Human NKp44$^+$IL-22$^+$ cells and LTi-like cells constitute a stable RORC$^+$ lineage distinct from conventional natural killer cells

Natasha K. Crellin,[1] Sara Trifari,[1] Charles D. Kaplan,[1] Tom Cupedo,[2] and Hergen Spits[1]

[1]Department of Immunology, Genentech, South San Francisco, CA 94080
[2]Erasmus University Medical Center, 3000 CA Rotterdam, Netherlands

Lymphoid tissue inducer (LTi) cells are required for lymph node formation during fetal development, and recent evidence implies a role in mucosal immunity in the adult. LTi cells share some phenotypic features of conventional natural killer (NK; cNK) cells; however, little is known to date about the relationship between these two cell types. We show that lineage$^-$ (Lin$^-$) CD127$^+$RORC$^+$ LTi-like cells in human tonsil are precursors to CD56$^+$CD127$^+$RORC$^+$NKp46$^+$ cells, which together comprise a stable RORC$^+$ lineage. We find that LTi-like cells and their CD56$^+$ progeny can be expanded and cloned ex vivo without loss of function and without conversion into cNK cells. Clonal analysis reveals heterogeneity of cytokine production within the CD127$^+$ LTi-like population. Furthermore, we identify within the tonsil a cNK precursor population that is characterized as Lin$^-$CD117$^+$CD161$^+$CD127$^-$ cells. Overall, we propose that CD127$^+$RORC$^+$ cells, although they share some characteristics with cNK cells, represent a functionally and developmentally distinct lineage.

CORRESPONDENCE
H. Spits:
hergen.spits@amc.uva.nl

Abbreviations used: cNK, conventional NK; KIR, killer cell immunoglobulin-like receptor; LTi, lymphoid tissue inducer; mRNA, messenger RNA; MSC, mesenchymal stem cell.

Lymphoid tissue inducer (LTi) cells are required for the formation of lymph nodes during embryogenesis (Mebius, 2003). LTi cells interact with stromal cells and, via production of the cytokines lymphotoxin β and TNF, induce stromal cell production of chemokines and expression of the adhesion molecules ICAM1 and VCAM1 (Mebius, 2003). This, in turn, attracts and retains other lymphocytes, thereby initiating the formation of a lymph node primordium. In the mouse fetus, LTi cells express c-kit (CD117) and lymphotoxin α and β but lack CD3 and other lineage markers, and most of these cells express CD4 although CD4$^-$ LTi cells have also been described (Mebius et al., 1997, 2003; Yoshida et al., 1999). The human counterpart of LTi was identified recently as lineage$^-$ (Lin$^-$; CD3$^-$CD19$^-$CD14$^-$) CD127$^+$RORC$^+$ cells, which are present in fetal lymph node anlagen (Cupedo et al., 2009). Functionally, these cells induce ICAM1 and VCAM1 on mesenchymal stem cells (MSCs) in a TNF- and LT-β–dependent manner, supporting the notion that they represent the human equivalent of mouse LTi cells.

Evidence suggests that LTi cells and conventional NK (cNK) cells are developmentally related cell types. Mouse fetal CD3$^-$CD4$^+$ LTi cells were shown to develop into NK1.1$^+$ cells but not into T and B cells, suggesting that LTi cells are precursors of cNK cells (Mebius et al., 1997). More recently, it was demonstrated that development of both LTi and cNK needs the Helix loop helix protein Id2 (Yokota et al., 1999; Eberl et al., 2004), whereas E12 and/or E47, which are encoded by the E2A locus, inhibit the generation of both LTi cells and cNK cells (Boos et al., 2007). However, differences exist in transcriptional control of development of these cells, as RORγt (RORC in the human), a nuclear hormone receptor critical for development of LTi cells, is not needed for cNK cell development (Satoh-Takayama et al., 2008). Furthermore, there are differences in cytokine requirements of LTi cells and cNK because LTi cell development requires the gamma common receptor but is IL-15 independent

H. Spits' present address is Academic Medical Centre, 1105 AZ Amsterdam, Netherlands

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(Satoh-Takayama et al., 2008), whereas cNK cells need IL-15. Thus the current data suggests that LTi cells and cNK cells are developmentally related yet distinct lineages.

Recently, we identified a novel cell type that expresses RORC and CD127, as well as NK cell–associated markers NKp46 and CD56, but were functionally different from cNK cells in that they lacked expression of granzyme B and perforin, were noncytotoxic, and failed to produce IFN-γ upon activation (Cupedo et al., 2009). Rather, these $CD56^+$ LTi-like cells were able to secrete IL-17 and IL-22 after activation. A cell type that is very similar to the $CD56^+CD127^+$ LTi cells was described by Cella et al. (2009) and was called NK22 because of the expression of NK cell markers NKp44 and CD56 and the production of IL-22 upon activation. Like the $CD56^+$ LTi cells, NK22 cells expressed RORC, were not cytotoxic, and were present in human tonsils, the human intestinal lamina propria, and Peyer's patches (Cella et al., 2009). In the mouse, cells with similar characteristics have been found in the gut. These cells express RORγt like LTi cells, but they also express the NK cell marker NKp46 and are able to secrete IL-22 but not IFN-γ (Satoh-Takayama et al., 2008; Luci et al., 2009; Sanos et al., 2009). An outstanding question is to which lineage $CD56^+CD127^+$ cells belong. One possibility is that these cells belong to the same lineage as LTi cells, whereas another is that $NKp46^+CD56^+CD127^+$ and cNK cells can interconvert. The latter possibility was suggested by the finding that human LTi cells are very similar to immature stage 3 NK cells, which were described to be precursors of cNK cells (Freud et al., 2006). We show in this paper that $Lin^-CD127^+RORC^+$ LTi-like cells, although sharing some characteristics with cNK cells, represent a functionally and developmentally distinct lineage.

RESULTS AND DISCUSSION

Human $CD127^+$ LTi-like cells are precursors of $CD56^+CD127^+$ cells, and have similar phenotype to NK22 cells

We have previously demonstrated that fetal LTi cells are capable of interacting with MSCs and inducing the expression of the adhesion molecules ICAM1 and VCAM1. We also found cells with a similar phenotype to that of the $CD127^+$ fetal lymph node cells in postnatal tonsil, but we did not test the LTi activity of those cells in that study (Cupedo et al., 2009). Fig. 1 A shows that both Lin^- ($CD14^-CD19^-CD3^-$TCR-$\alpha\beta^-$TCR-$\gamma\delta^-$) $CD127^+CD56^+$ and $Lin^-CD56^-CD127^+$ LTi-like cells (referred to as $CD127^+$ LTi-like cells), isolated from human tonsil, induced ICAM1 and VCAM1 on MSCs, whereas cNK cells had some ICAM-inducing capacity which was much lower than of $CD127^+$ LTi-like cells and did not induce VCAM1 expression. The observation that tonsil $CD56^+CD127^+$ cells have functional LTi activity underlines the functional differences between these cells and cNK cells.

It has been suggested that expression of NKp44 identifies a population of $RORC^+$IL-22–producing NK cells (Cella et al., 2009). Fig. 1 B shows that $Lin^-CD56^+CD127^+$ and $CD127^+$ LTi-like cells in human tonsils expressed high levels of NKp44 and were positive for CD161, whereas cNK cells expressed NKp44 on only a small subset in the tonsil and were negative for CD161. The $CD127^+$ populations expressed CD117, which was not present on cNK cells, but were negative for CD34 (unpublished data). Furthermore, cNK cells expressed higher levels of NKp46 and NKG2D than the $CD127^+$ populations. Tonsil LTi cells, but not cNK, also expressed CD25, the IL-2 receptor α chain, whereas the IL-15Rα chain on tonsil $CD127^+$ LTi-like cells was expressed at levels equivalent to those on cNK cells (unpublished data). Our data show that the phenotype of $CD56^+CD127^+$ cells is very similar to that of NK22 cells, suggesting that these represent two highly overlapping if not identical cell types.

Recently, we found that fetal $CD56^-$ LTi-like cells developed into $CD56^+CD127^+$ cells after 2 wk of culture with IL-15. Although these data suggested a precursor progeny relationship of LTi and $CD56^+CD127^+$ cells, we could not exclude the possibility that the $CD56^+$ cells were derived from a small number of highly proliferating $CD56^+$ cells contaminating the starting $CD56^-CD127^+$ population. To test whether tonsil $CD56^-$ LTi cells also develop into $CD56^+$ cells and to exclude the possibility that the appearance of $CD56^+$ cells in cultures with IL-15 is the result of outgrowth of contaminating cells, we labeled highly purified tonsil $CD56^-CD127^+$ cells with CFSE and stimulated with IL-2, IL-7, or IL-15. These cytokines were all capable of maintaining LTi viability and proliferation ex vivo, although less proliferation was observed when stimulated with IL-7 alone. In the absence of these cytokines the cells did not survive. After 6 d of culture, the great majority of the LTi cells up-regulated CD56 (Fig. 1 C). Most importantly, CD56 was also up-regulated on CSFE-bright nondividing cells, which proves that the appearance of $CD56^+$ cells is not the result of outgrowth. Additional kinetics experiments showed that this up-regulation is underway by day 4 of culture (unpublished data). We cannot completely exclude the fact that cells that did not up-regulate CD56 were more prone to cell death and that, therefore, these cells were underrepresented in our cultures. However, the observations that the cell viability was high (10–15% dead cells) in all conditions and that the cell numbers remained the same or increased during the cultures argue against selective death of $CD56^-$ cells. Our data indicate that $CD56^+$ cells did not arise from a small contaminating population of rapidly dividing $CD56^+$ cells and, thus, this data supports a direct developmental relationship between $CD127^+$ LTi-like cells and $CD56^+CD127^+$ cells.

Cultured $CD56^+CD127^+$ and $CD127^+$LTi-like cells maintain high RORC expression

Immature stage 3 NK cells have been shown to develop into cNK cells (Freud et al., 2006). Because considerable phenotypic similarities exist between LTi-like cells and stage 3 iNK cells, we examined whether $CD127^+$ LTi-like cells can convert into cNK cells. Using a rigorous sorting strategy, we isolated $CD56^+CD117^-$ cNK cells, $Lin^-CD56^+CD117^+CD127^+$ cells, and $Lin^-CD56^-CD117^+CD127^+$ LTi-like cells from human tonsils. The use of CD117, in conjunction with CD127 as selecting markers, combined with the rigorous exclusion of

Lin^+ and/or TCR^+ cells, was crucial for preventing contamination with T cells. These sorted populations were expanded ex vivo using a feeder mixture consisting of irradiated allogeneic PBMCs, JY EBV-transformed lymphoblastoid cells, PHA, and IL-2, which was previously used to expand cNK cells (Phillips et al., 1991). Under these conditions, $CD127^+$ LTi-like cells expanded 30–200-fold, and after resting they could be successfully restimulated and further expanded. Importantly, expanded LTi-like cell lines maintained expression of RORC messenger RNA (mRNA), as did $CD56^+CD127^+$ cells, whereas $CD56^+$ cNK cells remained negative (Fig. 2 A). At the end of the first feeder cycle, LTi-like cell lines were ~30–60% $CD56^+$ (Fig. 2 B). After subsequent feeder cycles, CD56 expression increased until ~80–100% of the cells were $CD56^+$. After expansion, all populations expressed similar levels of NKp46. CD127 was no longer detectable by flow cytometry; however, LTi-like cell lines continued to express CD127 message (Fig. S1) and to respond to IL-7 (unpublished data).

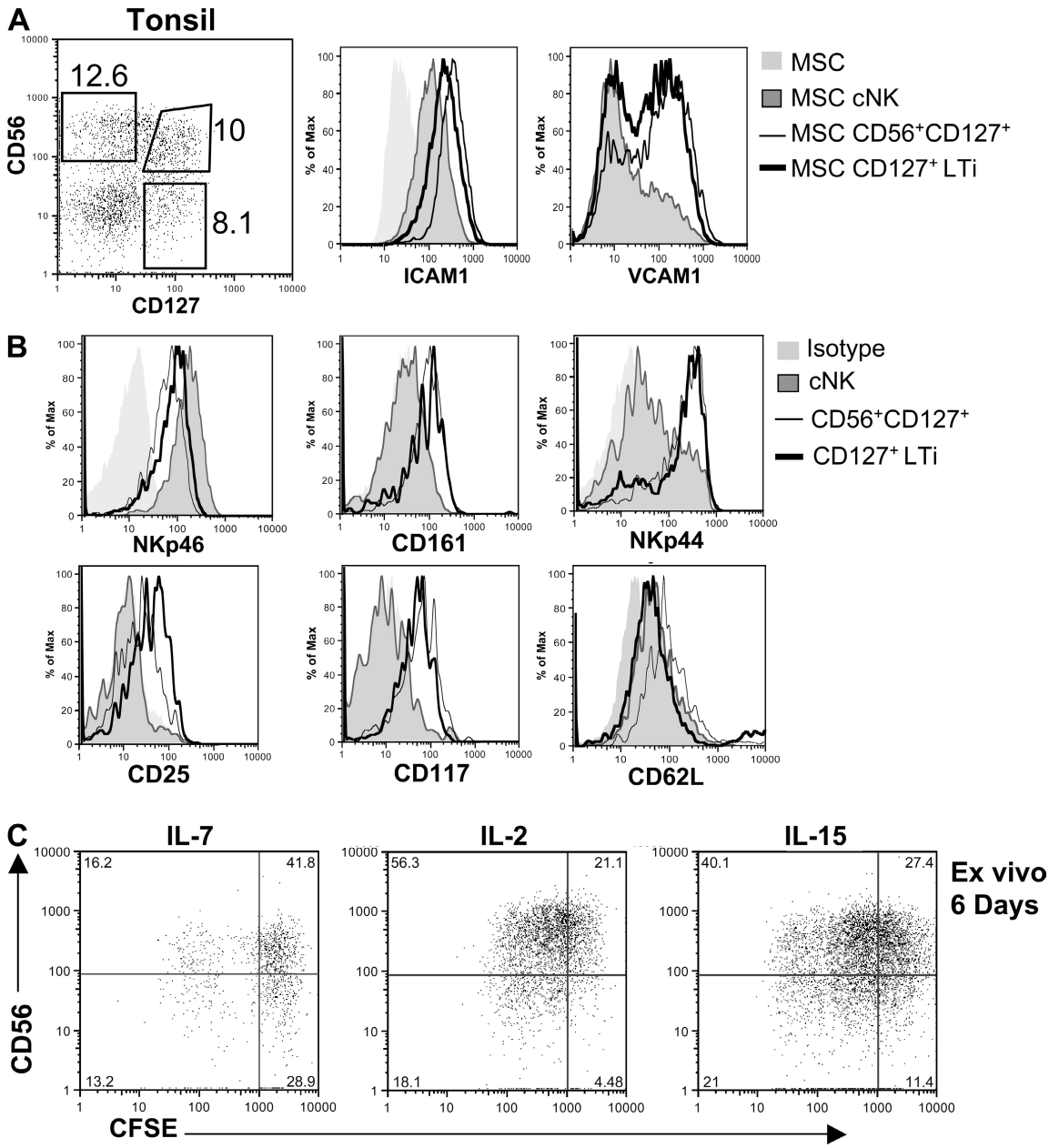

Figure 1. Human $CD127^+$ LTi-like cells are precursors of $CD56^+CD127^+$ cells and have similar phenotype to NK22 cells. (A) Lin^-, $CD56^+$ cNK, $CD56^+CD127^+$, or $CD127^+$ LTi-like cells, were sorted from tonsil and were co-cultured with human MSCs for 4 d with 10 ng/ml IL-7. Expression of ICAM1 and VCAM1 on MSCs was quantified by flow cytometry. (B) Expression of the indicated molecules was quantified using flow cytometry on Lin^- tonsil cells. (C) Flow cytometry–sorted $CD127^+$ LTi cells were labeled with CFSE and stimulated with IL-7, IL-2, or IL-15 for 6 d, followed by flow cytometry to assess CD56 expression. One donor out of at least two analyzed is shown in A, B, and C.

The down-regulation of CD127 is not unexpected, as it is well known that T cells lose CD127 expression after activation and expansion (Xue et al., 2002). Expanded cNK cells expressed CD56, but in contrast to cell lines derived from $CD127^+CD56^-$ and $CD127^+CD56^+$ cells, they lacked CD117.

To exclude potential uncertainties regarding the use of bulk populations, we performed a clonal analysis of $CD127^+$ LTi-like cells from two donors. $Lin^-CD117^+CD127^+$ LTi-like cells were purified and cloned by limiting dilution at one and three cells per well of a 96-well round-bottomed plate. Approximately 8–10% of the wells seeded with one cell per well showed robust expansion and 10–20 clones from each donor were characterized further. All clones expressed high amounts of transcripts of RORC and AHR (Fig. 2 C), a transcription factor which is associated with Th17 cells and IL-22 production (Trifari et al., 2009), and a positive correlation

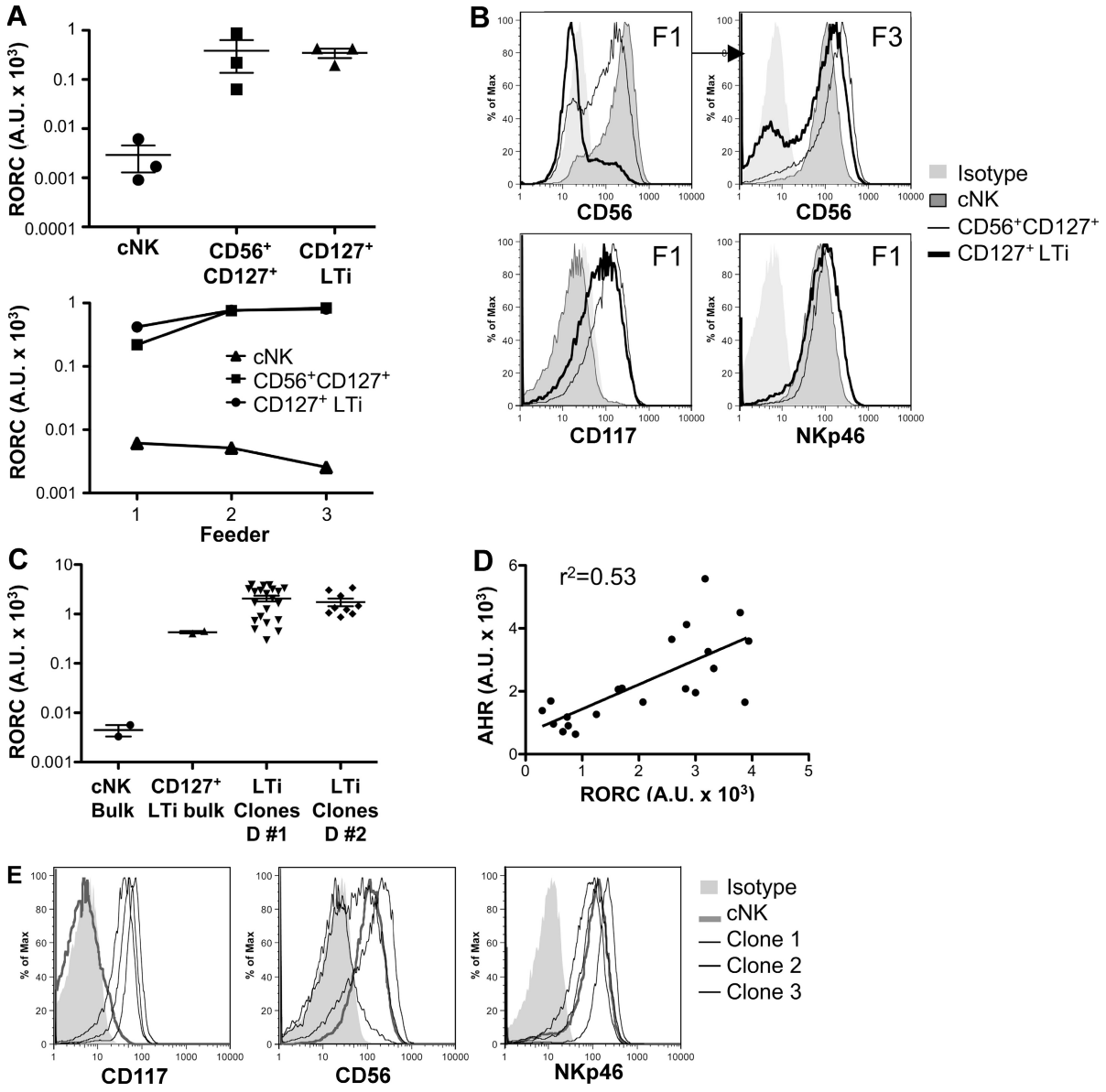

Figure 2. Cultured $CD56^+CD127^+$ and $CD127^+$ LTi-like cells maintain high RORC expression. Flow cytometry–sorted tonsil Lin^- cNK, $CD117^+CD56^+CD127^+$, and $CD117^+CD127^+$ LTi-like cells were expanded ex vivo using a feeder cell mixture. (A) After expansion, RORC mRNA normalized to 18S expression. The top represents the first expansion (F1), whereas the bottom follows RORC expression in a single donor over three rounds of expansion. (B) Flow cytometry of the indicated molecules on expanded cell lines after one (F1) or three (F3) rounds of expansion. (C) Single cell clones of $CD117^+CD127^+$ LTi-like cells were generated, and RORC mRNA of clones from two donors is shown, normalized to 18S expression. Bulk cell lines from the same donors are shown in parallel. (D) Correlation between AHR and RORC mRNA from LTi-like cell clones. (E) Flow cytometry of the indicated molecules on LTi-like cell clones. Each dot in A, C, and D represents a single clone or bulk cell line. In B, a single donor representative of five is shown. In E, three representative clones are shown. The mean (horizontal bars) and SEM (error bars) are shown in A and C.

existed between RORC and AHR expression levels (Fig. 2 D). Furthermore, all clones maintained expression of CD117 and NKp46 and most expressed CD56, which is consistent with observations of expanded CD127$^+$ LTi-like cell lines (Fig. 2 E). In contrast, parallel cNK cell lines were negative for CD117 and did not express RORC.

LTi cell lines and clones maintained their ability to interact with MSCs and induce the expression of adhesion molecules, whereas cNK cell lines failed to mediate this activity (Fig. S2). Up-regulation of adhesion molecules could be mimicked by addition of exogenous TNF and lymphotoxin, and blocking TNF signaling through addition of TNFRII-Ig inhibited the up-regulation of adhesion molecules on MSCs induced by CD127$^+$ LTi-like cell lines (Fig. S2). Collectively, our data demonstrate that LTi-like cells and their CD56$^+$ descendants can be expanded ex vivo and retain their phenotypic and functional characteristics. Most importantly, neither the lines nor the clones convert into cNK cells in vitro.

Heterogeneous cytokine production from expanded and clonal CD127$^+$ LTi-like cells

In vitro expanded LTi-like cells have a similar cytokine profile after stimulation as ex vivo cells (Fig. 3 A). Stimulation with PMA/ionomycin was used to reveal the intrinsic capacity of cells to produce cytokines. IL-22 was produced in much higher amounts than IL-17, suggesting that the preferential production

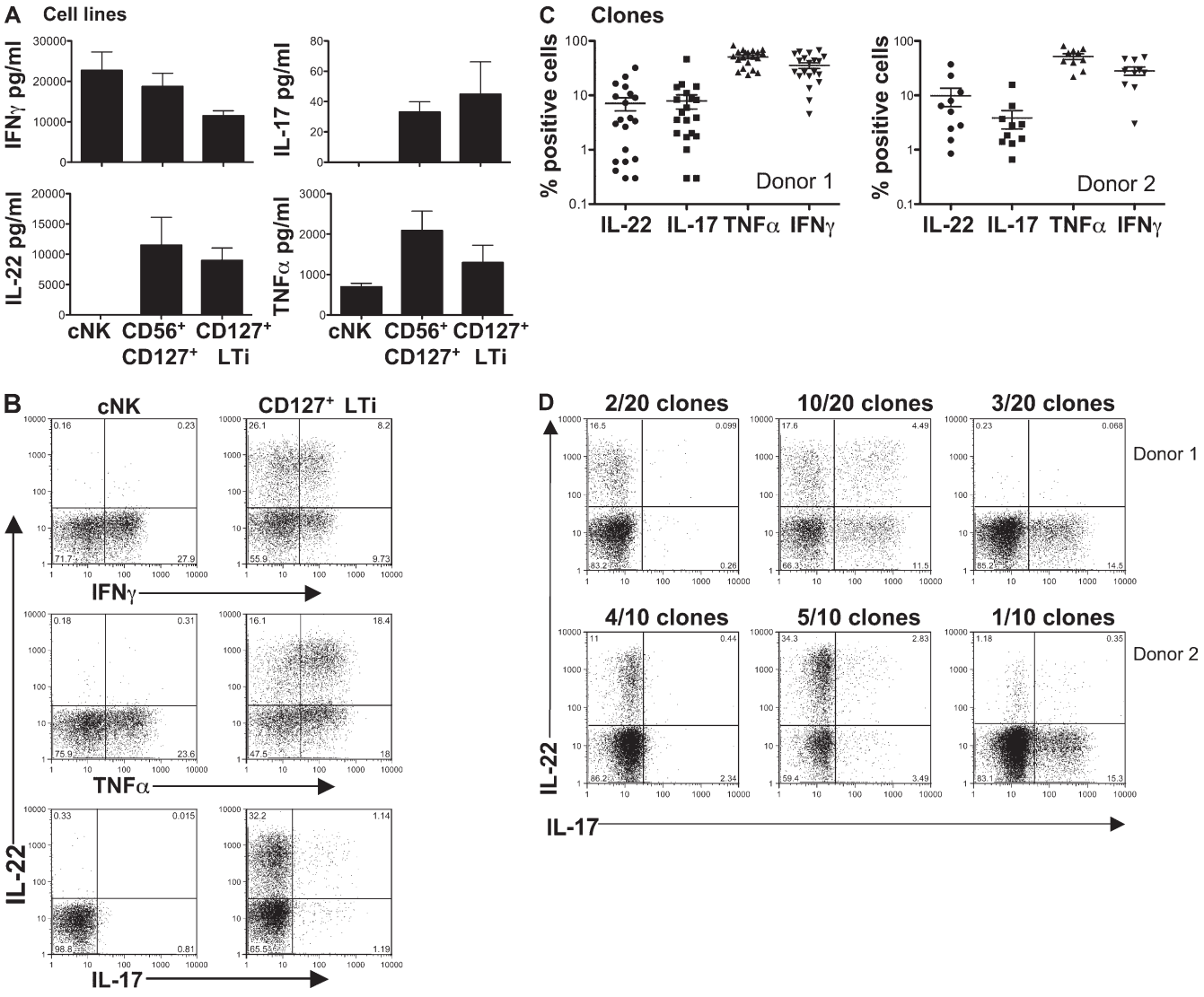

Figure 3. Heterogeneous cytokine production from expanded and clonal CD127$^+$ LTi-like cells. (A) Cell lines were stimulated with PMA and ionomycin for 24 h, supernatants were collected, and cytokine production was analyzed by Luminex assays and ELISA. (B) Intracellular cytokine analysis of expanded cNK and CD127$^+$LTi-like cell lines, stimulated for 6 h with PMA and ionomycin. (C and D) Intracellular cytokine analysis of LTi cell clones, stimulated for 6 h with PMA and ionomycin. In A, the mean value of three donors is plotted. B shows a single donor representative of at least three donors. In C, each dot represents a separate clone, with two donors shown. In D, three representative clones are shown from each donor. Error bars in A and C show SEM. Horizontal bars in C show means.

of IL-22 that was noted in freshly isolated cells was maintained through in vitro activation and expansion. After expansion, LTi-like cell lines produced amounts of IL-22 that were similar to those of the CD56$^+$CD127$^+$ cell lines upon activation, which is in contrast to the lower amounts produced by freshly isolated CD127$^+$ LTi-like cells (Fig. 3 A; Cupedo et al., 2009). However, in contrast to fresh LTi cells, both CD56$^+$CD127$^+$ and LTi-like cell lines produced IFN-γ after expansion (Fig. 3 A). The IFN-γ–producing cells within the LTi cell lines may represent differentiated cNK cells that had lost the capacity to produce IL-22. However, the observation that activated IFN-γ–producing LTi cell lines were also positive for IL-22, TNF, and IL-17 (Fig. 3 B) argues against this possibility.

The cytokine production profile of LTi-like cell clones was similar to that of LTi cell lines (Fig. 3 C). Like the LTi lines, the clones acquired the capacity to produce IFN-γ. There was no inverse correlation between IFN-γ production and RORC mRNA in the clones (unpublished data), indicating that IFN-γ production was not associated with a decrease of expression of RORC. Clones derived from donor 1 had higher IL-17 production after stimulation than did clones derived from donor 2. Although most clones produced both IL-17 and IL-22 after stimulation, some clones produced IL-22 but not IL-17, whereas other clones produced IL-17 but not IL-22 (Fig. 3 D). These data indicate that some heterogeneity, with respect to IL-22 and IL-17 production, exists within RORC$^+$ LTi cell clones. There was no correlation between CD56 expression and cytokine production profile. Over time, clones largely maintained their phenotype, although with in vitro culture an increase in IL-17 production was sometimes observed (Fig. S3).

It has been suggested that the capacity to produce IL-17 distinguishes LTi cells from NK22 cells (Colonna, 2009). However, our analysis suggests the presence of cells that can produce both IL-17 and IL-22, and even IL-17 only, within the CD127$^+$RORC$^+$ population which are also NKp44$^+$ ex vivo.

After expansion, LTi-like cell lines have low cytotoxic activity

We, and others, have documented that freshly isolated LTi-like cells from the tonsil, like their fetal counterparts, are negative for granzyme and perforin (Cella et al., 2009; Cupedo et al., 2009; Sanos et al., 2009). Because CD56$^+$ LTi-like cell lines were capable of producing IFN-γ (Fig. 3), we assessed whether they acquired cytotoxic activity. After expansion, LTi-like cell lines and clones expressed perforin, but at much lower levels compared with cNK cell lines, and did not acquire expression of killer cell immunoglobulin-like receptors (KIRs; Fig. 4, A and B; and not depicted). In contrast to LTi-like cell clones, LTi-like cell lines did express some granzyme B but at much lower than cNK cell lines (Fig. 4, A and B). In a cytotoxic assay with K562 cells as targets, cultured LTi-like cells achieved 50% lysis at an effector/target ratio of 19:1, which is ∼13-fold higher than that required for cNK cell lines (Fig. 4 C), suggesting that in vitro–cultured LTi-like cells acquired a moderate

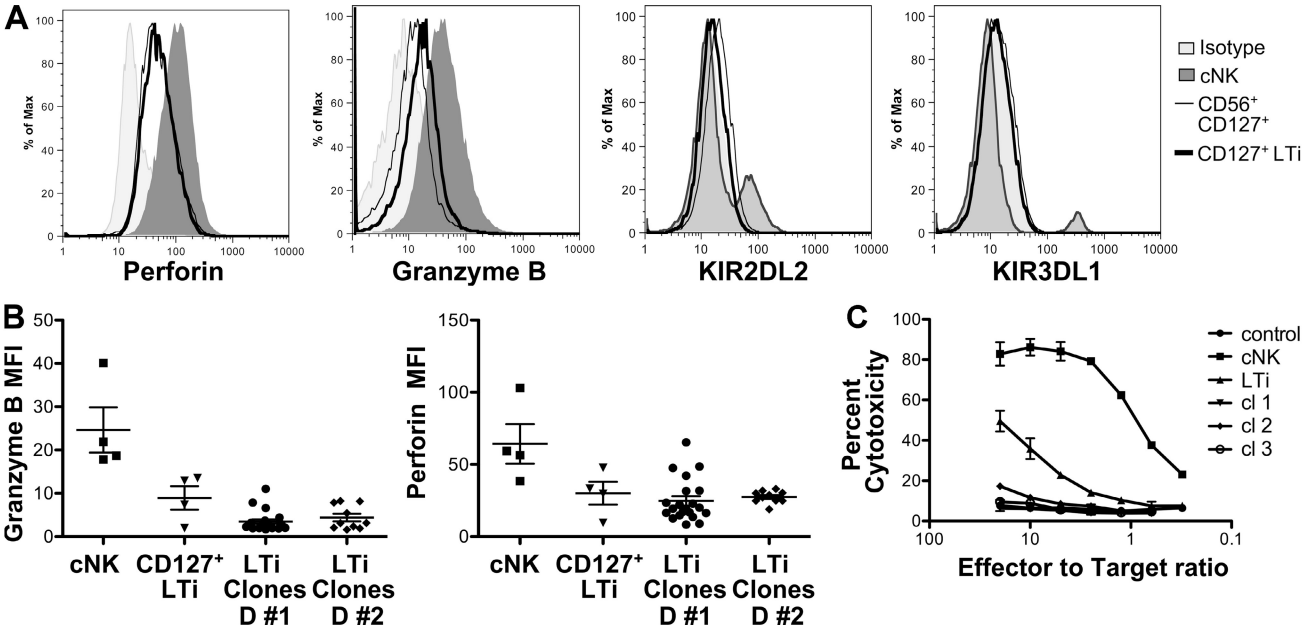

Figure 4. After ex vivo expansion, LTi cell lines have low cytotoxic activity. (A) Flow cytometry was performed for intracellular perforin, granzyme B, and cell surface KIR expression on cNK cell lines and CD127$^+$ LTi-like cell lines expanded ex vivo. (B) Geometric mean fluorescence intensity of intracellular granzyme B or perforin staining is shown for cNK or CD127$^+$ LTi-like cell lines and LTi-like clones after ex vivo expansion. (C) K562 cells were labeled with a fluorescent dye and co-cultured with cNK or CD127$^+$ LTi-like cell lines or LTi-like clones at the indicated ratios for 5 h, and cytotoxicity was determined by propidium iodide staining. Unlabeled K562 cells were added at the indicated ratios to assess nonspecific cell death (control). One of four donors analyzed is shown in A. Each dot represents a separate donor or clone in B. The mean of cell lines from two donors and three clones from a single donor are shown in C. Horizontal bars in B show the means. Error bars in B and C show SEM.

cytolytic activity. However, this activity was much lower than that of cNK cells. The clones derived from LTi-like cells did not display any cytotoxicity in this assay. Collectively, our data support the notion that LTi and their $CD127^+CD56^+$ progeny represent a lineage whose developmental pathway is distinct from cNK cells.

$CD117^+CD161^+CD127^-$ iNK cells contain cNK precursors

It has been reported that circulating $CD34^+CD45RA^+$ cells that are also resident in the lymph node can differentiate into NK cells (Freud et al., 2005). Close inspection of those in vitro–generated NK cells reveals some similarities with LTi-like cells, in that they expressed CD117 and CD161, and part of the cells expressed CD25 (Freud et al., 2005, 2006), raising the possibility that some of those in vitro–generated "NK" cells are in fact LTi-like cells. Indeed, recently the same group reported that the stage 3 iNK cells, which were hypothesized to be downstream of $CD34^{dim}CD45RA^+$ precursor cells, produced IL-22, suggesting that at least part of these iNK are identical to LTi-like cells (Hughes et al., 2009). Given that $CD127^+CD117^+$ cells cannot differentiate into cNK, the cNK precursor within the $CD161^+CD117^+$ iNK population described by Freud et al. (2006) should be negative for CD127. To test this hypothesis, we purified CD127 positive and negative cells from the

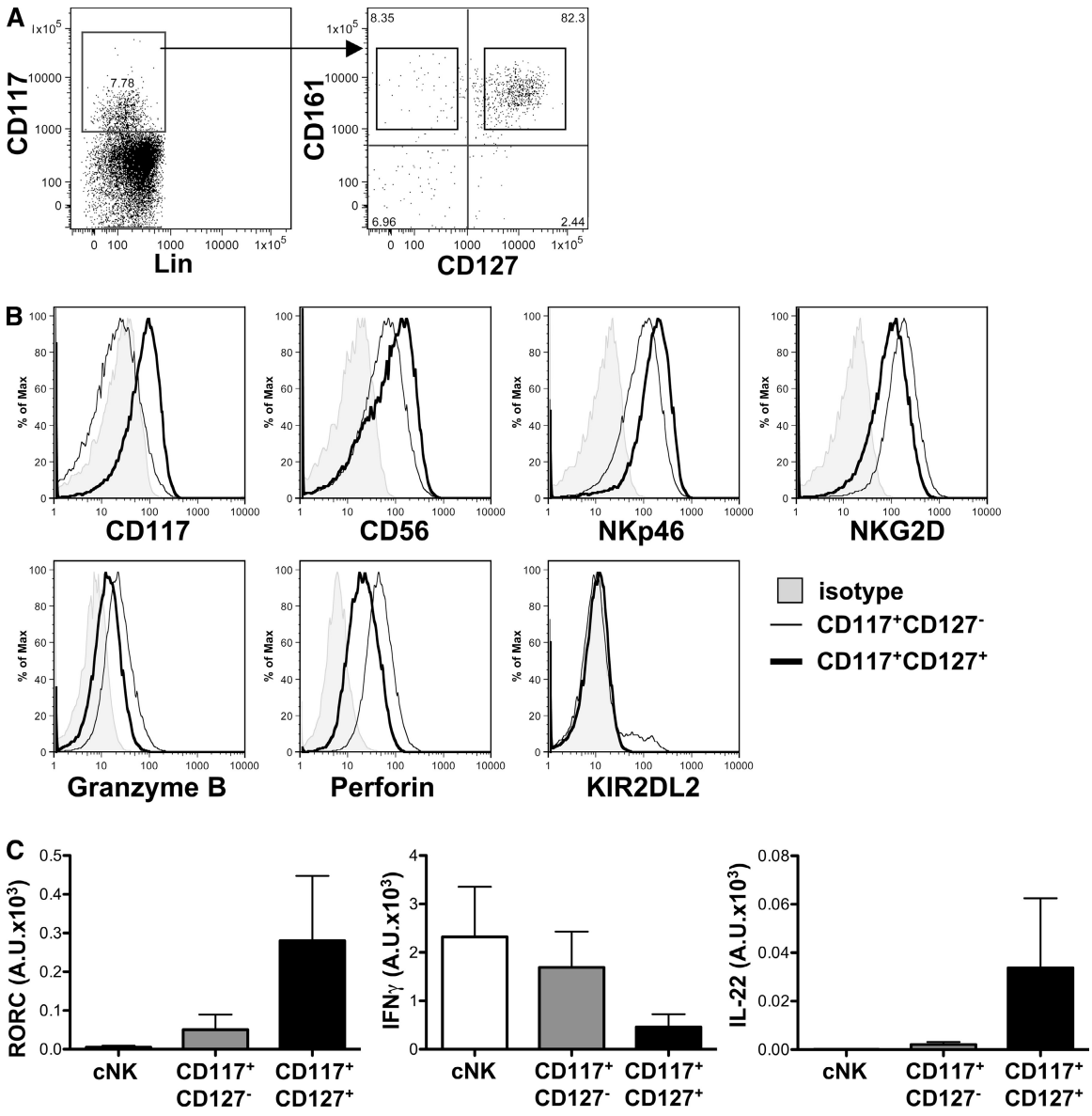

Figure 5. $CD117^+CD161^+CD127^-$ iNK cells contain cNK precursors. (A) $Lin^-CD117^+CD161^+CD127^-$ and $CD117^+CD161^+CD127^+$ cells were sorted from human tonsils. (B) Isolated populations were expanded ex vivo, and expression of indicated molecules was assessed by flow cytometry. (C) RORC, IL-22, and IFN-γ mRNA after ex vivo expansion, normalized to 18S expression. A single donor representative of two is shown in A and B, and C represents the mean and SEM of at least two donors.

$Lin^-CD161^+CD117^+$ population and expanded them in vitro (Fig. 5 A). Fig. 5 B shows that, as expected, $CD127^+CD117^+$ cells maintain the expression of CD117 and had low levels of perforin and granzyme B. In contrast, the expanded $CD127^-CD117^+$ cells lost CD117, were positive for granzyme B and perforin, and had some KIR expression (Fig. 5 B). Both populations expressed NKp46, NKG2D, and CD56. Consistent with the cell surface phenotype, RORC and IL-22 mRNAs were highly expressed in expanded $CD117^+CD127^+$ cells, expressed at low levels in $CD117^+CD127^-$ cells, and not expressed in cNK cell lines (Fig. 5 C). In contrast, IFN-γ mRNA was expressed in all populations but most highly expressed in expanded cNK cells, whereas $CD117^+CD127^-$ cells had an intermediate expression (Fig. 5 C). These data indicate that $CD127^-$ cNK precursors are found within the iNK cell population. Our data strongly suggest that iNK cells, as defined by Freud et al. (2006) and Hughes et al. (2009), may contain a mixture of LTi cells and immature cNK cells and do not necessarily contain common NK/LTi cell precursors. If such a precursor exists, it might be present in the $CD34^+CD45RA^{\pm}$ population (Freud et al., 2005, 2006).

The conclusion that cNK and $RORC^+$ $CD127^+$ LTi-like cells belong to different lineages is consistent with an accompanying paper (see Satoh-Takayama et al. in this issue). These researchers found that development of $NKp46^+RORC^+$ IL-22–producing cells, like that of LTi cells, requires IL-7 and is IL-15 independent. By using cell fate mapping, these researchers also observed that cNK cells never express RORC during their development. Because it is very likely that mouse $NKp46^+RORC^+$ cells, which also express CD127, are the mouse homologue of the human $CD56^+CD127^+RORC^+$ cells described in this paper, the data of this group are in agreement with our conclusion that human $CD127^+RORC^+$ cells are unable to differentiate into cNK cells. Nonetheless, $CD127^+$ LTi-like cells and their $CD56^+$ descendants seem to be related to cNK cells because apart from the fact that these cell types share expression of certain markers, development of both lineages in the mouse can be blocked by E2A proteins (Boos et al., 2007) and consequently requires the E protein inhibiting factor Id2. These two lineages may serve comparable functions in the immune response. It is well established that cNK cells are necessary for the initial defense against viruses (Lee et al., 2007). It is possible that LTi-like cells and their $RORC^+$ progeny are involved in the innate immune response against extracellular bacteria, much like the IL-22 and IL-17–producing T cells, which also express RORC and are instrumental in the adaptive immune response against bacteria (Aujla et al., 2008; Ishigame et al., 2009; Martin et al., 2009). Indeed, we have observed that LTi cells express toll-like receptors and are capable of responding to TLR stimulation (unpublished data). Non–T cell–derived IL-22 is required for protection from *Citrobacter Rodentium* infection in the intestine (Zheng et al., 2008), and IL-22–producing $RORC^+$ cells contribute to this response (Satoh-Takayama et al., 2008; Cella et al., 2009). The $RORC^+$ innate lymphocytes may constitute a heterogeneous group of cells, as indicated by our clonal analysis. In the intestinal lamina propria only IL-22–producing cells may be present, but is very well possible that similar cells producing IL-17 rather than IL-22 are prevalent in other mucosal sites such as the lung. We have also found that LTi cells and $CD56^+RORC^+$ cells are able to produce IL-5 and IL-13 (unpublished data) and $RORC^+$ cells might exist in certain tissues that are dedicated to producing these cytokines rather than IL-17 and/or IL-22. Such cells may function in the innate immune response against parasites.

MATERIALS AND METHODS

Cell purification and sorting. Human tonsils were digested for 30 min in 0.5 mg/ml collagenase IV (Invitrogen) at 37°C and processed to form a single cell suspension. $CD3^+$ and $CD19^+$ cells were depleted using kits (STEMCELL Technologies Inc.). For flow cytometry sorting, cells were labeled with PE-conjugated anti-CD127 (BD), PE-Cy7–conjugated anti-CD56 (BD), APC-conjugated anti-CD117 (eBioscience), Pacific blue–conjugated anti-CD19 (eBioscience), and FITC-conjugated anti-CD3, TCR-αβ, TCR-γδ, and CD14 (eBioscience) antibodies. In some conditions, PE-Cy5–conjugated anti-CD161 (BD) was also used. Cells were sorted on a FACSAria (BD) to >95% purity. Tonsils were obtained from Bio Options, and blood samples were obtained from the Genentech blood donors program after written informed consent. Ethical approval for the use of this material was obtained from the Western Institutional Review Board.

MSC assay. Human MSCs (Thermo Fisher Scientific) were cultured according to the supplier's instructions. MSCs were plated in a 48-well plate at a density of 5×10^4 cells per well. LTi-like or cNK cells were added at a 1:1 ratio and co-cultured for 4 d in the presence of 10 ng/ml IL-7. ICAM1 and VCAM1 expression on MSC was determined by flow cytometry. Where indicated, 10 ng/ml TNF or 100 ng/ml $LT\alpha1\beta2$ were added at the start of culture. 100 ng/ml TNFRII-Ig was used to block TNF signaling and was added on days 0 and 2.

Expansion of LTi cell lines and LTi-like cell clones. Flow cytometry–sorted cells were stimulated with irradiated allogeneic PBMCs (5,000 rad) and irradiated JY EBV-transformed B cells (7,500 rad) in the presence of 1 μg/ml PHA (Roche) and 100 U/ml IL-2 (Novartis). IL-2 was replenished every 3 d. LTi-like cell clones were generated using limited cell dilution and expanded using the same method.

Cell culture. Cells were cultured in Yssel's medium (Gemini Bio-Products; or in house prepared) plus 1% human AB serum (Gemini Bio-Products). Recombinant human IL-7 and IL-15 were used at 10 ng/ml (R&D Systems). For cytokine production, 5×10^5 cells per well were plated in a 200-μl volume in 96-well round-bottom plates.

Cytotoxic activity assay. K562 cells were labeled with a fluorescent dye using the Live/Dead cell–mediated cytotoxicity kit (Invitrogen) and co-cultured with effector cell lines at the ratios indicated for 5 h. Cytotoxicity was determined by propidium iodide staining using flow cytometry.

Quantitative real-time PCR. Total RNA was extracted using either the RNeasy mini or micro kit (QIAGEN) according to the manufacturer's instructions. Complementary DNA was reverse transcribed using the high-capacity complementary DNA archive kit (Applied Biosystems). Primers and probes were all predesigned Taqman Gene Expression Assays from Applied Biosystems. Real-time PCR was performed on the 7500 Real Time System (Applied Biosystems) machine. The following assays were used: RORC (Hs01076112), AHR (Hs00169233), 18S (Hs99999901), and IL-22 (Hs00220924). All samples were normalized using 18S expression level and expressed in arbitrary units.

Flow cytometry and intracellular cytokine staining. Cell surface staining was performed using Alexa Fluor 647–conjugated anti-NKp46 or

-NKp44, Pe-Cy7–conjugated anti-CD25, and FITC-conjugated anti-CD62L (all obtained from BD). KIRs were detected using FITC-conjugated anti-KIR2DL2 or -KIR3DL1 (BioLegend). Cells were fixed and permeabilized as described later in this section and stained with PE-conjugated anti–granzyme B and FITC-conjugated anti-perforin (eBioscience). Intracellular staining was performed on ex vivo expanded cell lines and clones stimulated for 6 h with 10 ng/ml PMA and 500 nM ionomycin (EMD) in the presence of 5 μg/ml BFA (BD) for the final 4 h of culture. Cells were stained with LIVE/DEAD (violet) viability assay (Invitrogen) and then fixed in 3% paraformaldehyde in PBS. Cell permeabilization, staining, and subsequent washings were performed using the Fix/perm wash buffer (eBioscience). The following antibodies were used (BD): PECY7-conjugated anti–IFN-γ, and PE-Cy7–conjugated anti-TNF. PE-conjugated anti–IL-17A (eBioscience) and Alexa Fluor 647–conjugated anti–IL-22 (prepared in house) were also used. Data were acquired on an LSRII (BD) and analyzed with FlowJo software (Tree Star, Inc.). Monoclonal mouse anti–human IL-22 antibodies were generated in house by immunizing mice with human IL-22Fc fusion protein. Clone 3F11 was chosen based on its ability to detect intracellular human IL-22 (Trifari et al., 2009). The antibody was then conjugated with Alexa Fluor 647 fluorochrome using a Labeling kit (Invitrogen) according to the manufacturer's instructions. During analysis, dead cells were eliminated using the live/dead violet dye and doublets were eliminated based on light scattering parameters.

Luminex and ELISA. Supernatants were collected, and IL-17A, IFN-γ, and TNF were measured by Bioplex bead-based assays (Bio-Rad Laboratories) and read by a Luminex System (Luminex). IL-22 was measured by ELISA (R&D Systems) according to the manufacturer's protocol.

Online supplemental material. Fig. S1 shows CD127 mRNA expression on expanded cell lines. Fig. S2 shows up-regulation of adhesion molecules on MSCs induced by expanded LTi-like cell lines and clones. Fig. S3 follows cytokine production of individual clones over successive expansions in vitro. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091509/DC1.

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The authors declare that potential financial conflicts of interest may exist in that N.K. Crellin, S. Trifari, C.D. Kaplan, and H. Spits are or were employees of Genentech, a company that develops and markets drugs. The authors have no additional financial interests.

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