Identification of diverse innate lymphoid cells in human decidua

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Innate lymphoid cells (ILCs) are developmentally related cells that play an important role in innate defenses and tissue remodeling. So far, only natural killer (NK) cells have been identified and functionally characterized in human decidua where they contribute to induction of immune suppression, neo-angiogenesis, and tissue building/remodeling. The presence of other ILC subsets in human decidua has not been yet characterized. Here we identify in human decidua, during early pregnancy, two subsets of decidual group 3 ILC (ILC3), including lymphoid tissue inducer (LTi)-like cells and natural cytotoxicity receptors (NCRs)[+] ILC3 and interferon-(IFN)γ-producing ILC1, different from NK cells. Decidual LTi-like cells produced interleukin -17 (IL-17) and tumor necrosis factor (TNF), while NCR[+] ILC3 released IL-22 and IL-8. Importantly, NCR[+] ILC3 and LTi-like cells established functional interactions with stromal cells. Decidual LTi-like cells differentiated into NCR[+] ILC3, whereas they marginally contributed to NK cell generation. Our data suggest that decidual ILC3 may play a role in innate defenses and in vessel and tissue building, thus contributing to maintenance of pregnancy.

INTRODUCTION

Innate lymphoid cells (ILCs) are an extended family of developmentally related hematopoietic cells, which contribute to host immune defenses. Differentiation of ILC depends on the expression of the transcriptional repressor inhibitor of DNA binding 2 (ID2) and on the common cytokine receptor γ-chain. This shared requirement implies the existence of a common ILC progenitor. On the basis of their functional and developmental characteristics, three main groups of ILC have been identified: ILC1, ILC2, and ILC3.[1,2] ILC1 include natural killer (NK) cells and other interferon (IFN)γ-producing cells. NK cells play a fundamental role in host defenses, thanks to their ability to kill virus-infected and tumor cells. In addition, they rapidly release cytokines and chemokines involved in early inflammatory responses.[3,4] Human NK cell function is regulated by an array of inhibitory receptors, including killer Ig-like receptors (KIRs) and CD94/NKG2A, and activating receptors including NKp46, NKp30, and NKp44 (collectively known as natural cytotoxicity receptors (NCRs)), NKG2D,

DNAM-1, and CD16.[5–9] During NK cell differentiation, other transcription factors (TFs) besides ID2 are required.[10–14] While the nuclear factor interleukin (IL)-3-regulated (also known as E4BP4) TF is involved in early commitment to the NK cell lineage, the T-box expressed in T cells (Tbet/Tbx21) and Eomesodermin (Eomes) TFs are crucial for the acquisition of NK functional properties.[15,16] Recently, other IFNγ-producing ILC1, different from NK cells, have been identified including non-cytotoxic CD127^{+} CD117^{−} CD56^{−} CD94^{−} NCR^{−} Tbet^{+} IFNγ^{+} cells [17] and intraepithelial CD127^{−} CD56^{+} NKp44^{+} CD103^{+} ILC1.[18] ILC2 require GATA-binding protein 3 and RAR-related orphan receptor α TFs for their development. They are characterized by CD127, CD161, and chemoattractant receptor-homologous molecule expressed on T helper 2 cells (CRTH2) expression, and produce T helper 2-associated cytokines.[19] Finally, group 3 ILC includes different cell types, defined by the requirement of RORγt TF for their development and function and by their ability to produce IL-17 and/or IL-22.[2,20] Lymphoid tissue inducer (LTi) cells, the first

described ILC3, play a key role in the formation of secondary lymphoid organs (SLO) during fetus development.[20] Notably, cells with similar phenotypic characteristics were identified also in adult SLO and were named LTi-like cells. In humans, LTi/LTi-like cells express CD127 and CD117.[2,21] A third cell population belonging to group 3 ILC, termed NK22 or NCR$^+$ ILC3, has been recently identified in mucosal tissues. The NCR$^+$ ILC3 population shares common features with NK cells and ILC3, as they express NCRs (NKp44^{high}NKp46^{low}), RORγt TF, and produce IL-22.[22–25]

Regarding the possible role of ILC during pregnancy, so far only NK cells have been identified and functionally characterized in human decidua, where they are thought to contribute to the establishment of an immunosuppressive environment and to play a role in tissue building.[26,27] Notably, a successful pregnancy requires an early inflammatory phase necessary for implantation, while later the establishment of a regulatory/immunosuppressive phase is needed to prevent fetal rejection.[28] During the first trimester, decidual NK (dNK) cells represent as much as 50–70% of decidua-infiltrating lymphocytes (DILs).[29] Previous studies indicated that dNK cells may derive from CD34$^+$ hematopoietic precursors present in situ (dCD34) and/or from immature NK cells already present in endometrial tissue, and/or from peripheral blood NK cells migrated to decidua during the early phase of pregnancy.[30–32] dNK cells display unique phenotypic characteristics, are poorly cytolytic,[33,34] and, unlike peripheral blood NK cells, release cytokines/chemokines involved in neo-angiogenesis, tissue remodeling, and placentation.[26,29,35,36] In addition, dNK cells have been shown to induce regulatory T (Tregs) cells in vitro, i.e., cells that are thought to play a major role in the inhibition of maternal immune response and in tolerance induction.[37]

In the present study, we identified two additional decidual ILC3 populations, namely LTi-like cells and NCR$^+$ ILC3 and an ILC1 subset that differs from NK cells. Decidual LTi-like cells produced IL-17 and tumor necrosis factor (TNF), while NCR$^+$ ILC3 produced IL-22 and IL-8, whereas decidual ILC1 primarily released IFN-γ. LTi-like cells underwent in vitro differentiation into NCR$^+$ ILC3, whereas both NCR$^+$ ILC3 and LTi-like cells only marginally contributed to NK cell generation. Our data suggest that not only dNK cells, but also decidual ILC3 and ILC1 may play a relevant role both in innate defenses and in tissue building/remodeling during the early phases of pregnancy.

RESULTS
Human decidua contains different ILC subsets
Different ILC subsets are present in different organs and are characterized by the secretion of cytokines such as IL-17A, IL-22, IFN-γ, and TNF that promote/mediate tissues remodeling, lymphoid organogenesis, inflammation, and antimicrobial immunity.[2] We investigated whether cells producing these cytokines were present in human decidua. To this end, DILs were stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomicyn/IL-23. After 18 h, the cytokines produced by

different cell subsets were analyzed by using the gating strategy shown in **Figure 1a**. Lineage negative (Lin$^-$) cells (CD45$^+$ CD34$^-$CD19$^-$CD14$^-$CD3$^-$) were gated as CD56$^+$CD94$^+$ (bona fide NK cells), CD56$^+$CD94$^-$, or CD56$^-$CD94$^-$. As shown in **Figure 1b,d**, CD56$^+$CD94$^+$ NK cells produced primarily IFN-γ. CD56$^+$CD94$^-$ cells included IFNγ-positive cells and cells that were IFNγ-negative but produced IL-22 and IL-8. In addition, a small fraction of CD56$^+$CD94$^-$ cells produced IL-17A and TNF. The CD56$^-$CD94$^-$ cell population express IL-17A and TNF, but not IL-22. None of the three cell subsets produced IL-2 (**Figure 1b**). Previous studies revealed that dNK cells, identified as CD3$^-$CD56$^+$ cells, released high amounts of IL-8, a chemokine playing an important role in pregnancy.[26,35] Our present results indicate that high percentages of IL-8$^+$ cells belong to a CD56$^+$CD94$^-$ cell subset, rather than to CD56$^+$CD94$^+$ NK cells. Moreover, decidual CD56$^+$CD94$^-$IL-8$^+$ cells also produced IL-22, similar to ILC3 identified in tonsils.[38,39] Conversely, CD56$^+$CD94$^-$ IFNγ-producing cells may represent ILC1 distinct from "conventional NK" cells.[18] Our results also suggest that decidual tissue may contain ILC1 and ILC3 also among CD56$^-$ cell population. Indeed, we identified CD56$^-$ cells expressing IL-17A and TNF, putative LTi-like cells, and IFN-γ^+ cells that may represent ILC1[17] (**Figure 1b–d**). Therefore, in order to verify whether cell populations identified in human decidua indeed included ILC3 and ILC1, a more detailed phenotypic characterization was required.

Characterization of decidual ILC3 and ILC1
The putative decidual ILCs were further analyzed for the expression of CD127, CD117, and NKp44, i.e., markers allowing a more precise identification of ILC subsets. CD56$^+$ CD94$^+$ NK cells were negative for CD127 and CD117 and, when sorted, they produced only IFN-γ and TNF (**Figure 2a** and **Supplementary Figure S1a** online). CD127 and CD117 were expressed only by a fraction of CD56$^+$CD94$^-$ cells that homogenously expressed also NKp44, as previously described for NCR$^+$ILC3.[1,2] When sorted, CD56$^+$CD94$^-$CD127$^+$ CD117$^+$ cells produced high amount of IL-8 and IL-22, while they were negative for IFN-γ (**Supplementary Figure S1a**). Thus, CD56$^+$CD94$^-$CD127$^+$CD117$^+$NKp44$^+$ cells represent decidual NCR$^+$ group 3 ILC and are hereinafter referred to as NCR$^+$ILC3. Moreover, among Lin$^-$CD56$^-$ cells, we identified a subset of CD127$^+$CD117$^+$ cells that produced IL-17A and TNF, similarly to LTi-like cells previously described [1,2] (**Figure 2a** and **Supplementary Figure S1a**). Thus, Lin$^-$CD56$^-$CD127$^+$CD117$^+$ cells likely represent decidual LTi-like cells, and are hereinafter referred to as LTi-like cells (**Figure 2a**). Moreover, as shown above (**Figure 1b**), we observed that IFN-γ expression was not restricted to CD56$^+$CD94$^+$ NK cells. Indeed, also CD56$^+$CD94$^-$ (CD127$^-$)CD117$^-$ cells produced IFN-γ (**Supplementary Figure S1b**). These cells expressed Tbet and Eomes, but not RORγt TF. Notably, similarly to intraepithelial ILC1 (iILC1) identified in gut,[18] they were CD103$^+$NKp44$^{-/low}$. Interestingly, in decidual CD3$^-$CD56$^-$(CD127$^-$)CD117$^-$ subset,
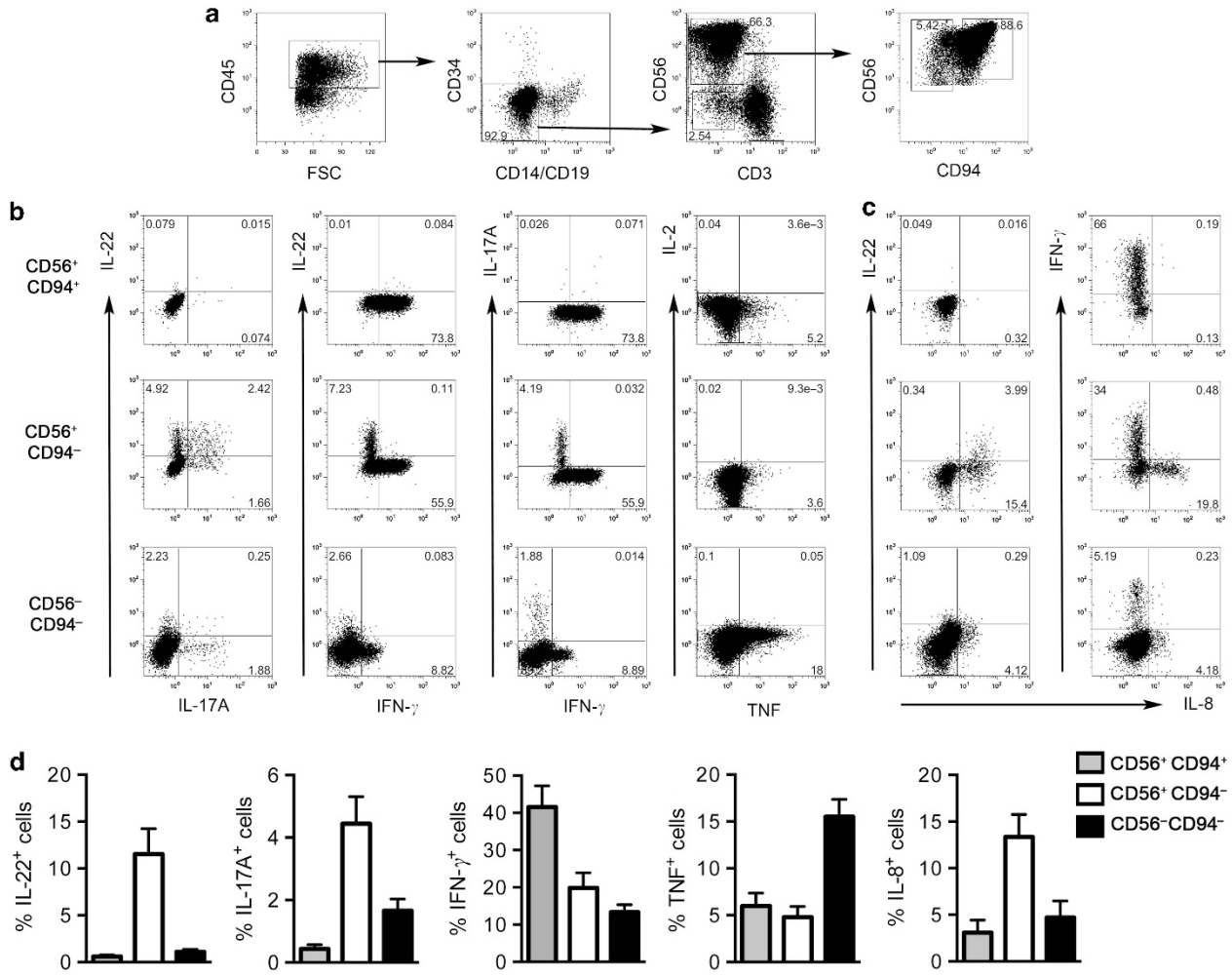
Figure 1 Cytokine profile of decidual ILCs. Decidua-infiltrating lymphocytes (DILs) were stimulated *ex vivo* and analyzed by flow cytometry for the expression of the specified markers and cytokines. (**a**) Gating strategy according to surface markers expression. (**b–d**) Intracellular analysis of the indicated cytokines, after 18-h stimulation with PMA/ionomicyn/IL-23. We identified three different cell populations according to the expression of the surface markers indicated in panel **a**: Lin⁻ (CD45⁺CD34⁻CD14⁻CD19⁻CD3⁻) –CD56⁺CD94⁺ or –CD56⁺CD94⁻ or –CD56⁻CD94⁻ cells. (**b**, **c**) One representative experiment out of six performed. (**d**) Mean ± s.e.m. of cytokine-positive cells.

we could also identify IFN-γ⁺Tbet⁺Eomes⁻RORγt⁻ cells, similar to ILC1 described by Bernink *et al.* (**Supplementary Figure S1b**).[17] These data indicate that ILC1 different from NK cells are present in human decidua.

A fundamental requirement to define both NCR⁺ILC3 and LTi-like cells is the expression of RORγt TF. As shown in **Figure 2b,c**, unlike NK cells, decidual NCR⁺ILC3 and LTi-like cells expressed RORγt. On the contrary, NK cells expressed the Eomes TF. In order to further characterize the subset assignment of decidual ILC, we next assessed different markers associated with NK cells, NCR⁺ILC3, or LTi-like cells. As shown in **Figure 2d**, both NCR⁺ILC3 and LTi-like cells expressed higher levels of IL-23R, IL-1R1, CCR6, and RANKL compared with NK cells. In line with previous reports, decidual LTi-like cells expressed higher levels of CD25 as compared with NK cells and NCR⁺ILC3, while all cell subsets were CD62L negative and displayed bimodal expression of

CD7.[21,24] In addition, NCR⁺ILC3 displayed lower LFA-1 expression them NK and LTi-like cell subsets.[40] On the contrary, NCR⁺ILC3 expressed higher levels of CXCR5, CD161, and NKp44 compared with NK cells and LTi-like cells. Conversely, NK cells expressed much higher levels of NKp46, NKG2D, KIRs, perforin, and granzyme B compared with NCR⁺ILC3 and LTi-like cells. Finally, CRTH2 was barely detectable in all subsets, which suggested that decidual ILC may not contain ILC2.[19] Taken together, these data further confirm that both NCR⁺ILC3 and LTi-like cells are present in human decidua during the first trimester of pregnancy.

Decidual NCR⁺ILC3 and LTi-like cells respond to cytokine stimulation

It has been shown that upon stimulation with a combination of IL-7, IL-1β, and IL-23, ILC3 release cytokines including IL-22.[38,41] As shown in **Figure 3a,b**, decidual NCR⁺ILC3
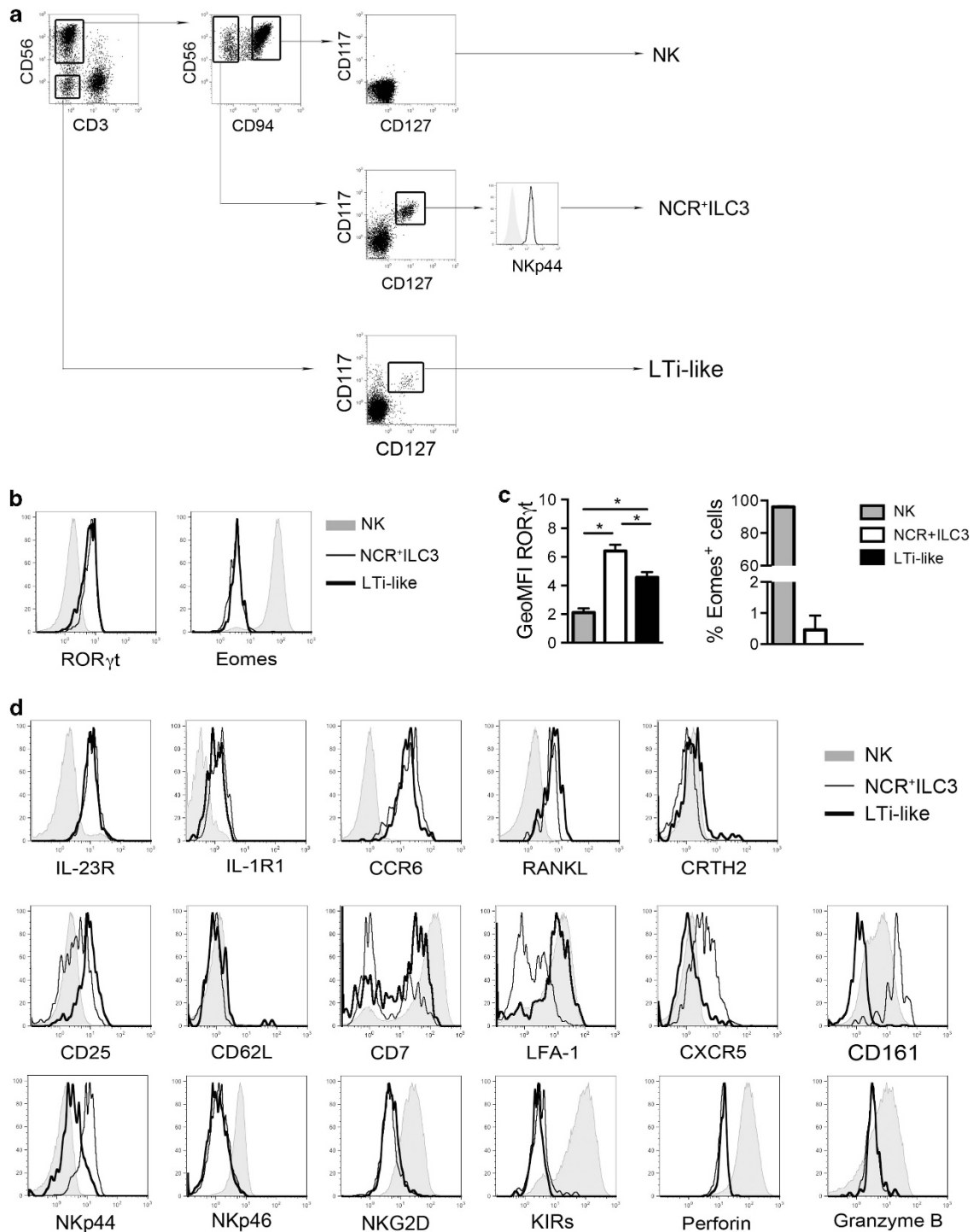
Figure 2 Phenotypic analysis of decidual ILCs. DILs were analyzed *ex vivo* by flow cytometry for the expression of the specified markers. (**a**) Identification of different ILC subsets by surface marker analysis. (**b–c**) Intranuclear expression of the indicated transcription factors in NK cells (Lin⁻CD56⁺CD94⁺; filled gray profile), NCR+ ILC3 (Lin⁻CD56⁺CD94⁻CD127⁺CD117⁺; thin black line), and LTi-like cells (Lin⁻CD56⁻CD127⁺CD117⁺; thick black line). (**b**) One representative experiment out of seven performed. (**c**) Mean ± s.e.m. of RORγt geoMFI and percentage of Eomes-positive cells. * = P ≤ 0.05. (**d**) Surface expression of the indicated markers in NK cells (filled gray profile), NCR⁺ILC3 (thin black line), and LTi-like cells (thick black line). One representative experiment out of five performed.

produced IL-22 in response to IL-7/IL-1β/IL-23 stimulation. In addition, NK cells and LTi-like cells did not produce IL-22 in response to IL-7/IL-1β/IL-23 stimulation (not shown). Upon interaction with stromal cells, ILC3 have been shown to induce the expression of ICAM1 and VCAM1 adhesion molecules.[21,24] Thus, we investigated whether decidual NCR⁺ILC3 and LTi-
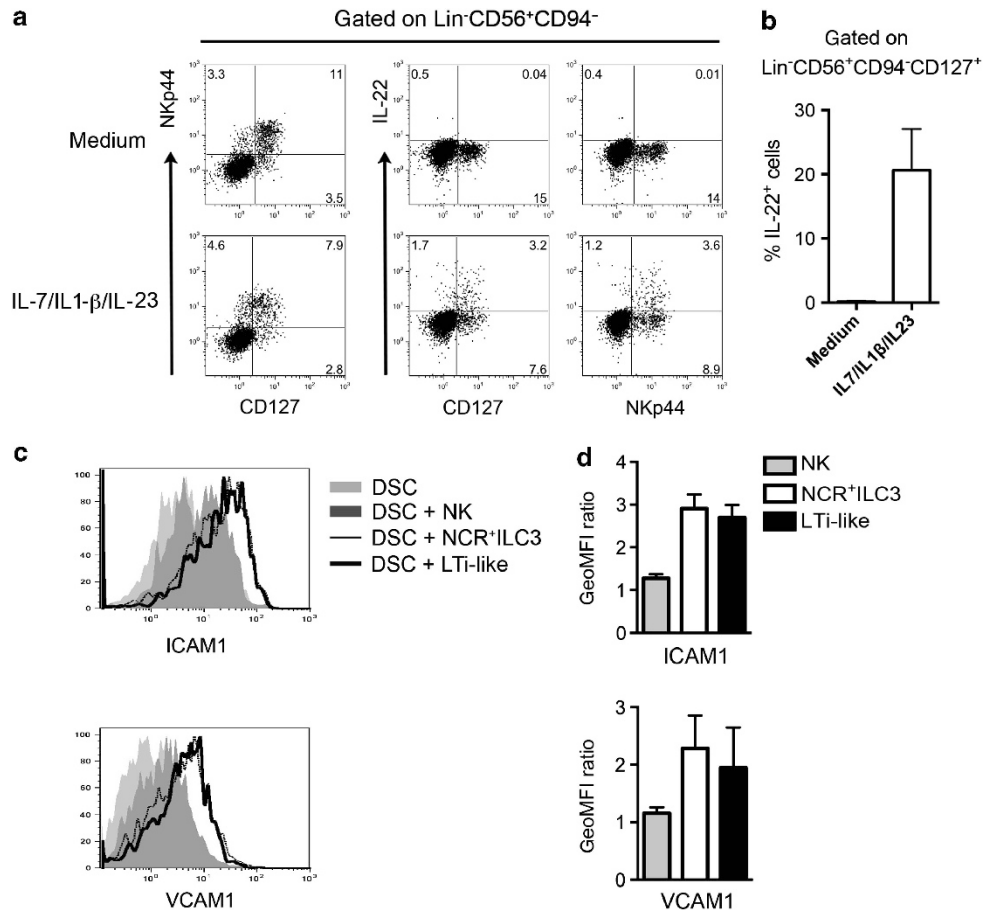
Figure 3 Decidual ILC3 produce IL-22 in response to cytokine stimulation and induce upregulation of adhesion molecules on decidual stromal cell (DSC). (a, b) DILs were left untreated (medium) or stimulated with IL-7/IL-1β/IL-23 for 18 h and analyzed for IL-22 production and CD127 and NKp44 expression after gating on Lin⁻CD56⁺CD94⁻ cells. (a) One representative experiment out of three performed. (b) Mean ± s.e.m. of IL-22-positive cells; cells were gated on Lin⁻CD56⁺CD94⁻CD127⁺ cells. (c, d) DSC were cultured for 7 days in IL-7 in the presence or in the absence of sorted NK cells (Lin⁻ CD56⁺CD94⁺), NCR⁺ILC3 (Lin⁻CD56⁺CD94⁻CD127⁺CD117⁺), and LTi-like cells (Lin⁻CD56⁻CD127⁺CD117⁺). (c) Expression of ICAM1 and VCAM1 on DSC was analyzed by flow cytometry. One representative experiment out of three was performed. (d) ICAM1 and VCAM1 expression, calculated as ratio of geoMFI of DSC + cells to DSC alone. Mean ± s.e.m. of three independent experiments.

like cells could induce upregulation of these adhesion molecules on decidual stromal cells (DSCs). Sorted NK cells, NCR⁺ILC3, and LTi-like cells were cocultured with DSCs. After 7 days, DSCs displayed higher expression of ICAM1 and VCAM1 when cultured with NCR⁺ILC3 and LTi-like cells as compared with DSCs cultured with NK cells (**Figure 3c,d**). These data reveal that cells with LTi activity are present in human decidua.

ILC3 proliferate and partially differentiate toward NK cells
We analyzed whether decidual ILC3 could undergo *in vitro* proliferation in the presence of cytokines previously shown to promote survival and proliferation of ILC3 isolated from tonsil.[21,41] The decidual NCR⁺ILC3 and LTi-like cells represent a small fraction of total CD45⁺ cells. However, their numbers/10⁶ CD45⁺ cells in decidua were comparable with, if not higher, than that of tonsil ILC3 subsets (**Supplementary Figure S2a**). In the presence of IL-2 and IL-7, we observed only marginal proliferation of decidual

NCR⁺ILC3. Moreover, the combined use of IL-7/IL-2 and IL-7/IL-1β could slightly increase cell proliferation. Much higher proliferation occurred in the presence of feeder cells (irradiated allogeneic peripheral blood mononuclear cells + LCL 221*G cells) and IL-2 or IL-15 (**Supplementary Figure S2b**). Notably, NCR⁺ILC3 and LTi-like cells displayed a much higher proliferative capacity in comparison with NK cells (**Supplementary Figure S2c**).

IL-15-expanded NK cells maintained their phenotype and upregulated NKp44 expression, compared with freshly isolated cells (**Figure 4a,b**). As previously described, CD127 was no longer detectable after culture on both NCR⁺ILC3 and LTi-like cells (**Figure 4a**).[21] Cultured NCR⁺ILC3 expressed CD117, CCR6, RANKL, NKp44 and upregulated NKp46 (**Figure 4a,b**). Interestingly, a consistent fraction of cultured LTi-like cells seemed to differentiate toward NCR⁺ILC3 as suggested by the acquisition of CD56 and NKp44 (**Figure 4a,b**). In addition, a fraction of cultured NCR⁺ILC3 and CD56⁺
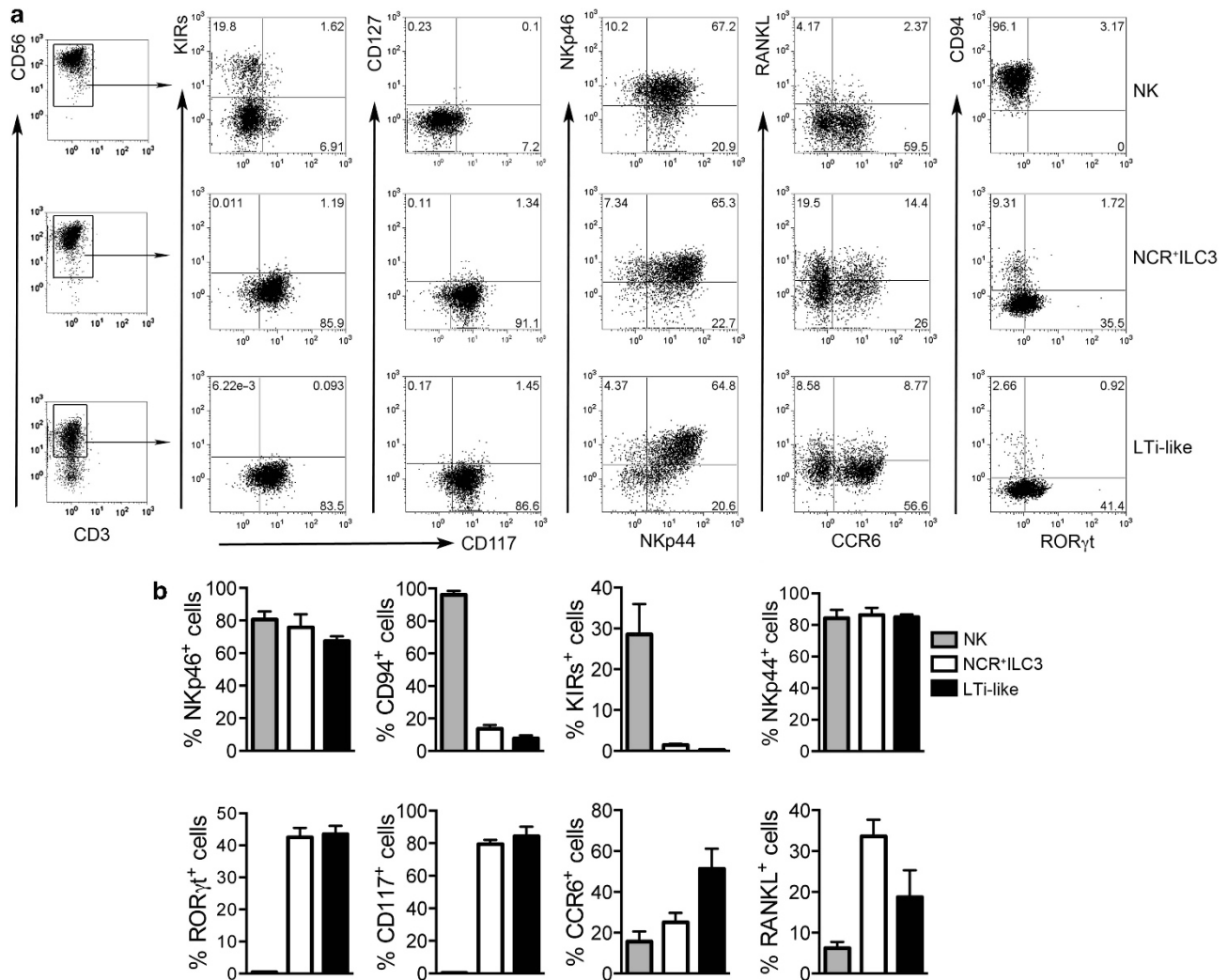
Figure 4 Phenotypic analysis of decidual ILCs after culture. NK cells, NCR$^+$ILC3, and LTi- like cells were sorted from decidua and cultured for 20 days in the presence of IL-15. Phenotypic analysis of indicated markers after gating on CD56$^+$ cells. (**a**) One representative experiment out of eight was performed. (**b**) Mean ± s.e.m. of percentage of positive cells.

LTi-like cells acquired CD94, a typical feature of NK cells (**Figure 4a,b**). However, NCR$^+$ILC3 and CD56$^+$ LTi-like cells expressed low levels of LFA-1 (acquired during NK cell differentiation [39,40]) and were KIR negative (**Figure 4a,b** and **Supplementary Figure S2d**). These results suggest that LTi-like cells could in part differentiate toward NCR$^+$ILC3 and that LTi-like cells and NCR$^+$ILC3 could partially differentiate toward CD56$^+$CD117$^+$CD94$^+$LFA-1^{low}KIR$^-$ cells that might represent immature NK cells.[42] Similar results were obtained in cultures with IL-2 (**Supplementary Figure S2e–f**). In order to verify the differentiation potential of NCR$^+$ILC3 and LTi-like cells, limiting dilution experiments were performed, in comparison with NK cells. As shown in **Supplementary Figure S3a**, NCR$^+$ILC3 and LTi-like cells displayed a higher cloning efficiency than that of NK cells. All NK clones maintained their initial surface phenotype, thus, they expressed CD94 and remained CD117 negative

(**Supplementary Figure S3b,d**). In line with the results obtained in bulk cultures (see **Figure 4a ,b**), a fraction of LTi-like clones acquired CD56 and NKp44, indicating that they can undergo differentiation toward NCR$^+$ILC3 (**Supplementary Figure S3c** and data not shown). In addition, some NCR$^+$ILC3 and CD56$^+$ LTi-like clones partially acquired CD94, but not KIRs. The presence of CD94$^+$ cells in ILC3 cultures might be due to NK cell contamination. However, similarly to freshly isolated ILC3, the CD94$^+$ cells obtained from ILC3 cultures expressed CD117 (**Supplementary Figure S3b–d** and data not shown). Fresh and cultured NK cells did not express CD117 and displayed lower cloning efficiency than NCR$^+$ILC3 and LTi-like cells. All these findings suggest that, upon culture with cytokines, decidual ILC3 can give rise to CD117$^+$CD94$^+$ cells that may represent putative immature NK cells.[42,43] Notably, some ILC3-derived clones maintained their initial phenotype and did not acquire
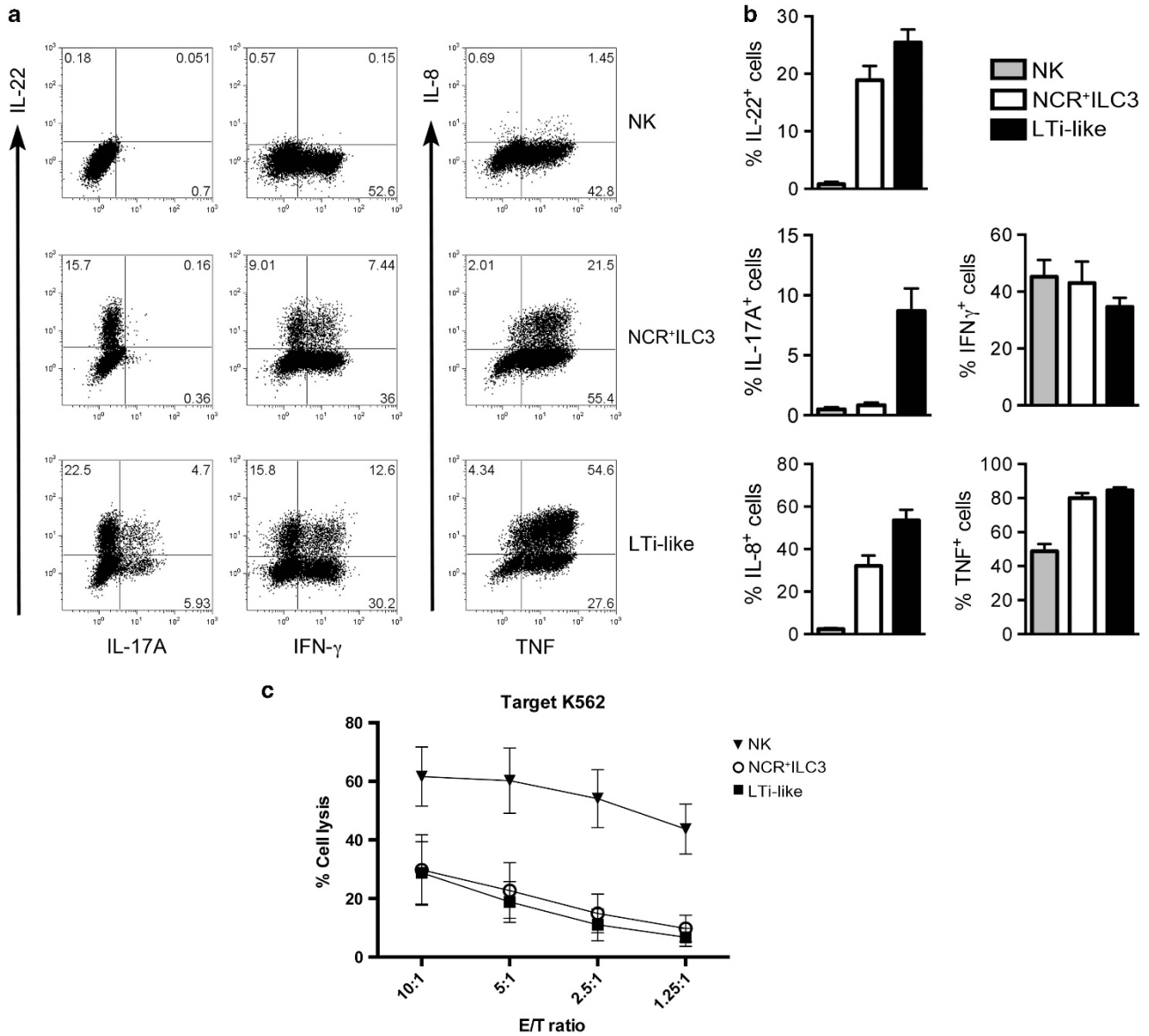
Figure 5 Functional characterization of cultured decidual ILCs. NK cells, NCR$^+$ILC3, and LTi-like cells were sorted from decidua and cultured for 20 days in the presence of IL-15. (**a**, **b**) Intracellular cytokine analysis of NK cells, NCR$^+$ILC3, and LTi-like cells stimulated for 4 h with PMA/ionomicyn/IL-23. (**a**) One representative experiment out of seven performed. (**b**) Mean ± s.e.m. of percentage of positive cells. (**c**) Cytolytic activity of NK cells, NCR$^+$ILC3, and LTi-like cells was evaluated by ^{51}Cr-release assay against K562 target cells. The effector/target (E/T) ratios are indicated. Mean ± s.e.m. of percentage of cell lysis obtained in four independent experiments.

the expression of CD94. This suggests that decidual NCR$^+$ILC3 and LTi-like cells are heterogeneous.

Functional analysis of cultured decidual ILCs

We next investigated whether cells cultured in IL-15 had modified their cytokine profile. As shown in **Figure 5a,b**, NK cells, upon PMA/ionomicyn/IL-23 stimulation, maintained the ability to produce primarily IFN-γ and TNF and did not produced IL-22, IL-17A, or IL-8. Similar to NK cells, cultured CD56$^+$CD94$^-$CD127$^-$CD117$^-$CD103$^+$ ILC1 expressed IFN-γ and TNF and mainly maintained their phenotype with the exception of CD94 acquisition (**Supplementary**

Figure S4a–b). On the other hand, cultured NCR$^+$ILC3 and LTi-like cells enhanced their production of IL-22 and IL-8 as compared with their fresh counterparts, while the production of IL-17A remained restricted to LTi-like cells. In addition, cultured NCR$^+$ILC3 and LTi-like cells also expressed IFN-γ and TNF (**Figure 5a,b**). While NCR$^+$ILC3 maintained their phenotypic and functional characteristics, most LTi-like cells not only acquired CD56 and NKp44, but also a cytokine profile typical of NCR$^+$ILC3. Similar results were obtained in cells cultured with IL-2 (**Supplementary Figure S5a–b**). In view of the ability of NCR$^+$ILC3 and LTi-like cells to partially differentiate toward CD94$^+$ NK cells, we

also assessed whether they could mediate cytolytic activity. IL-15-cultured NCR$^+$ILC3 and LTi-like cells displayed only marginal cytolytic activity against K562 target cells (**Figure 5c**). In particular, NCR$^+$ILC3 and LTi-like cells displayed 25% target cell lysis at an effector/target (E/T) ratio of 10:1. The same levels of cytolysis was mediated by NK cells and ILC1 at a ratio of 0.3:1, which reveals approximately 30-fold difference in cytolytic activity (**Figure 5c** and **Supplementary Figure S4c**). Similar results were obtained with cells cultured in IL-2 (**Supplementary Figure S5c**). Thus, in line with low perforin and granzyme B expression (**Supplementary Figure S2f**), both cultured NCR$^+$ILC3 and LTi-like cells are poorly cytolytic. This confirms that these cells could not differentiate toward mature cytolytic NK cells.

A small subset of CD56$^-$ LTi-like cells maintain their phenotypic and functional characteristics after culture

As shown above, cultured LTi-like cells could differentiate toward NCR$^+$ILC3 or even acquire CD94 expression. However, a small fraction (10%) of LTi-like cells did not acquire the expression of CD56 antigen after culture both with IL-15 or IL-2 (**Figure 6a**). After culture, CD56$^-$-persisting cell population acquired CD161, NKp44, and partially NKp46 (**Figure 6b** and **Supplementary Figure S6a**). Notably, after culture, CD56$^-$ cells maintained high expression of both RORγt and CD117, similarly to freshly isolated LTi-like cells (**Figure 6c** and **Supplementary Figure S6b**). In addition, upon PMA/ionomicyn/IL-23 stimulation, CD56$^-$ cells retained the ability to secrete IL-17A and TNF and also produced IL-8 and IL-22 (**Figure 6d** and **Supplementary Figure S6c**). We investigated whether the remaining CD56$^-$ cells maintained their features or rather acquired NCR$^+$ILC3 features upon additional culture. Thus, from LTi-like cells cultured for 20 days, we sorted CD56$^-$ cells and cultured them for additional 10 or 20 days. As shown in **Figure 6e**, CD56$^-$ cells could maintain their CD56$^-$CD117$^+$(NKp44$^{-/+}$) surface phenotype after 10 days of culture. However, after 20 days in two out of five experiments, we observed that a fraction of cells became CD56$^+$ CD117$^+$NKp44$^+$ (not shown). Finally, in limiting dilution cultures of freshly isolated LTi-like cells, plated at three cells per well, we obtained clones that did or did not acquire CD56 (**Supplementary Figure S3c,e**). Taken together, these data
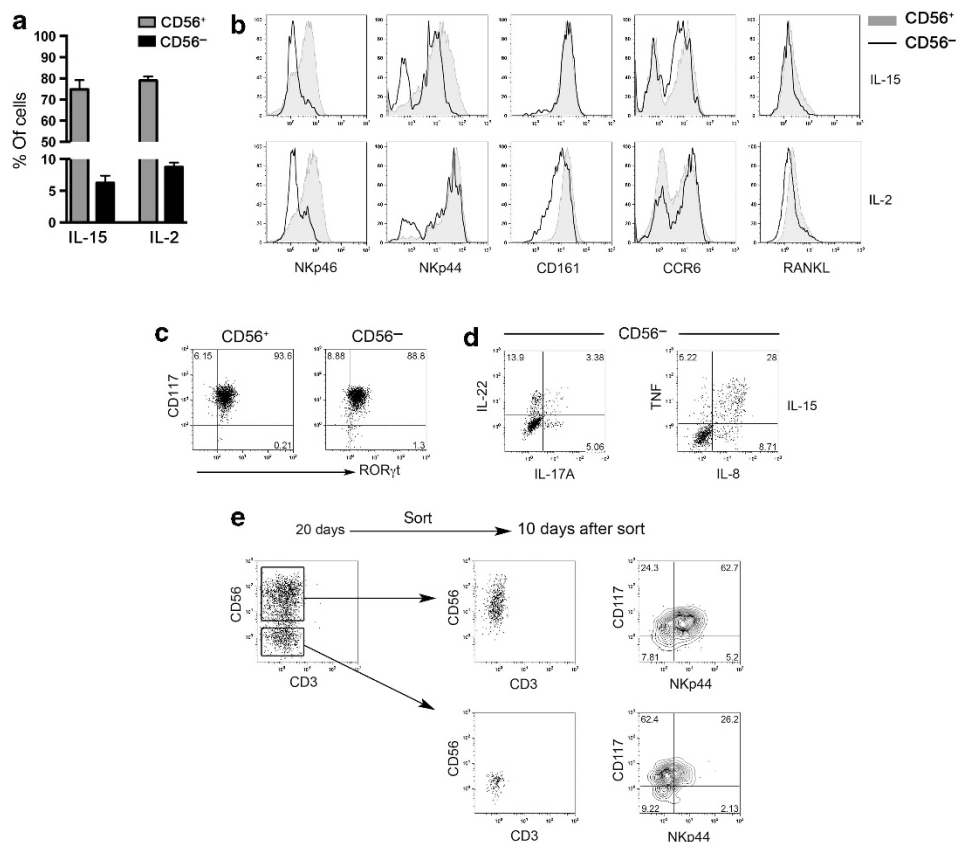

Figure 6 Analysis of cultured CD56$^+$ and CD56$^-$ decidual LTi-like cells. LTi-like cells were sorted from decidua and cultured for 20 days in the presence of IL-15 or IL-2. (**a**) Mean ± s.e.m. of percentages of CD3$^-$CD56$^+$ (gray bars) and CD3$^-$CD56$^-$ (black bars) cells. (**b**) Surface expression of the indicated markers in CD3$^-$CD56$^+$ (filled gray profile) and CD3$^-$CD56$^-$ cells (black line). One representative experiment out of six performed. (**c**) Analysis of RORγt and CD117 expression on CD3$^-$CD56$^+$ and CD3$^-$CD56$^-$ cells cultured in IL-15. One representative experiment out of six performed. (**d**) Analysis of cytokine production after 4-h stimulation with PMA/ionomicyn/IL-23 by CD3$^-$CD56$^-$ cells cultured in IL-15. (**e**) After 20 days of culture in IL-15, LTi-like cells were sorted again according to CD56 expression and further cultured. After 10 days, cells were analyzed by flow cytometry for the indicated markers. One representative experiment out of three performed.

suggest that LTi-like cells are heterogeneous, indeed they may contain cells with capability to maintain their phenotypic and functional features as well as to differentiate toward other ILC subsets.

DISCUSSION

In the present study, we show that different ILC populations are present in human decidua during the first trimester of pregnancy. Indeed, in addition to NK cells, decidua contains other IFN-γ-producing ILC1. Moreover, we identified two ILC3 populations, NCR$^+$ILC3 and LTi-like cells, characterized by the ability to produce IL-22/IL-8 and TNF/IL-17A, respectively. Finally, we show that, while NCR$^+$ILC3 substantially maintain their phenotypic characteristics after culture in IL-15 or IL-2, the majority of LTi-like cells acquire the phenotypic and functional features of NCR$^+$ILC3.

Different ILC populations have been recently identified in different tissues, where they play a role in innate responses and contribute to tissue homeostasis and lymphoid tissue generation.[2,44] The finding that ILC are also present in human decidua, during the first trimester of pregnancy, suggests that these cells may participate in the immune responses necessary for the maintenance/success of pregnancy. The absolute cell number of decidual ILC3 is very small compared with that of NK cells. However, decidual ILC3 have a frequency comparable, if not higher, with that of tonsil ILC3, suggesting that these cells, similarly to tonsil ILC3,[21,38,45] may play a role in decidual tissues. During early pregnancy, the balance between inflammation and tolerance is critical. Indeed, it has been shown that pro-inflammatory cytokines contribute to tissue remodeling and neo-angiogenesis, thus favoring embryo implantation. The inflammatory phase is followed by a regulatory phase characterized by an increase in Tregs and other regulatory cells that prevent immune-mediated fetal rejection.[28] Our present results indicate that NCR$^+$ILC3 and LTi-like cells present in decidua are capable of producing pro-inflammatory cytokines including IL-8, IL-22, IL-17A, TNF, and IFN-γ. These results suggest that ILC3 and ILC1 may be involved in the inflammatory phase of early pregnancy required for embryo implantation. In addition, our data suggest that peculiar functional capabilities, previously thought to be confined to other innate or adaptive immune cells, may be assigned, at least in part, also to ILC populations. For example, it has been proposed that Th17 cells present in the uterus may mediate immune responses against extracellular microbes.[28] Since decidual ILC produce IL-17A and IL-22, it is conceivable that they contribute to antimicrobial defenses during pregnancy.

For a successful pregnancy, building and remodeling of vessels is a fundamental process, which is favored by the presence of cells releasing pro-angiogenic factors, including IL-8, vascular endolthelial growth factor, and heparin-binding-EGF growth factor.[26,27,35,46,47] Different studies reported that dNK cells release these soluble factors, in particular IL-8, during the first trimester of pregnancy. However, in light of our present study, also decidual NCR$^+$ILC3 appear to contribute to IL-8 production. These data are in line with previous reports

showing that tonsil NCR$^+$ILC3 produce IL-8.[2,38,39] Importantly, after culture in IL-15 or IL-2, decidual NCR$^+$ILC3 and LTi-like cells can upregulate their IL-8 production.

The developmental relationship among different ILC is still a matter of debate. In particular, since the phenotypic features of human-immature NK cells and ILC3 partially overlap, the relationship between NK cells and ILC3 is unclear.[21,24,44,48,49] It has been proposed that NCR$^+$ILC3 may simply represent an immature stage of NK cell differentiation. Alternatively, they could represent lineage-specified cells. Notably, NCR$^+$ILC3 and LTi-like cells isolated from decidua failed to differentiate into cytolytic mature NK cells, in spite of the partial expression of CD7 (marker of NK cell precursors). Our present results indicate that LTi-like cells are heterogeneous. Indeed, only a small percentage (10%) of LTi-like cells retained their phenotypic characteristics (Lin$^-$CD56$^-$CD94$^-$CD117$^+$RORγt$^+$) upon culture, while the majority of them underwent differentiation toward NCR$^+$ILC3 (Lin$^-$CD56$^+$CD94$^-$NCR$^{+/-}$RORγt$^+$ IL-22$^+$). This finding supports the notion of the existence of a developmental relationship between decidual LTi-like cells and NCR$^+$ILC3, as suggested by previous reports in other organs.[2,21] It cannot be excluded that, similar to NK cells, also NCR$^+$ILC3 and LTi-like cells may derive from a hematopoietic precursor present in decidua. Interestingly, previous studies showed that dCD34 precursors express ID2 TF, which is required for ILC development.[30,36] These data suggest that dCD34$^+$ cells contain not only precursors of dNK cells, but also of NCR$^+$ILC3 and LTi-like cells. Further studies are required to clarify the origin of decidual ILC3.

In conclusion, our study provides clear evidence that different ILC populations are present in human decidua and also sheds light on the developmental relationship among dNK cells, NCR$^+$ILC3, and LTi-like cells. The ability of ILC1 and ILC3 to release pro-inflammatory cytokines suggests that they may exert an innate antimicrobial role, thus contributing to defenses against infectious agents. In addition, ILC3 may contribute to both tissue building/remodeling and neo-angiogenesis through IL-8 production. It is possible that altered ILC differentiation and function may result in pregnancy failure consequent to infection or to deficient tissue and vessel formation.

METHODS

Isolation and culture of DILs and DSCs. We obtained decidua (d) samples at 9–12 weeks of gestation from singleton pregnancies of mothers requesting termination of pregnancy for social reasons at AOUSM-IST (Genova, Italy). Tonsils were obtained from patients undergoing tonsillectomy at the Giannina Gaslini Institute (Genova, Italy). The relevant institutional review boards approved the study and all patients gave their written informed consent according to the Declaration of Helsinki. We isolated cell suspensions from decidual tissue with GentleMacs (Miltenyi Biotec, Bergisch Gladbach, Germany) and cells were then filtered as previously described.[34] Decidua-infiltrating lymphocytes were isolated by Fycoll (Cedarlane, Burlington, Ontario) gradient centrifugation. DILs were subsequently analyzed by flow cytometry or stimulated for cytokine production. To obtain single-cell suspension, tonsils were cut in small pieces, minced, and filtered through a 100-μm strainer before density gradient

centrifugation. Tonsil mononuclear cell suspensions were analyzed by flow cytometry. In order to isolate NK cells, NCR$^+$ILC3, or LTi-like cells, DILs were sorted as described in the results section on FACSAria (BD Bioscience, San Jose, CA), purity was routinely >95%. We plated sorted NK cells, NCR$^+$ILC3, or LTi-like cells at 1,000 cells per well for bulk cultures or using a limiting dilution approach from 200 to 1 cells per well for clonal assays. Cells were cultured in 96-well plates (Corning, Tewksbury, MA) in RPMI-1640 medium (Lonza, Basel, Switzerland) supplemented with 10% (vol/vol) fetal calf serum (Lonza), 1% (vol/vol) glutamine, and 1% (vol/vol) penicillin, neomycin, and streptomycin antibiotic mixture (Lonza and Cambrex, Charles City, IA), with 50 ng ml^{-1} IL-7, 50 ng ml^{-1} IL-1β, (Miltenyi), or 1,000 U ml^{-1} IL-2 (Peprotech, Rocky Hill, NJ), or with feeder cells (irradiated allogeneic peripheral blood mononuclear cells + LCL 221*G cells) and 10 ng ml^{-1} IL-15 (Miltenyi) or 100 U ml^{-1} IL-2. To obtain DSCs, we plated fresh decidual cell suspensions and cultured them in the presence of serum-supplemented RPMI medium, as previously described.[50] After 7 days, we removed non-adherent cells to separate the adherent cell fraction (DSC). Half the medium volume was replaced twice a week. When the cultures nearly reached confluence, they were harvested by treatment with trypsin/EDTA solution (Lonza) and re-plated at 5×10^5 cells per 75-cm^2 tissue culture flasks. DSCs were used in the experiments only after 1–2 expansion passages to ensure depletion of monocytes/macrophages. To perform coculture experiments, DSC and ILC were plated at the ratio of 1/5 in the presence of IL-7.

Flow cytometry analyses and monoclonal antibodies. Cells were stained with the following monoclonal antibodies: IL-17A-FITC; IL-17A-APC; IL-2-APC; NKp44-biotin; CD335 (NKp46)-APC; CD294 (CRTH2)-PE; CD314 (NKG2D)-PE; CD117-APC; CD3-PE and CD25-PE purchased from Miltenyi, Bergisch Gladbach, Germany; IFNγ-Alexa647; CD62L-PE; CD45-APC-H7, and CCR6-A647 purchased from BD Bioscience; IL-22-PE; TNFα-eFluor450; IFNγ-PerCP-Cy5.5; RORγt-PE; CD14-eFluor450; CD19-eFluor450; CD14-APC-eFluor480; CD19-APC-eFluor480; RORγt-APC; Eomes-AlexaFluor647; CD34-PerCP-Cy5.5, and CXCR5-PE purchased from eBioscence, San Diego, CA; CD94-FITC; CD127-BrilliantViolet421; NKp44-APC; CD335-PacifiBlue; CD254 (RANKL)-PE; CD161-PerCP-Cy5.5; CD117-PerCP-Cy5.5; CD127-PerCP-Cy5.5; CD7-FITC; CD11a(LFA-1)-PerCP and -FITC; CD103-PE purchased from BioLegend, San Diego, CA: IL-8-PE; IL-23R-biotin; IL-1R1-PE and CD335(NKp46)-PE purchased from R&D, Minneapolis, MN; CD103-FITC purchased from ImmunoTools, Friesoythe, Germany; CD106 (VCAM1)-PE purchased from Ancell, Bayport, MN; CD56-PC7, CD158a,h-PE; CD158e1,e2-PE; CD158b1/b2,j-PE and CD3-ECD purchased from IL-BeckmanCoulter; Streptavidin-Alex-Fluor700; GranzymeB-PE and Live/dead fixable aqua dead purchased from Invitrogen/Molecular Probes (Carlsbad, CA). ICAM1 was kindly provided by A. Poggi.

For intranuclear staining of TF, cells were stained for surface markers, fixed, and permeabilized with Fixation/Permeabilization buffer (eBioscience) and permeabilization buffer (eBioscience), respectively, according to the manufacturer's instructions. For intracellular cytokine detection, cells were stimulated as indicated in the result section in the presence of 25 ng ml^{-1} PMA (Sigma), 1 µg ml^{-1} Ionomycin (Sigma-Aldrich, St. Louis, MO), 50 ng ml^{-1} IL-23, 50 ng ml^{-1} IL-7, and 50 ng ml^{-1} IL-1β (Miltenyi) in the presence of GolgiStop (BD). After stimulation, cells were stained for surface markers, fixed, and permeabilized with Cytofix/Cytoperm (BD) and with Perm/Wash (BD), respectively according to the manufacturer's instructions. All samples were analyzed on Gallios Flow Cytometer (Beckman Coulter, Brea, CA). Data analysis was done using FlowJo software (TreeStar, Ashland, OR).

Cytolytic activity. We analyzed cytotoxicity of in vitro (20 days)-derived NK cells, NCR$^+$ILC3, or LTi-like cells in a 4-h ^{51}Cr-release

assay against K562 target cell line at different effector/target (E/T) ratios and experiments were performed in duplicates; data are expressed as percentage of target cell lysis.

Statistical analysis. Statistical analysis was performed with Graphpad Prism (La Jolla, CA) software using Friedman test and Dunn's multiple comparison; $P < 0.05$ (*); $P < 0.01$ (**).

AUTHOR CONTRIBUTIONS
P.V. and E.M. designed the study, performed experiments, analyzed data, and wrote the manuscript; D.C. and F.L. performed the experiments; F.C. provided samples and assisted in experiments; P.L.V. provided samples; L.M. and M.C.M. supervised the research and wrote the manuscript.

DISCLOSURE
The authors declared no conflict of interest.

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