fetal intestinal Nkp44+ ILC3 expressed CD103, whereas this marker was restricted to CD103+ ILC1 in adult gut and tonsils (Supplemental figure 1C). Interestingly, the frequency of CORTH2+ group 2 ILCs in the fetal intestines decreased in post-natal intestinal tissues. Furthermore, whereas in adult non-infamed intestinal resection specimen the frequencies of CD103+ ILC1 and CD127+ ILC1 were the same, lamina propria CD127+ ILC1 expanded dramatically in inflamed ileum resected from Crohn’s disease patients, outnumbering intraepithelial CD103+ ILC1, which expanded less than twofold (Figure 1E, Supplemental Figure 1D). No significant changes in frequency were observed for cNK cells between non-infamed and inflamed Crohn’s ileum (Supplemental figure 1E).

Together, these data confirm that CD103+ ILC1 are restricted to the intraepithelial compartment (Fuchs et al., 2013), whereas CD127+ ILC1 are found within the lamina propria. Furthermore, CD127+ ILC1 outnumbered CD103+ ILC1 in inflamed gut tissues of Crohn’s disease patients.
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Figure 1. Phenotype, function, and distribution of CD127+ and CD103+ ILC1 in human tonsil, intestine, and mesenteric lymph node. (a) Flow cytometric analysis of the expression of CD103+ ILC1 and CD127+ ILC1 in freshly isolated tonsil mononuclear cells, which are depleted from T cells (CD3+) and B cells (CD19+) by magnetic bead-based separation, in freshly isolated intestinal epithelial cell (IE) and lamina propria (LP) fraction of resection specimen, and freshly mesenteric lymph nodes (MLN). Subsets were gated on CD3- and CD45+ followed by gating on either CD56, CD103 and Nkp44 for CD103+ ILC1, or selecting for Lin- (CD1a- CD3- CD14- CD19- CD34- CD123- TCRαβ- TCRγδ- BDCA2- FcεR1- CRTH2-) and CD161+, CD127+ and Nkp44 and c-Kit for CD127+ ILC1, both indicated in blue box. Numbers in gates (outlined areas) or quadrants indicate percent cells in each. Distribution of ILC1 subsets in tissues is quantified in bar diagrams as percentage (%) of the total CD45+ CD3+ lymphocyte population. Data shown for tonsil is representative of at least 15 experiments, with one to two donors each, and intestinal data is representative of at least 6 experiments, one donor each. (b) Flow cytometric analysis of CD127+ ILC1 (black line), CD103+ ILC1 (dashed line), and cNK cells (grey filled) of indicated surface molecules. Gating strategy used is as in figure 1a. Data shown is representative of at least 4 experiments. (c) Expression of RORC, TBX21, AHR, IFNG, and EOMES in the CD127+ ILC1, Nkp44+ ILC3, Nkp44+ ILC3, and CD103+ ILC3 subsets of freshly isolated tonsils, presented relative to the expression of ACTB (which encodes β-actin). Data shown is combined data of at least 3 experiments, each with 1 to 2 donors each. (d) IFNγ-production by CD127+ ILC1 (black), and CD103+ ILC1 (white), and cNK cells (grey), cultured for 4 days, either alone or IL-15 and/or IL-12, and IL-12 with IL-18. (e) Mean frequency (as percentage of total ILCs per sample) of CD103+ ILC1, CD127+ ILC1, CRTH2+ ILC2, Nkp44+ ILC3, and Nkp44+ ILC3 within fetal intestine (N=12), non-inflamed control intestine (N=6), and Crohn’s disease intestine (N=6). In these experiments IE and LP fractions were combined before analysis. * P < 0.05, ** P< 0.01 (analysis of variance).

IL-23 and IL-1β are sufficient to drive differentiation of CD127+ ILC1 towards ILC3

Differentiation of ILC3 towards IFNγ-producing CD127+ ILC1 under influence of IL-12 may contribute to the increase in frequency of CD127+ ILC1 in inflamed mucosal tissues as seen in the inflamed intestines of individuals with Crohn’s disease (Bernink et al., 2013; Vonarbourg et al., 2010). We then asked whether IFNγ-producing ILC1 have also the potential to differentiate towards IL-22 producing ILC3. To address this question, we sorted CD127+ ILC1, Nkp44+ ILC3, Nkp44+ ILC3, CD103+ ILC1, and cNK cells (Figure 2A, left column), and cultured these cells for 4 days in the presence of feeder cells with IL-2 and IL-12, or IL-2 and IL-18. As documented previously (Bernink et al., 2013; Cella et al., 2010), IL-2, IL-12 and IL-1β maintained the ILC3 phenotype, and promoted the acquisition of Nkp44. Furthermore, IL-2 and IL-12 was sufficient to induce differentiation of Nkp44+ and Nkp44+ ILC3 towards CD127+ ILC1 (Bernink et al., 2013), and CD127+ ILC1 cultured in the presence of IL-2 and IL-12 maintained their phenotype. (Figure 2A). Although CD127+ ILC1 showed only a modest expression of IL-1R and IL-23R compared to ILC3 (Supplemental Figure 2), CD127+ ILC1 differentiated towards ILC3 when cultured with IL-1β, IL-2 and IL-23 (Figure 2A). As a consequence, CD127+ ILC1 lost their potential to produce large amounts of IFNγ, and instead started to produce IL-22 (Figure 2B and C). Neither CD103+ ILC1 nor cNK cells differentiated into ILC3 or produced IL-22 (Figure 2A-C).
Figure 2. IL-2, IL-23 and IL-1β drive ILC3 differentiation. (a) Purified CD127+ ILC1, Nkp44+ ILC3, Nkp44+ ILC3, CD103+ ILC1, and cNK cells from tonsil were cultured for 4 days either with IL-2 and IL-12 or with IL-2, IL-23, and IL-1β. Cells were phenotyped for the expression of c-Kit and Nkp44. Numbers in quadrants indicate percent cells in each. Data shown is representative of 4 experiments. (b) Expression of intracellular IFNγ measured by flow cytometry of either IL-2 and IL-12 (dashed line) or IL-2, IL-1β and IL-23 (black line) cultured subsets in (a), following stimulation with PMA plus ionomycin. Data shown is representative of 3 experiments. (c) Expression of intracellular IL-22 measured by flow cytometry of either IL-2 and IL-12 (dashed line) or IL-2, IL-1β and IL-23 (black line) cultured subsets in (a), following stimulation with PMA plus ionomycin. Data shown is representative of 3 experiments.

IL-22 and IL-17 production is restricted to lymphocytes that express the transcription factor RORγt (Ivanov et al., 2006), whereas IFNγ production is associated with high expression of the transcription factor T-bet (Lazarevic et al., 2010). Given the plasticity of the CD127+ ILC subsets in their cytokine expression pattern, we asked whether the transcription factor program adapted accordingly. As such, we purified ILC subsets from tonsils and intestinal resection specimens and cultured these cells for 4 days in the presence of feeder cells with IL-2 and IL-12, or IL-2, IL-23, and IL-1β (Supplemental Figure 3A-F). Indeed, Nkp44+ ILC3 lost their RORγt expression upon exposure to IL-2 and IL-12, whereas T-bet was up-regulated (Supplemental Figure 3A and B). Also, we observed a change into an IL-17-like transcription factor profile in CD127+ ILC1 when exposed to IL-2, IL-23 and IL-1β (Supplemental Figure 3A-B). Notably, neither in ILC3 nor in CD127+ ILC1 we observed Eomes (Supplemental Figure 3C). This was in sharp contrast to cNK cells and CD103+ ILC1, which did not express RORγt, but rather maintained or enhanced their expression of T-bet and Eomes (Supplemental Figure 3C and F).

The observation that CD127+ ILC1 acquire phenotypic and functional features of ILC3 may be the result of outgrowth of a small number of ILC3 contaminating the purified ILC1. In that case the ILC3 should have undergone many cell divisions. To address this, we labeled sorted CD127+ ILC1 with a cell tracer dye and cultured them in the presence of IL-2, IL-23 and IL-1β. Electronic gating on cells that expressed high levels of the cell tracer, and thus did not proliferate, revealed that these cells expressed c-Kit and Nkp44, indicating that the non-proliferated fraction of CD127+ ILC1 differentiated towards ILC3 (Figure 3A). This observation argues against the possibility that outgrowth of contaminating ILC3 in CD127+ ILC1 cultures accounted for the observed phenotype. Furthermore, we did not detect any difference in viability between the non- and proliferating cell fractions (Figure 3B). Further supporting our hypothesis, we sorted out ILC3-derived ILC1 and observed that these cells could re-differentiate towards ILC3 in the in the presence of IL-2, IL-23 and IL-1β (Supplemental Figure 4).

To further confirm that the CD127+ ILC1 can differentiate to ILC3, we generated clones of purified ILC1 (>97%) in the presence of feeder cells and IL-2, IL-1β and IL-23. No difference was observed in the plating efficiencies between the two ILC3 populations and ILC1 under these conditions (Figure 3C). The burst size after 14 days of culture was on the average 4000 cells per well (Figure 3D), allowing for a phenotypic evaluation of the progeny of the individual ILC1. Interestingly most ILC1-derived clones showed a heterogeneous phenotype as exemplified in the left panel of Figure 3E; the right panel shows an overview of the distribution of nine individual clones within the progeny of one single cell. We observed cells with a phenotype of ILC1, Nkp44+ ILC3, and Nkp44+ ILC3 (Figure 3E). In addition, we observed clones that were partly c-Kit+ c-Kit- Cells with a similar phenotype were also observed in ex vivo isolated ILC (Bernink 2013), and have yet to be characterized in more detail. The different phenotypes we observed within a clone correlated with different functional capacities, since c-Kit+ cells isolated from the progeny of a single ILC1 produced mainly IL-22 and little IFNγ, whereas c-Kit- Nkp44+ cells isolated from the same cell samples produced IFNγ and little IL-22 (Figure 3F). These data support the notion that a sizeable fraction of CD127+ ILC1 can differentiate into IL-22 producing ILC3 in the presence of the cytokines IL-2, IL-23 and IL-1β.
ILC1 differentiate towards ILC3 in vivo

Previously, we demonstrated that CD127+ ILC1 accumulated at the onset of intestinal inflammation in mice that were reconstituted with a human immune system (HIS) (Bernink et al., 2013). In order to evaluate whether the observed ILC1 to ILC3 differentiation in vitro also occurred in humanized mice without inflammation, we engrafted sub-lethally irradiated lymphopenic mice (NOD SCID IL2gc−/−; NSG) with human hematopoietic stem cells (HIS-mice). First we analyzed the ILC1 and ILC3 composition in blood, spleen, lung, small intestine and colon, which were reconstituted on average up to 80% with human cells at the age of 8 weeks (Figure 4A and B, Supplemental Figure 5A). Next, we injected humanized mice intravenously (i.v.) with expanded ILC1 as described previously (Bernink et al., 2013), which were labeled with a cell tracer dye (Supplemental Figure 5B). This allowed us to trace the fate of ILC1 in vivo under homeostatic conditions. Analyses of blood, spleen, liver, lung, small intestine and colon revealed that cell tracker-positive ILCs were clearly detectable in all organs 4 days after injection (Figure 4C, Supplemental Figure 5B). Interestingly, the ILC distribution-profile of cell tracer-positive ILCs is similar to the human ILC profile derived from the injected hematopoietic stem cells (Figure 4B), which was particularly emphasized in the small intestine, as also NKp44+ ILC3 were detectable (Figure 4D).

ILC in mice and humans are very similar in functional respects although phenotypic differences are obvious. It has been documented also in mouse that ILC3 can lose RORγt and upregulate T-bet and their capacity to produce IFNγ (Cella et al., 2010; Vonarbourg et al., 2010). Based on our findings we expected that these murine ILC3-derived ILC1 (also called ex-RORγt+ ILC3) can differentiate back to ILC3 under homeostatic conditions. To examine this, we reconstituted lymphopenic mice (Rag2−/− Il2rg−/−) with murine NKp46+ NK1.1+ RORγt-fate-map (fm)+ ILC3s, which had downregulated RORγt expression (ex-RORγt+ ILC3) (Klose et al., 2013) (Supplemental Figure 5C). Analysis of small and large intestine 6 weeks after injection revealed that, ILC3-derived ILC1 upregulated RORγt expression (Figure 4E). Together, these data indicate that in the absence of inflammation, CD127+ ILC1 can switch towards ILC3 in vivo.

Retinoic acid accelerates ILC3 differentiation and IL-22 production

The vitamin A metabolite retinoic acid (RA) has recently been reported to enhance IL-22 production by ILC3 in mice (Mielke et al., 2013). Here we investigated whether RA contributes to the differentiation of ILC1 towards ILC3. The subunits for the receptor for RA, retinoic acid receptor α (RARA), γ (RARG) and RXRG were readily detectable in freshly isolated CD127+ ILC subsets (Figure 5A), suggesting that these cells are responsive to RA. Purified NKp44+ ILC3 and CD127+ ILC1 stimulated with the combination of IL-2, IL-1β and IL-23 upregulated NKp44, and this effect was further enhanced by the addition of RA (Figure 5B), suggesting that RA accelerated differentiation. In line with these results, we observed that RA enhanced upregulation of ROIRγt in CD127+ ILC1 cultured with IL-2, IL-23 and IL-1β as compared to IL-2 alone (Figure 5C).
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CD45 cells. Then gating strategy was used as in Figure 1a. (b) Using gating strategy as in Figure 4a, blood, spleen, lung, small intestine, and large intestine were analyzed for the expression of c-Kit and NKp44. (c) Humanized mice were retro-orbitally inoculated with expanded and with cell tracker labeled ILC1 (*1x10^5–5x10^5 cells per mouse) 4 days before analysis. Percentage of cell tracker positive ILCs compared to total hCD45 ILCs in analyzed organs varied between 0 and 0.5%. Left: large intestine, right: bar diagram indicating % of cell tracker positive ILCs per organ. (d) Using gating strategy as in Figure 4a and 4c, blood, spleen, lung, small intestine, and large intestine were analyzed for the expression of c-Kit and NKp44. Data shown is representative of 3 experiments, each with 2 humanized mice (1-3 donors each), and reconstituted cells are from 1 donor each. (e) Ex-ROßy+ ILC3 (defined as Lin−RORγt−fam−NKp46+ NK1.1+) and NK/ILC1 (defined as Lin−RORγt−fam−NKp46−NK1.1−) (Supplemental Figure 5C) were isolated from eight week old Rorcγt−Cre<sup>x Rosa26R-YFP</sup> mice on a C57BL/6 background (H-2b), and were transferred intravenously into 8 week old Rag2<sup>-/-</sup> Il2rg<sup>-/-</sup> mice, which were kept for 6 weeks under SPF conditions before analysis of ROßy expression in small and large intestine. Ex-ROßy+ ILC3: N=3; NK/ILC1: N=2.

Next, we measured IL-22 protein by ELISA in cultures that started with purified CD127+ ILC1. Culture with RA alone did not result in any production of IL-22. However, when cultured for 7 days in combination with IL-2, IL-23 and IL-1β, increased amounts of IL-22 were found in the culture supernatants of CD127+ ILC1, and further increased in the presence of RA (Figure 5D), indicating that following differentiation of ILC1 to NKp44+ ILC3, these cells started to produce IL-22. RA did not induce a notable production of IL-22 in either CD103+ ILC1 or cNK cells (Supplemental Figure 6). Cells cultured in the presence of RA showed a reduced proliferating capacity (Figure 5E). Thus, although RA enhanced differentiation of ILC1 into functional ILC3, it also reduced expansion of the ILC3 pool.

Differentiation of CD127+ ILC1 to ILC3 is controlled by RORγt

Given the plasticity among the CD127+ ILCs in terms of transcription factor and cytokine expression profile, we asked whether differentiation of CD127+ ILC1 to ILC3 was controlled by the transcription factor RORγt. To address this question, we cultured purified tonsil CD127+ ILC1 and ILC3 for 4 days with IL-2, IL-23 and IL-1β in the presence or absence of SR1001, a synthetic RORγt ligand, which induces suppression of the receptor’s transcriptional activity (Solt et al., 2011). SR1001 inhibited differentiation of CD127+ ILC1 to ILC3 compared to the DMSO control, as reflected by their hampered up-regulation of c-Kit and NKp44 and reduced IL-22 production, whereas the cell number did not differ (Figure 6A-B). SR1001 variably affected IL-22 production by NKp44+ ILC3, compared to the DMSO control as measured by ELISA (Figure 6B), but SR1001 did not substantially affect the ability of ex vivo isolated NKp44+ ILC3 to produce IL-22.

Together, these data indicate that RORγt is important for the differentiation from CD127+ ILC1 towards IL-22 producing ILC3 but may not be involved in IL-22 production after completion of the differentiation process. ORγt expression in small and large intestine. Ex-ROßy+ ILC3: N=3; NK/ILC1: N=2.
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**Figure 6.** CD127+ ILC1 derived differentiation to ILC3 is controlled by RORγt. (a) Freshly isolated CD127+ ILC1, NKp44– ILC3, and NKp44+ ILC3 from tonsil are cultured for 4 days with IL-2, IL-23, and IL-1β, and RA in the presence of either SR1001 or DMSO. Cells are phenotyped for the expression of c-Kit and NKp44. Numbers in quadrants indicate percent cells in each. On the right, bar diagrams indicate absolute cell number and percentage of c-Kit+ NKp44+ cells of each subset cultured in either SR1001 or DMSO. (b) IL-22 production by CD127+ ILC1, NKp44– ILC3, and NKp44+ ILC3 from tonsil cultured for 4 days with IL-2, IL-23, IL-1β, and RA. Data shown is representative of 3 experiments with 1 donor each. * P < 0.05 (Mann-Whitney test).

ILC are instructed by dendritic cells

The finding that CD127+ ILC1 can differentiate into ILC3 raises the question which cell type may induce this differentiation. We examined the frequencies of HLA-DR+CD11c+ dendritic cells (DCs) in Crohn’s ileum compared to non-inflamed control. We observed that a significantly greater proportion of DCs in the inflamed gut expressed CD14 and that the CD14 expression levels were higher than on CD14– DC from non-inflamed tissue (Figure 7A). Interestingly, while there was no detectable difference in the expression of IL23p19 between CD14+ and CD14– DCs, the latter did show increased expression of IL12p35 (Figure 7B), indicating a potential role for these cells in the differentiation into CD127+ ILC1. In order to investigate the differential potential of these two DC subsets in promoting and maintaining the ILC1 phenotype, we cultured CD127+ ILC1 or NKp44+ ILC3 in the presence of autologous CD14+ or CD14– DCs. CD127+ ILC1 maintained their phenotype when cultured with CD14+ DCs but upregulated c-Kit and NKp44 to adopt an ILC3 phenotype when cultured with CD14– DCs.
DISCUSSION

Whereas group 2 and group 3 ILC subsets have been well characterized, group 1 ILCs are less clearly defined. Recently, we and another research group identified human ILC subsets that shared the capacity to produce IFNγ (Bernink et al., 2013; Fuchs et al., 2013). These subsets were distinct from each other; one expressed high amounts of CD127, whereas the other expressed the integrin CD103 and only low amounts of CD127. Here we compared these two subsets side by side. We confirmed
that CD103+ ILC1 are located within the epithelium of the intestine, whereas CD127+ ILC1 reside in the lamina propria. Both populations were present only in very low numbers in fetal intestine, which suggests that colonization by microbiota may trigger their appearance in the intestine. We found that CD127+ and CD103+ ILC1 were equally distributed in both tonsil and non-inflamed intestinal resection specimen. In inflamed resection specimens of individuals with Crohn’s disease, we observed a dramatic increase in frequency of CD127+ ILC1 whereas the proportions of CD103+ ILC1 also increased but to a substantially lesser extent.

In mice a distinct subset of ILC1 has been recently characterized which also lacked Eomes but were NKp46+ Tbet- and RORyt-fm, meaning that these cells never expressed RORyt during their development (Klose et al., 2014). This ILC1 subset was mainly present in the mucosal tissues, such as the intestine (Klose et al., 2014). Although the human CD127+ ILC1 subset described here also lack Eomes, they also lacked expression of the natural cytotoxicity receptors (NCRs) NKp46 and NKp44. CD103+ ILC1 have been described to express perforin and granzymes, and mostly co-express Eomes and Tbet, and therefore may represent a subset of cNK cells specifically located in the epithelium. Thus the human cell types described here are probably distinct from the mouse RORyt fate-map ILC1 and the human equivalent of these cells has yet to be identified.

Previous studies in human and mouse systems have provided evidence that ILC3, particularly in the intestine, have the ability to upregulate T-bet and downregulate RORyt, both in vitro and in vivo (Bernink et al., 2013; Klose et al., 2013; Vonarbourg et al., 2010). Here we reported that human IFNγ-producing CD127+ T-bet- c-Kit+ NKp44+ ILC1 can differentiate into IL-22-producing NKp44+ ILC3. This differentiation was induced by IL1β and IL-23, coincided with increased levels of RORyt and decreased levels of T-bet and was amplified by RA. Since RA has been shown to directly upregulate RORyt (van de Pavert et al., 2014), those findings raised the possibility of an involvement of RORyt in this process. This notion is reinforced by the finding that SR1001, an antagonist binding to the ligand binding domain of RORyt (Solt et al., 2011), inhibited ILC3 differentiation. Not only the frequency of NKp44+ ILC3, but also the accumulation of IL-22 during the differentiation process was reduced by the RORyt inhibitor. Importantly, SR1001 did not inhibit IL-22 production by ex vivo isolated NKp44+ ILC3, suggesting that the reduction of IL-22 production in the ILC1 differentiation cultures was the result of inhibition of the differentiation process of ILC1 into ILC3 and not of the effector function of ILC3 after completion of the differentiation process. However, it should be noted that not all CD127+ ILC1 differentiated towards ILC3 in the presence of IL-2, IL-1β and IL-23. Between 2-15 percent of the cells kept their ILC1 phenotype. Whether these cells constitute a distinct subset or whether a certain equilibrium is maintained between the subsets remains an open question and needs to be further investigated.

Using HIS-mice we previously observed that CD127+ ILC1 expanded in frequency promptly upon inflammation in the gut (Bernink et al., 2013). We proposed that the accumulation of ILC1 under those conditions is the result of a rapid inflammation-induced differentiation of NKp44+ ILC3, which dominate under homeostatic conditions, into ILC1. This conversion was dependent on IL-12 (Bernink et al., 2013; Vonarbourg et al., 2010). Gut inflammation triggers the influx of IL-12 producing phagocytes (Goldszmid et al., 2012; Neurath, 2014; Schultness et al., 2012). In line with those findings we observed in intestinal resection specimen from people with Crohn’s disease an elevated frequency of IL12 expressing CD14+ DCs compared to non-inflamed control, and ex-vivo isolated CD14+ DC cultures in the presence of NKp44+ ILC3 were sufficient to drive ILC3 to ILC1 differentiation. CD14 DCs dominate under homeostatic conditions, and these cells were sufficient to drive ILC1 to ILC3 differentiation. Our observation that human ILC1 can also differentiate into ILC3 in reconstituted HIS mice and that mouse ILC3-derived ILC1 (or ex-ILC3) differentiate into ILC3 in lymphopenic mice, raises the possibility that after resolution of inflammation the “inflammatory” IFNγ-producing CD127+ ILC1 revert to “homeostatic” IL-22-producing ILC3. Further evaluation of the CD14+ population revealed approximately 25% of these cells expressed CD103, which marks an RA-producing tissue resident DC population. Direct comparison of monocyte-derived DCs and RA-producing CD103+ DCs (Bakdash et al., 2014) revealed that the latter were superior in driving ILC1 to ILC3 conversion compared to the mDCs. Thus, DCs respond differently to environmental cues, which enable them to instruct the ILCs.

Several studies in mice support the idea that DCs are important in instructing ILCs. In one study it was demonstrated that basal production of IL-1β by a subset of gut-resident macrophages in mice was essential for ILC3 homeostasis locally and in MLN. Crosstalk between these macrophages and ILC3 instructed CD103+ DCs to produce RA, which in turn contributed to intestinal homeostasis (Mortha et al., 2014). Another study demonstrated that CD103+ DCs are a cellular source of IL-23, which have been demonstrated to interact and activate ILCs in a transient manner (Kinnebrew et al., 2012).

Our data suggest that RA is an important regulator of ILC1 to ILC3 conversion. RA is present in high concentrations in the gastrointestinal tract (Veldhoen and Brucklacher-Waldert, 2012). Epithelial derived RA has been shown to influence processes of immune cells located in the lamina propria. For example, RA drives differentiation of monocytes towards CD103+ DCs, which acquired a tolerogenic phenotype and thereby able to drive regulatory T cell differentiation (Iliev et al., 2009). RA also has a direct effect on mouse ILC3 by promoting the up-regulation of IL-22 (Mielke et al., 2013), and in another study it was demonstrated that the gut-resident ILC3 composition is directly influenced by RA, favoring ILC3 over ILC2 (Spencer et al., 2014). In line with these reports we observed in our in vitro cultures that RA, in combination with IL-2, IL-23 and IL-1β, induced human ILC3 differentiation and enhanced IL-22 production. This, together with the notion that high titers of RA are readily produced by epithelial cells and lamina propria-resident CD103+ DCs, may explain our observation that adult gut-resident CD127+ ILCs predominantly comprised the NKp44+ ILC3 compartment and lacked CRTH2+ ILC2. These NCR+ ILC3 expressed high levels of IL-22 under homeostatic conditions compared to NKp44+ ILC3 and ILC1, possibly in order to maintain the integrity of the epithelial barrier during homeostasis (Sonnenberg and Artis, 2012).
We found in intestinal resection specimen of Crohn’s disease patients an increased frequency of IFNγ producing CD127+ ILC1, which was inversely proportional to the reduced frequency of NKp44+ ILC3. Intestinal inflammation, such as Crohn’s disease, is accompanied by an influx of pro-inflammatory IL-12 producing phagocytes at the site of infection (Goldszmid et al., 2012), which may drive the differentiation of ILC3 towards CD127- ILC1. IFNγ in turn, has been reported to orchestrate the replacement of resident mononuclear phagocytes by circulating pro-inflammatory monocytes (Goldszmid et al., 2012), reinforcing the pro-inflammatory milieu at the site of infection. Furthermore, it was reported that IFNγ signals directly on Paneth cells in the intestinal crypts, instructing them to release antimicrobial compounds, which was coupled to the extrusion and death of Paneth cells (Farin et al., 2014). Concordantly, prolonged IFNγ responses do harm the host, resulting in severe gut inflammation (Buonocore et al., 2010; Klose et al., 2014; Vonarbourg et al., 2010). Therefore, it is tempting to speculate that persistently increased numbers of ILC1 contribute to the disease process. If this is the case it might be possible that enforcing differentiation of these ILC1 to ILC3 may have a therapeutic effect in Crohn’s Disease. In this respect it is noteworthy that ILC1 isolated from surgical specimens of Crohn’s disease patients also convert to NKp44+ ILC3.

Taken together, an efficient mechanism presents itself by which ILC can quickly adapt to changes inflicted by pathogens without the need of recruiting new cells from the circulation. Furthermore, the identification of environmental cues and transcription factors that drive this plasticity may be used to develop future targets for potential therapeutic interventions.

**METHODS**

**Tissue collection**

All tissues were collected after subjects provided informed consent, with approval of tissue-specific protocols by the Medical Ethical Committee of the Academic Medical Centre, Amsterdam. Tonsils were obtained from pediatric tonsillectomies. Inflamed intestinal ileum of patients with Crohn’s disease was freshly obtained and processed after surgical resection. Non-inflamed ileum, referred to as ‘non-inflamed control’, was collected after surgical resection of colon cancer, where the ileum was considered unaffected. Subjects were excluded if they had undergone chemo- or radiotherapy before resection. Patients’ age range from 18 to 68 years. Human fetal tissues were obtained from elective abortions at the Stichting Bloemenhove clinic in Heemstede, the Netherlands, upon on the receipt of informed consent. The use of human abortion tissues was approved by the Medical Ethical Committee of the Academic Medical Center, Amsterdam. Gestational age, determined by ultrasonic measurement of the diameter of the skull or femur, ranged from 14 to 17 weeks.

**Human immune system mice**

CD34+ CD38- HSCs isolated from human fetal liver (0.2×10⁵ – 2×10⁶ cells) were transplanted intrahepatically into sublethally irradiated (1.0 Gy) newborn NSG mice (younger than 1 week of age). Peripheral blood was collected from a facial vein every 3 to 4 weeks after transplantation to determine the kinetics of human cell engraftment. At 2 months of age mice were retro-orbital inoculated with approximately 1x10⁵ – 5x10⁵ expanded and cell tracker labeled CD127- ILC1 (as described in Bernink et al.). After 4 days mice were sacrificed and organs were harvested for analyses.

**Transfer experiments**

ILC populations were prepared from week old Rorc(Δt)-Cre+ x Rosa26R-YFP mice on a C57BL/6 background (H-2b) (as described in (Vonarbourg et al., 2010)). Ex-RORγt+ ILC3 were defined as Lin RORγt+ NKp46+ NK1.1+ cells and the NK/ILC1 population was defined as Lin RORγt- NKp46+ NK1.1- cells. Using a BD FACS Aria III cell sorter, cells were sorted into Eppendorf tubes filled with 500 µL Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FCS, 10mM Sodium Pyruvate plus nonessential amino acids, 80 mM 2-Mercaptoethanol, 8 mg/ml Glutamine, 100 U/ml Penicillin, 0.4 mg/ml Gentamicin and 100 mg/ml Streptomycin. Cells were subsequently washed in PBS and 8,000 cells were transferred intravenously into 8 week old Rag2-/- Il2rg-/- mice. Mice were kept for 6 weeks under SPF conditions at which time they were analyzed.

**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-µm cell strainer and mononuclear cells were isolated with Ficoll-Paque Plus medium (GE Healthcare).

Intestinal lamina propria was incubated for 30 min with PBS containing 5 mM EDTA at 37 °C to separate epithelial cells from lamina propria cells. Lamina propria was then cut into small pieces and digested for 30 min at 37 °C with RPMI (Gibco) containing Liberase TM (125 µg/ml). Cell suspensions were filtered through a 70-µm nylon mesh, and intraepithelial and lamina propria mononuclear cells were isolated with Ficoll-Paque Plus medium (GE Healthcare). In some experiments IEL and LP fractions were analyzed separately, and in some experiments both fractions were combined before analysis. Mesenteric lymph nodes were mechanically disrupted and were passed through a 70-µm cell strainer and mononuclear cells were isolated with Ficoll-Paque Plus medium.

Isolation of Lamina Propria Lymphocytes (LPL) in mice were performed as described in (Sanos and Diefenbach, 2010). In short: small intestines were removed, Peyers’ patches were excised, and the intestines were flushed with ice-cold PBS and cut longitudinally. Epithelium was dissociated by incubation for 40 min at 37°C in Hank’s Balanced Salt Solution without Ca²⁺/Mg²⁺ (HBSS) with 5 mM EDTA and 10 mM HEPES and subsequent vortexing. Remaining tissue, containing LPL, was minced with a scalpel and digested in HBSS (with Ca²⁺/Mg²⁺) containing Collagenase D (0.5 mg/ml), DNase I (0.5 mg/ml) and Dispase (0.5 U/ml) for 1h at 37°C under constant shaking. Cells were detached by thorough vortexing and filtered through a 70 µm filter. Leukocytes were enriched using a Percoll Gradient centrifugation. Cells were resuspended in PBS containing 2% FCS and stained for 30 min on ice with fluorochrome-conjugated antibodies.
Flow cytometry analysis and sorting
The following antibodies to human proteins were used. From BioLegend: fluorescein isothiocyanate (FITC)-conjugated anti-CD1a (HI149), anti-CD3 (OKT3), anti-CD94 (DX22), anti-CD123 (6H6) and anti-FcErIiz (AER-37); phycoerythrin- conjugated anti-CD161 (HP-3G10), anti-KIR3DL1 (D9S), and anti-CD144 (P44-8); peridinin chlorophyll protein–cyanine 5.5– conjugated anti-CD117 (104D2); Alexa Fluor 647–conjugated anti-NKp44 (P44-8); allopheocyanin– conjugated anti-NKG2D (1D11) and anti-T-bet (eBio4B10); Alexa Fluor 700–conjugated anti-CD56 (HC56). From Beckman Dickinson: FITC- conjugated anti-CD14 (M499), anti-CD16 (3G8), anti-CD19 (B98), anti-CD34 (581), anti-CD56 (NCAM16.2), anti-TCRβ (IP26) and TCRγ (B1); phycoerythrin-conjugated anti-CD16 (3G8); Alexa Fluor 647–conjugated anti-CRT1H2 (CD294; BM16); allopheocyanin-indotricarbocyanine (Cy7)–conjugated anti-CD45 (2D1). From other manufacturers: phycoerythrin-Cy7–conjugated anti-CD127 (R34.34; Beckman Coulter), phycoerythrin-conjugated anti-RORγt (AFKJ5-9; ebioscience); and FITC-conjugated anti-BCA2 (CD303, AC144; Milteny). The cell proliferation dye was Cell-Trace Violet (34557; Invitrogen). The following antibodies to mice proteins were used. CD3-biotin (145-2C11), CD5-biotin (S3-7.3), CD19-biotin (MB19-1), Gr-1-biotin (RB6-8C5), NKp46-PE (29A1.4), NK1.1-PEcy7 (PK136), CD4-PerCP-Cy5.5 (GX1.5), RORγt-APC (B2D), H2-Kb-PerCP-Cy5.5 (AF6-88.5.5.3) (all ebiosciences), CCR6-BV421 (29-2117, BioLegend) Streptavidin-V500 (BD Bioscences). For phenotypic analyses by flow cytometry, data were collected with an LSR-Fortessa instrument (BD Biosciences). For sorting by flow cytometry an ARIA IIU (BD Biosciences) was used. Data was analyzed with FlowJo software (TreeStar). Peripheral blood and tonsil mononuclear cell samples were depleted of T cells and B cells by labeling with FITC-conjugated anti-CD3 and anti-CD19 (described above) plus anti-FITC microbeads (Milteny). Intracellular cytokine staining Cell cultures were stimulated for 6 h with PMA (10 ng/ml; Sigma) and ionomycin (500 nM; Merck) in the presence of GolgiPlug (BD Biosciences) for the final 4 h of culture. A Cytofix/Cytoperm kit (BD Biosciences) was used for cell permeabilization, staining and subsequent washing. The following antibodies were used: allopheocyanin–ann conjugated IL-17 (BL168; BioLegend), and phycoerythrin- conjugated anti-IL-22 (142928; R&D Systems) and anti-IFN-γ-pe-cy7 (B27; BD Bioscience). Data were acquired with an LSRFortessa instrument (BD Biosciences) and analyzed with FlowJo software (TreeStar).

Quantitative real-time PCR
RNA was isolated with NucleoSpin RNA XS kit (Macherey-Nagel) according to the manufacturer’s protocol. Complementary DNA was synthesized with the High-Capacity cDNA Archive kit (Applied Biosystems). PCR was done on a LightCycler 480 Instrument II (Roche) with SYBR Green I master mix (Roche). Primers sets used are shown in Supplemental table 2.

Cell cultures and RORγt inhibition
For short-term cultures (2 to 7 days), CD103+ ILC1, cNK cells, and Lin’ CD127+ ILC populations were cultured in some experiments with or in some experiments without 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. For ILC3 polarization experiments IL-23 (50 ng/ml; R&D Systems), and IL-1β (50 ng/ml; R&D Systems), and in indicated experiments retinoic acid (RA) (1µM; Sigma-Aldrich) were supplemented to cultures. For ILC1 polarizing experiments IL-12 (50 ng/ml; R&D Systems) was supplemented to cultures. Inhibition of RORγt was achieved by the addition of the synthetic RORγt-ligand SR1001 (10µM; Sigma-Aldrich) to cell cultures with freshly isolated CD127+ ILC1 and ILC3, which were cultured in the presence of IL-2 (10 U/ml; Novartis) IL-23 (50 ng/ml; R&D Systems), and IL-1β (50 ng/ml; R&D Systems and RA (1µM; Sigma-Aldrich) and RA (1µM; Sigma-Aldrich).

Generation of mDC and RA-mDC
RA-mDCs and mDCs were generated as described in (Bakdash et al., 2014). In brief, monocytes were isolated from PBMCs using density centrifugation, then cultured for 6 days in IMDM (Gibco, Paisley, UK) containing gentamicin (86 µg/l; Duchefa, Haarlem, The Netherlands) and 10% FCS (Gibco), supplemented with GM-CSF (500 U/ml; Schering-Plough, Uden, The Netherlands) and IL-4 (10 U/ml; Milteny Biotech, Bergisch Gladbach, Germany). RA-DCs were generated in the additional presence of 1 µM of retinoic acid (Sigma-Aldrich).

Aldefluor assay
The ALDH activity of DCs was determined by the ALDEFLUOR staining kit (Aldagen) following manufacturer’s instructions.

Cytokine production
Freshly isolated CD103+ ILC1, CD127+ ILC1 and cNK cells (2000 cells per well in a 96-well plate) were stimulated for 4 days with combinations of IL-2 (10 U/ml; Novartis), IL-12 (50 ng/ml; R&D Systems), IL-18 (50 ng/ml; R&D Systems), IL-1β (50 ng/ml; R&D Systems and RA (1µM; Sigma-Aldrich) and RA (1µM; Sigma-Aldrich). Cell cultures and RORγt inhibition

Statistical Analysis
Statistical significance was determined with ANOVA, Student’s t-test, or Mann-Whitney test. Prism GraphPad software was used.
REFERENCES


### SUPPLEMENTAL DATA

#### Supplemental table 1 – lineage markers

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Chapter 3  

IL-12 and IL-23 Control Plasticity of CD127+ ILC1 and ILC3

(A) Flow cytometric analysis of the expression of CD103+ ILC1 and CD127+ ILC1 in fetal intestinal mononuclear cells. Both subsets were gated on CD3- and CD45+ followed by either CD56, CD103 and NKP44 for CD103+ ILC1, or by Lin- (CD1a- CD3- CD14- CD19- CD94- CD34- CD123- TCRβ- TCRγδ- BOCA2- FcεR1+ CRTH2) and CD161-, CD127-, and NKP44 and c-Kit for CD127+ ILC1, both indicated in blue box. Numbers in gates (outlined areas) or quadrants indicate percent cells in each. Data shown is representative of at least 5 experiments, with one to three donors each.

(B) Flow cytometric analysis of the expression of CD127+ ILCs in mononuclear cells of fetal and adult intestinal tissues. Cells were gated as in figure 1a, and then CD127+ ILC1 (dashed line), NKP44+ ILC3 (black line) and NKP44- ILC3 (grey line) were stained for CD103. Numbers in gates (outlined areas) indicate percent cells in each. Data shown is representative of at least 5 experiments, with one to three donors each. Under: quantification of percentage CD103+ cells in each ILC population of total ILC pool.

(D) Flow cytometric analysis of the expression of CD103+ ILC1 and CD127+ ILC1 in control and inflamed (Crohn’s) intestinal mononuclear cells. Gating strategy is as in 1a, and numbers in gates (outlined areas) or quadrants indicate percent cells in each. Top is epithelial fraction and bottom is lamina propria fraction. Shown experiment is representative of 6 experiments.

(E) Flow cytometric analysis of the expression of CD56- CD94+ cNK cells in Crohn’s and control ileum. Bars indicate cells of 5 patients each.

Supplemental figure 2. Flow cytometric analysis of the expression of IL-1R, IL-23R, CD25, CD122, and CD132 on freshly isolated CD127+ ILC1 (black), NKP44+ ILC3 (dashed), NKP44- ILC3 (blue), and isotype (grey, filled). Data shown is representative of 2 experiments.
IL-12 and IL-23 Control Plasticity of CD127+ ILC1 and ILC3

Supplemental figure 3. IL-2, IL-23 and IL-1β induce the upregulation of RORγt. Sorted CD127+ ILC1, CD103+ ILC1, Nkp44+ ILC3, and cNK cells from tonsil were cultured for 4 days with either IL-2 and IL-12 (black line) or with IL-2, IL-23, and IL-1β (dashed line), followed by intracellular staining for RORγt, T-bet, or Eomes. Filled histograms are isotypes for RORγt, T-bet, Eomes. Data shown is representative of 4 independent experiments with 1 to 2 donors each. (d-f) IL-2, IL-23 and IL-1β up-regulate RORγt and IL-2 and IL-12 down-regulate RORγt in intestinal CD127+ ILCs. Highly purified CD127+ ILC1, and Nkp44+ ILC3 from intestinal resection specimen are cultured for 4 days with either IL-2 and IL-12 (black line) or with IL-2, IL-23, and IL-1β (dashed line), followed by intracellular staining for RORγt (d), T-bet (e), or Eomes (f). Data shown is representative of 2 independent experiments, each with one donor.

Supplemental figure 4. Purified ILC3 were cultured in the presence of IL-2 and IL-12 for 7 days. ILC3-derived ILC1 were then sorted out and cultured in the presence of IL-2 IL-23 and IL-1β. Cultures were analyzed for the expression of c-Kit and Nkp44 after 7 days. Gating strategy is as in figure 1a, and numbers in gates (outlined areas) or quadrants indicate percent cells in each. Data shown is representative of 5 experiments, each with one donor, and combined data is quantified in bar graphs on the right.
cells are from 1 donor each. (c) Sorting strategy of Lin^− RORγt-fm^+ NKp46^− NK1.1^+ cells (ex-ILC3) and Lin^− RORγt-fm^− NKp46^− NK1.1^+ (ILC1/NK) from intestines of Rorc(γt)-Cre^+ x Rosa26R-YFP mice. Lamina propria lymphocytes were selected by electronic gating, followed by selecting for lineage- cells, which were either RORγt-fm^+ and RORγt-fm^−. Then cells were selected for NKp46 and NK1.1.

Supplemental figure 6. Freshly isolated CD127^+ ILC1, CD103^+ ILC1, and cNK cells from tonsil were cultured for 4 days either with IL-2 alone or with IL-2, IL-23, IL-1β, and RA. Il-22 production was measured by ELISA. Data shown is representative of at least 3 independent experiments.
Supplemental figure 7. Dendritic cells instruct differentiation of CD127+ ILC1 and ILC3. (a) Gating strategy of the isolation of fetal intestinal DCs. CD3 CD45+ mononuclear cells were gated on HLA-DR and CD11c. Cells were sorted based on CD14 expression. LPS (30 ng/ml) activated CD14+ and CD14- DCs were co-cultured with allogenic tonsil derived ILC1 (gating strategy as in figure 1a). ILCs were analyzed for the expression of c-Kit and NKp44 after 4 days. Numbers in quadrants indicate percentage in each. On the right, bar diagrams indicate percentage ILC3. Data shown is representative of 3 independent experiments. (b) Aldehyde dehydrogenase activity of mDC and RA-mDC with (grey, filled) or without DEAB (black line) as determined by Aldefluor assay after stimulation with LPS. Data shown is representative of 2 experiments. (c) Expression of IL12p35 and IL23p19 of monocyte derived DCs (black) and RA-mDCs (white) after activation with LPS or Poly I:C for 4 hours, presented relative to the expression of ACTB (which encodes β-actin). Data shown is representative of 4 independent experiments, each with 1 to 3 donors.