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Innate-Like Effector Differentiation of Human Invariant NKT Cells Driven by IL-7

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Conventional MHC-restricted T lymphocytes leave thymus with a naive phenotype and require Ag-dependent stimulation coupled to proliferation to acquire effector functions. Invariant (i)NKT cells are a subset of T lymphocytes considered innate because they display an effector memory phenotype independent of TCR stimulation by foreign Ags. We investigated the effector differentiation program followed by human iNKT cells by studying cells from a relevant set of fetal thymus and umbilical cord blood samples. We find that human fetal iNKT cells have already started a differentiation program that activates the epigenetic and transcriptional control of \(\text{Ifng}\) and \(\text{id4}\) genes, leading to birth to cells that express these cytokines upon TCR signaling but independently of proliferation in vitro. Both ex vivo and in vitro analysis of fetal and neonatal iNKT cells delineate an effector differentiation program linked to cell division in vivo, and they identify IL-7 as one of the crucial signals driving this program in the apparent absence of Ag stimulation. Consistent with these data, human fetal and neonatal iNKT cells are hyperresponsive in vitro to IL-7 in comparison to conventional T cells, owing to an increased expression and signaling function of the IL-7 receptor \(\alpha\)-chain. The innate nature of human iNKT cells could thus derive from lineage-specific developmental cues that selectively make these cells efficient IL-7 responders following thymic selection. The Journal of Immunology, 2008, 180: 4415–4424.

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Abbreviations used in this paper: iNKT, invariant NKT; \(\alpha\)-GalCer, \(\alpha\)-galactosyl ceramide; AB, adult blood; CB, umbilical cord blood; CIRE, conserved intronic regulatory element; FT, fetal thymus; \(T_{EM}\), effector memory T; TREC, TCR excision circle.
Mouse iNKT cells develop in postnatal thymus and undergo a linear differentiation program consisting of proliferation, the up-regulation of the activation marker CD44, the acquisition of effector functions beginning with IL-4 followed by IFN-γ, and the expression of the terminal differentiation marker NK1.1 (16, 17). The final NK-differentiation step takes place in both thymus and periphery and is regulated by a number of molecules, including IL-15, which plays a critical role in the establishment and homeostasis of the mature mouse NKT cell compartment (18, 19). Mouse iNKT cells colonize the periphery already armed with effector functions because, unlike conventional T cells, they activate the expression of IL-4 and IFN-γ already in thymus by a process that involves both epigenetic derepression of the ifng and il4 cytokine genes and expression of constitutive cytokine transcripts (20, 21).

Unlike mouse iNKT cells, human iNKT cells can be detected in fetal thymus between 12 and 20 wk of gestation (22) and persist in postnatal thymus (23, 24). Human iNKT cells displaying the immature CD4⁺CD161⁻ phenotype predominates in thymus of any age and in neonatal blood (22–24). The immature iNKT cells undergo a postthymic maturation, leading to the appearance of mature CD4⁺CD161⁺ and CD4⁺CD161⁺ iNKT cells present in adults.

Human iNKT cells exhibit at birth an intriguingly dissociated phenotype: they display a primed/memory surface phenotype, but no effector cytokine expression upon activation ex vivo (25), calling into question the truly innate nature of these lymphocytes. We thus investigated thoroughly the effector competence of fetal and neonatal human iNKT cells in direct comparison with T cells. We find that human iNKT cells, unlike conventional T cells, have already started an effector differentiation program in fetal life, consisting in the epigenetic derepression of il4 and ifng loci and in the activation of GATA-3 and T-bet. Furthermore, we identify IL-7, and not IL-15, as a crucial signal driving the differentiation program of human iNKT cells.

**Materials and Methods**

**Donors and tissues**

Umbilical cord blood (CB) mononuclear cells were obtained after normal delivery, and peripheral blood was obtained from adult healthy donors. Fetal thymi (FT) were obtained at the 20–23 gestational wk, in accordance with the guidelines set forth by “L. Mangiagalli” Hospital ethical committee of Milano, Italy. The collection and use of human material were approved by the local ethical committee under investigational protocols, and a written informed consent was obtained from all of the tissue donors.

**Preparation of PBMCs and thymocytes**

Total mononuclear cells were separated from heparinized adult blood (AB) or CB by Ficoll-Hypaque (Pharmacia) density gradient centrifugation.
Thymus specimens were homogenized on a cell strainer (Falcon) to obtain a single-cell suspension.

**Isolation of iNKT cells and T cells**

CB iNKT cells were sorted by staining with anti-Vβ11-FITC, anti-Vα24-PE (26), and anti-CD3-APC (BD Biosciences). CB CD4+ naive T cells were sorted as CD3+CD8-CD45RA T cells using anti-CD8-PE, anti-CD45RA-FITC, and anti-CD3-APC (BD Biosciences). Adult CD4+ and CD4- iNKT cells were sorted by staining with anti-CD3, allophycocyanin, anti-CD4-PEcy5, and fixed/permeabilized and stained with anti-Ki67-PE mAb (BD Biosciences) before and after cell permeabilization to compensate for the TCR down-regulation induced by the stimulation with PMA/Ionomycin. Samples (at least 10^6 T cells) were acquired on a FC 500 flow cytometry system (Beckman Coulter) and analyzed with FlowJo software (TreeStar).

**Flow cytometry analysis**

iNKT cells were identified by staining with anti-Vα24-PE or -biotin, anti-Vβ11-FITC or -PE, anti-CD3-APC, and streptavidin-PEcy5. The expression of surface markers was assessed with anti-CD161-PE, anti-CD4-PE, anti-CD127-PE (Beckman Coulter), and anti-CD127-PECy5, and fixed/permeabilized and stained with anti-Ki67-PE mAb (BD Biosciences). Intracellular cytokines production was detected as described (28). Invariant TCR was stained with anti-CD3, anti-Vα24, and anti-Vβ11 Abs before and after cell permeabilization to compensate for the TCR down-regulation induced by the stimulation with PMA/Ionomycin. Samples (at least 10^6 T cells) were acquired on a FC 500 flow cytometry system (Beckman Coulter) and analyzed with FlowJo software (TreeStar).

**Stimulation with anti-CD3 + anti-CD28 beads**

Sorted iNKT or T cells were stimulated with anti-CD3 + anti-CD28-coated beads (Dynal Biotech) at a 1:3 T-cell-to-bead ratio. Culture supernatants were harvested at the indicated time points and completely replaced with fresh medium. The concentrations of secreted IFN-γ, IL-4, and IL-2 were determined with a Th1/Th2 cytokometric bead array kit (BD Biosciences).

**Quantitative RT-PCR**

Total RNA was extracted from primary or activated iNKT and T cells with an additional DNA digestion step (RNase-Free DNase set; Qiagen) and reverse transcribed. Real-time PCR was performed on cDNA with the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) using the following primers: UbcH5B sense 5'-CTTGGCAATTCATTCCCAAACAG-3', antisense 5'-TGATTCCGCTGACAACT-3'; IFN-γ sense 5'-CCGGCATATGCTGTTCGGTC-3', antisense 5'-CCAGATGACCCGGCTGAA-3'; T-bet sense 5'-CCCCGGCTGCATATCG-3', antisense 5'-TCTTGACAATTCATGAG-3'. IFN-γ and T-bet mRNA levels were evaluated using the LightCycler software version 3.5.28 (Roche Diagnostics) and the second derivative maximum algorithm. Specificity of the PCR product was confirmed by melting curve analysis. Serial dilutions of human CD4 cells were used to generate the standard curves.

**Enrichment of IL-4-producing neonatal iNKT cells in vitro**

CB mononuclear cells were expanded in vitro by culturing with 100 ng/ml α-galactosyl ceramide (αGalCer; Alexis) and rIL-2 for 10 days at 37°C.
Cells were then activated with PMA/ionomycin for 2 h, stained for surface IL-4 (IL-4 secretion cell-enrichment and detection kit, Miltenyi Biotec), followed by anti-IL-4-PE, anti-Vβ11-FTC, and anti-CD3-PECy5. Vβ11-IL-4+ iNKT cells were sorted and immediately frozen for CpG methylation mapping.

**Methylated CpG mapping of IL-4 gene**

Methylation of CpG motifs was performed as described (30). The bisulfite-specific nested PCR was performed using the following primers: IL-4 promoter outer sense 5′-gttattttatgtagaagttagg-3′; outer antisense 5′-TACCAAAATAAATCTACCTTCTACT-3′; inner sense 5′-GATAT TATTTTATTTATTTATGAGG-3′; inner antisense 5′-AACTATCC AAAATTTTAAATCCTCC-3′; intronic region of conserved intronic regulatory element (CIRE) outer sense 5′-GTTTTATTTTATTTTATGAGG-3′; inner antisense 5′-TCCATAAACAAATTTTCTAT-3′; inner antisense 5′-AACTCCCATTACTAAACAAAC-3′. The PCR products were subcloned into a TA vector (Invitrogen), and individual clones were sequenced.

**TCR excision circle (TREC) analysis**

DNA was extracted from sorted fetal thymic and CB iNKT cells and T cells. TREC number was determined by quantitative PCR using primers specific for GADPH to normalize DNA content. The TREC sequences were amplified using the primers sense 5′-CGTCTCAACAAAGGATGACACCA TTC-3′; antisense 5′-CATTCATTCTCACTCAGGTGCTCC-3′; probe 5′-FAMCGTCTCTCATTCAAGTCCCCACG(TAMRA)p-3′. Genomic GADPH was amplified using the primers sense 5′-ACCACAGTCCA TGCCCATCCT-3′; antisense 5′-GGCCATCACCCACAGT-3′; probe 5′-(FAM)CCACCCAGAAGACTGTGGATGGCC(TAMRA)p-3′. A standard curve was prepared by serial dilutions of a plasmid containing the amplified TREC sequence. To ensure similar PCR conditions, the standard plasmid was diluted in genomic DNA extracted from a TREC-negative cell line. PCR analyses were performed on 100 ng of DNA using TaqMan Universal PCR Master Mix (Applied Biosystems). The reactions were performed in an ABI Prism 7700 sequence detection system and data analyzed using the GeneAmp software (Applied Biosystems). The detection limits of this technique are 3 TREC copies/100 ng of DNA.

**Cell cultures with IL-7 in vitro**

Total fetal thymocytes and purified T cells (Pan T Cell Isolation Kit II, Miltenyi Biotec) from CB and AB were cultured for 14 days at a concentration of 1 × 10⁶ cells/ml in complete RPMI 1640 medium supplemented with 10% normal human serum (Euroclone) in the presence of rhIL-7 (Roche) or rhIL-15 (R&D Systems) at 20 ng/ml. Absolute numbers were derived from total cell counts, and iNKT and T cell frequencies were determined by flow cytometry. Intracellular cytokine production by iNKT and conventional T cells were determined as described above. For CD161 expression analysis, CD3+CD161+ T cells were sorted (purity >95%) by staining purified CB T cells with anti-CD161-biotin (Serotec) and streptavidin-PECy5, and incubated with either rhIL-7 or rhIL-15 (20 ng/ml). After 7 days, cells were stained with anti-Vα24-PE, anti-Vβ11-FTC, anti-CD161-biotin, and streptavidin-PECy5 and analyzed by flow cytometry.
Stat-5 phosphorylation by IL-7

Purified CB-derived T cells were incubated at a concentration of $1 \times 10^6$ cells/ml with 0.8 ng/ml IL-7. After 60 min, cells were harvested, stained with anti-V<sub>A24</sub>-biotin, anti-V<sub>P<sub>801</sub>-FITC, anti-CD3-APC mAbs, and streptavidin-PE-Cy5, and then fixed, permeabilized with ice-cold methanol for 10 min, and stained with anti-phospho-Stat-5 (Y694)-PE (BD Biosciences) at room temperature for 20 min. T cells were analyzed on a FACSCaliber cytometer (BD Biosciences).

Results

Human neonatal iNKT cells are poised to rapidly display effector functions

We first compared the effector phenotype and the functional competence of human iNKT cells from FT, CB, and AB (Fig. 1). First, human iNKT cells were present in 20–23-wk-old FT, where they increased 4-fold in frequency during the last trimester of gestation and less than 2-fold in the postnatal life (Fig. 1A), suggesting that the subset expanded substantially during fetal life and remained essentially stable after birth. Second, in FT and CB, human iNKT cells display already a CD45R0 primed/memory phenotype, accompanied however by an incomplete degree of NK differentiation, indicated by the low percentage of CD161 (Fig. 1C). These data show that neonatal iNKT cells are poised to rapidly display effector functions at birth independently of cell division in vitro.

Distinct transcriptional control of cytokine gene expression in neonatal iNKT and conventional T cells

In light of the above findings, we undertook a comparative molecular analysis of the transcriptional control of ifng and il4 gene expression in iNKT and T cells from CB and AB. Human neonatal iNKT cells are mostly CD4<sup>-</sup>, while adult iNKT cells are subdivided into CD4<sup+</sup> and CD4<sup>-</sup> subsets and display a T<sub>EM</sub> phenotype (25). iNKT and CD4<sup+</sup> T cells were therefore isolated from CB, while CD4<sup+</sup> iNKT, CD4<sup+</sup> iNKT, and CD4<sup+</sup> T<sub>EM</sub> cells (27) were sorted from AB. These cells were directly analyzed ex vivo, or after 12 h of activation in vitro with anti-CD3 + anti-CD28-coated beads, for the ability to secrete IL-4 and IFN-γ and for quantitative expression of IL-4, IFN-γ, GATA-3, and T-bet mRNA by real-time PCR.

As shown in Fig. 2A, neonatal iNKT cells, but not CD4<sup+</sup> T cells, produced IL-4 upon TCR + CD28 activation in vitro. The secretion of IL-4 by neonatal iNKT cells upon activation was even higher than that of both adult CD4<sup+</sup> and CD4<sup+</sup> iNKT subsets. Consistently, neonatal iNKT, but not T cells, expressed detectable constitutive IL-4 transcripts, which were further up-regulated upon TCR + CD28 stimulation. Both CD4<sup+</sup> and CD4<sup+</sup> adult iNKT cells maintained a level of constitutive IL-4 transcript similar to that of neonatal iNKT cells; however, their capacity to up-regulate IL-4 mRNA levels upon TCR + CD28 stimulation was markedly reduced as compared with that of neonatal iNKT cells, which was particularly clear in the CD4<sup+</sup> subset. Conventional T cells, in contrast, acquired the competence to produce IL-4 in vitro only upon differentiation, as shown by adult T<sub>EM</sub> cells; these cells, however, did not show detectable constitutive IL-4 mRNA, but expressed it only upon activation. Constitutive GATA-3 mRNA expression was markedly higher in neonatal iNKT than in T cells. The constitutive GATA-3 expression increased progressively from neonatal to adult iNKT cells and from naive T to T<sub>EM</sub> cells. Upon activation, the expression of GATA-3 was maintained at the same level only in neonatal iNKT cells, whereas it was down-regulated in the adult iNKT cells and in all T cell subsets analyzed.

As shown in Fig. 2B, neonatal iNKT cells, and not T cells, secreted IFN-γ upon sustained TCR + CD28 activation in vitro. At variance with IL-4 production, adult CD4<sup+</sup> and, to a lesser extent, CD4<sup+</sup> iNKT cells secreted more IFN-γ than did neonatal iNKT cells, resulting in a shift of the cytokine balance from an IL-4 = IFN-γ pattern displayed at birth, to a marked IFN-γ > IL-4 pattern displayed in adult life. In line with the cytokine secretion, neonatal iNKT and not T cells expressed detectable constitutive IFN-γ transcripts and up-regulated it markedly upon activation. Constitutive expression of T-bet mRNA was detected in neonatal iNKT and not in T cells, while TCR-CD28 activation up-regulated T-bet transcription in both cell subsets, even though the latter cells did not express IFN-γ, suggesting that factors other than T-bet might be required for IFN-γ production in naive T cells. Consistent with the age-related shift toward a Th1 pattern, both constitutive and inducible IFN-γ and T-bet mRNAs increased from neonatal to adult iNKT cells, particularly within the CD4<sup+</sup> subset. In analogy with IL-4, T cells acquired the production of IFN-γ only after birth, upon differentiation from naive to T<sub>EM</sub> cells, which was associated with the appearance of both constitutive and activation-inducible transcription of IFN-γ and T-bet.
Together, these findings indicate that iNKT cells have already activated the transcription of both *ifng* and *il4* loci during fetal life, and they argue for a distinct regulation of IFN-γ and IL-4 gene expression during fetal development between iNKT and conventional T cells. Furthermore, these findings also show a different regulation of *ifng* and *il4* gene expression in neonatal vs adult iNKT cells.

**Epigenetic derepression of effector cytokine genes in neonatal iNKT cells**

We next determined whether the distinct effector competence of neonatal iNKT cells could be attributed also to epigenetic modifications in the effector cytokine loci already present at birth. iNKT and CD4⁺ T cells were purified from CB and subjected to bisulfite-based cytosine methylation analysis to determine the degree of demethylation at critical CpG residues in the promoter and the CIRE of the human *il4* gene. These two regions are implicated in the transcriptional regulation of this cytokine (32). As shown in Fig. 3, A and B, CpGs in the *il4* promoter and CIRE showed a higher degree of demethylation in the chromosomes sequenced ex vivo from neonatal iNKT than from CD4⁺ T cells. The promoter was less methylated than was the CIRE, consistent with the 5′-to-3′ direction of gene demethylation.
The frequency of demethylated \textit{il}4 genes in primary CB iNKT cells was consistent with that of iNKT cells competent to secrete cytokines at birth upon TCR CD28 activation. The degree of demethylation of \textit{il}4 promoter and CIRE regions increased further in neonatal iNKT cells enriched for IL-4 secretion after expansion in vitro for 10 days with the specific Ag GalCer, confirming the importance of the demethylation of these regulatory sequences for \textit{il}4 gene expression. The differential methylation status of \textit{ifng} and \textit{il}4 loci in CB T and iNKT cells was further substantiated in activation experiments using anti-CD3 + anti-CD28-coated beads for 2 days in the presence or absence of the demethylating agent 5'-azacytidine (data not shown). The increase in the frequency of cells expressing IFN-\gamma or IL-4 induced by 5'-azacytidine was substantially lower in iNKT than in T cells, suggesting a higher basal level of demethylation at the \textit{ifng} and \textit{il}4 genes in iNKT cells.

Collectively, these results demonstrate that human iNKT cells have programmed the epigenetic derepression of effector cytokine loci already in fetal life, and they argue for a differential developmentally regulated epigenetic control of cytokine gene expression between iNKT and T cells.

\textit{Human iNKT cells undergo cell division in vivo during fetal life}

Cell division in vivo during fetal life could favor the epigenetic derepression of effector cytokine genes found in neonatal iNKT cells, and explain the expansion of the subset. We therefore determined the proliferative history of iNKT cells during fetal life. Conventional T and iNKT cells were sorted from fetal thymus and CB, and their TREC contents were determined by quantitative real-time PCR. As shown in Fig. 4, fetal thymic T and iNKT cells contained similar quantities of TRECs, suggesting comparable cell division in fetal thymus. CB iNKT cells contained less TRECs than did FT iNKT cells, compatible with four divisions during fetal life. CB T cells also contained fewer TRECs than did fetal thymic T cells, compatible with two cell divisions during fetal life and in line with published data (33). In CB, TREC content in iNKT cells was significantly lower than in T cells, indicating that iNKT cells undergo more cell divisions than do T cells during fetal life.

To assess whether iNKT cells were actively proliferating in FT or CB, iNKT and T cells from both of these compartments were stained for the intracellular expression of the cell cycle-related protein Ki67 (34). Fig. 4B shows that up to 30% of both iNKT or T cells are cycling in the fetal thymus. The frequency of actively cycling iNKT cells and T cells decreases markedly in the CB; nevertheless, the low fraction of Ki67-expressing iNKT cells remains significantly higher than in T cells.

Thus, human iNKT cells divide more than do conventional T cells during fetal life and become quiescent at birth, even though some degree of slow turnover seems to persist throughout life.
IL-7 recapitulates human iNKT cellular differentiation program in vitro

IL-15, and to a lesser extent IL-7, plays an essential role in controlling the maturation and size of murine iNKT cells (18, 19). We asked whether these cytokines could drive cell division, effector cytokine expression, and NK differentiation in human fetal and neonatal iNKT cells in vitro. We first verified the expression of IL-7Rα (CD127) and IL-2/15Rβ (CD122) in FT- and CB-derived iNKT and T cells. Among CD3^hi fetal thymocytes, substantially more iNKT cells expressed CD127 compared with T cells (Fig. 5, A and B). CD127 became expressed by almost all iNKT and T cells in CB. At variance with CD127, however, the expression of CD122 was infrequent among FT- and CB-derived iNKT and T cells.

Consistent with the distribution of receptors, iNKT cells from the two compartments expanded vigorously in response to IL-7 in vitro (Fig. 5C), but not in response to IL-15 (not shown). The growth of fetal thymic and neonatal T cells in response to IL-7 in vitro was markedly lower than that of iNKT cells.

iNKT or T cell proliferation induced by IL-7 in vitro was confirmed by measuring intracellular expression of Ki67 or by determining CFSE dilution (data not shown). IL-7-up-regulated in vitro the expression of CD161 in sorted CD161^- neonatal iNKT cells, but not in T cells, indicating the capacity of the cytokine to selectively induce the NK differentiation in human neonatal iNKT cells (Fig. 5D). Finally, culturing cells with IL-7 for 14 days induced in vitro the production of both IFN-γ and IL-4 in a higher proportion of FT- and CB-derived iNKT cells than T cells (Fig. 5, E and F). The experiments described in Fig. 5 were carried out with either total CB mononuclear cells or purified total T lymphocytes without significant differences (not shown).

Collectively, therefore, these results show that IL-7 recapitulates in vitro the differentiation program followed by human iNKT cells during fetal and, possibly, postnatal life.

Neonatal iNKT cells are hyperresponsive to IL-7

Although similar fractions of iNKT and T cells from the CB expressed CD127, iNKT cells grew and differentiated much more efficiently than T cells in response to IL-7 in vitro, suggesting a higher responsiveness of neonatal iNKT cells to this cytokine. Indeed, the mean fluorescence intensity of CD127 was significantly higher in CB iNKT than T cells, as shown in Fig. 6A. Furthermore, most of CB iNKT cells but only 50% of T cells responded to IL-7 signaling by phosphorylating Stat-5 (35), indicating an increased signaling via the IL-7/CD127 pathway in human iNKT cells at birth (Fig. 6, B and C). Remarkably, as shown in Fig. 6, D and E, IL-7 signaling triggered also the expression of intracellular IFN-γ and IL-4 in ~20% of CB iNKT cells, but not in T cells, as early as after 24 h of culture and independently of cell division (data not shown). Together, these results strongly argue for a major role for IL-7 in driving the proliferation, acquisition of effector functions, and NK differentiation of human fetal and neonatal iNKT cells in vivo, due to a higher membrane expression of IL-7Rα, resulting in turn in a stronger signaling cascade that leads to Stat-5 phosphorylation.

Discussion

This study shows that a sizable fraction (up to 20%) of human iNKT cells is competent at birth for the production of IFN-γ and IL-4 independently of cell division in vitro, and it displays a molecular setting for effector cytokine gene expression that is already similar to that of adult iNKT cells. We provide ex vivo and in vitro evidence that IL-7 is one of the signals crucial to drive the innate-like differentiation program of human iNKT cells in fetal life.

In mice, the IL-15 signaling pathway plays an essential role in the maturation and overall population size of iNKT cells in the thymus and periphery (18, 19). CD122 is indeed expressed at low levels already in thymic immature CD44^- NK1.1^- iNKT cell precursors, and it becomes progressively up-regulated upon subsequent differentiation into intermediate CD44^+ NK1.1^- and then fully mature CD44^+ NK1.1^- cells (18). Moreover, CD122 expression is maintained high in all peripheral mature mouse iNKT cells, irrespective of CD4 coreceptor expression (18). In contrast, CD127 is expressed similarly by mouse iNKT cells at any maturation stage; however, IL-7 seems critical for the development of iNKT cells, but it only plays a minor role in regulating the maturation and homeostasis of these cells (18). Our data show that only very small fractions of human fetal and neonatal iNKT cells express CD122, whereas they homogenously express CD127 and respond vigorously to IL-7 in vitro by proliferating and differentiating to mature CD161^- effector cells. Therefore, IL-7 dominates the human iNKT cell differentiation process during the fetal and perinatal life.

IL-7, however, might also play a functional role for human iNKT cells in adult life. In adult humans, in fact, CD127 is expressed by most iNKT cells, which grow in vitro in response to the cytokine, whereas CD122 is expressed only by a minor fraction of these cells, mainly in the CD4^- subset, implying a selective and age-specific role for IL-15 in the expansion/homeostasis of the human CD4^- iNKT cell subset ((23) and C. de Lalla, unpublished data). Overall, therefore, the control of maturation and size of iNKT cells would seem to be differently regulated by IL-7 and IL-15 in humans and mice.

We showed that fetal and neonatal iNKT cells are hyperresponsive to IL-7 in vitro compared with T cells due to the higher density of membrane IL-7Rα, which is associated with a stronger IL-7-dependent signaling. We propose that the high level of CD127 expression by human fetal iNKT cells is acquired in a lineage-dependent manner during the thymic selection, driven by unique molecular interactions between developing iNKT cells and DP thymocytes (15, 36). These characteristics would allow iNKT cells to outcompete T cells for limiting concentration of IL-7 in vivo, thereby explaining the greater proliferation and differentiation stage attained by iNKT cells in fetal life.

Human iNKT cells have indeed divided in vivo during fetal life significantly more than have conventional T cells. This is compatible with the 4-fold expansion of iNKT cells from fetal thymus to umbilical cord blood that we document in this study. The expression of Ki67 indicates that iNKT cells divide actively in fetal thymus; nevertheless, we cannot rule out that iNKT cell division continues in the periphery of the fetus, for example, in secondary lymphoid organs where IL-7 is available (37).

The IL-7-driven signaling and proliferation of human iNKT cells during fetal life should facilitate the epigenetic derepression of effecter cytokine loci, consistent with the role of DNA synthesis coupled with cell cycle in the remodeling of cytokine loci in T cells (2, 3). The opening of chromatin at effector cytokine loci in fetal iNKT cells is linked to the early activation of T-bet and GATA-3 and the expression of constitutive transcripts coding for IFN-γ and IL-4. Together, these molecular features allow human iNKT cells, unlike conventional naive T cells, to express cytokines at birth in the absence of cell division. T-bet and GATA-3 are induced in conventional naive T cells by TCR signaling and are sustained by the IFN-γ/Stat-1 and the IL-4/Stat-6 signal transduction pathways, respectively (6, 7). iNKT cells are normal in mice deficient in the IFN-γ/Stat-1 signaling axes (38), while Stat-6
seems dispensable for IL-4 expression by iNKT cells (39), suggesting that the expression of T-bet and GATA-3 in this subset might be differentially regulated in comparison to naïve T cells. How the two master gene regulators of Th1 and Th2 cytokines are activated in developing human iNKT cells is therefore an open question.

Given the activated transcriptional machinery at cytokine loci, it is unclear why human neonatal iNKT cells require a sustained TCR signaling (12 h) to secrete IFN-γ and IL-4, in comparison to adult iNKT cells. It is possible that a sustained TCR signaling serves to increase the number of cytokine mRNA molecules above a critical threshold for translation by inducing more transcription of cytokine genes, or to couple preexisting cytokine transcripts to the translation machinery via integrated stress response (40), or both.

Mouse thymic immature CD4<sup>4<sub>hi</sub></sup>CD161<sup>−</sup> iNKT cells produce IL-4 and progress to a prevalent IFN-γ production upon differentiating into the most mature CD4<sup>4<sub>lo</sub></sup>CD161<sup>+</sup> cells (16, 17). IFN-γ and IL-4 expression seems always coupled in human NKT cells as early as we could assess in development, even though most of these cells display at birth an immature CD161<sup>−</sup> phenotype. Nevertheless, human iNKT cells clearly modify their relative IL-4 secretion marker CD161 by iNKT cells is stimulated by the expression and, possibly, CD1d self-recognition. A differentiation of human fetal thymus.

The authors have no financial conflicts of interest.

Disclosures

The authors have no financial interests of interest.

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