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Cytotoxicity and IFN-γ production by human γδ T cells underlie their potent antitumor functions. However, it remains unclear where and how human γδ T cells acquire these key effector properties. Given the recent disclosure of a major contribution of the thymus to murine γδ T cell functional differentiation, in this study we have analyzed a series of human pediatric thymuses. We found that ex vivo–isolated γδ thymocytes produced negligible IFN-γ and lacked cytolytic activity against leukemia cells. However, these properties were selectively acquired upon stimulation with IL-2 or IL-15, but not IL-4 or IL-7. Unexpectedly, TCR activation was dispensable for these stages of functional differentiation. The effects of IL-2/IL-15 depended on MAPK/ERK signaling and induced de novo expression of the transcription factors T-bet and eomesodermin, as well as the cytolytic enzyme perforin, required for the cytotoxic type 1 program. These findings have implications for the manipulation of γδ T cells in cancer immunotherapy. The Journal of Immunology, 2014, 192: 000–000.

Interestingly, we (19) and others (20, 21) have shown that murine γδ T cells acquire their effector properties during thymic development, in a process regulated by TCR-γδ (and coreceptor) signaling (22). For example, IFN-γ–producing γδ T cells require TCR and CD27 signals for differentiation in the mouse thymus (19–21). This raises the question whether human γδ thymocytes can also complete their functional differentiation before being exported to the periphery. Although thymic commitment to the γδ T cell lineage is controlled by Notch signaling (23, 24), much less is known about the subsequent steps of functional differentiation of human γδ T cells (16, 25, 26). This will likely have major implications for their manipulation in cancer immunotherapy.

Building on these considerations, in this study we have used pediatric thymic tissue to address the molecular mechanisms of human γδ T cell differentiation toward antitumor lymphocytes. Our results reveal an NK-like mode of differentiation that is dependent on IL-2/IL-15 signals but surprisingly not on TCR activation. Interestingly, this process must take place in the periphery, because, unlike their murine counterparts, human γδ thymocytes are devoid of cytotoxic type 1 effector functions. Finally, our data disclose an MAPK/ERK-mediated differentiation pathway that may constitute an important target for future modulation of γδ T cell activity in the clinic.

Materials and Methods

Ethics

Thymic specimens were routinely obtained during pediatric corrective cardiac surgery, after parent’s written informed consent. The study was approved by the Ethics Board of the Faculdade de Medicina da Universidade de Lisboa.

Lymphocyte preparations

Thymic samples (from newborn to 9-y-old children) were processed by tissue dispersion and Histopaque-1077 (Sigma-Aldrich) density gradient. Peripheral blood was collected from anonymous healthy volunteers, diluted with 1 volume of PBS (Invitrogen Life Technologies), and separated on a Histopaque-1077 density gradient. γδ T cells were isolated (>95% purity) by magnetic cell sorting by positive selection (Miltenyi Biotec). Alternatively, CD1a+ γδ T cells or CD3+ CD4+ CD8− ‘TCRγδ+’ thymic progenitors were electronically sorted on a FACSAria cell sorter (BD Biosciences).
Cell culture

Isolated γδ T cells were cultured at 10^6 cells/ml at 37°C, 5% CO₂ in round-bottom 96-well plates with RPMI 1640 and 2 mM L-glutamine (Invitrogen Life Technologies) supplemented with 10% FBS (Invitrogen Life Technologies), 1 mM sodium pyruvate (Invitrogen Life Technologies), and 50 mg/ml penicillin and streptomycin (Invitrogen Life Technologies). Indicated cytokines were added when mentioned (all from PreproTech, 10 ng/ml).

To study the effects of chemical inhibitors of signal transduction, the MEK inhibitor UO126, the PI3K inhibitor LY294002, and the STAT5 inhibitor Ssi (all from Calbiochem) were added at 20 μM after 4 d in culture; CD3, CD4, and ATG-3 thymic progenitors were seeded at 2 × 10^5 cells/well into 48-well tissue culture plates (BD Biosciences) containing a subconfluent monolayer of OP9-DL1 cells. Cocultures were performed in cultered medium consisting of DMEM (Invitrogen Life Technologies) supplemented with 20% FCS, 100 IU/ml streptomycin, penicillin, and L-glutamine (Invitrogen Life Technologies) in the presence of 10 ng/ml IL-7 (PreproTech). Where mentioned, IL-2 (10 ng/ml; PreproTech) was added. Every 4–5 d, cells were harvested by forceful pipetting and transferred to a fresh confluent monolayer of OP9-DL1 cells.

In vitro tumor-killing assays

The MOLT-4 leukemia cell line was stained with CellTrace Far Red 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one-succinimidyl ester (1 μM; Molecular Probes/Invitrogen) and each batch of 3 × 10^5 tumor cells was incubated with 3 × 10^5 γδ T cells in RPMI 1640 for 3 h in a round-bottom plate with 96 wells. Cells were stained with annexin V–FITC (BD Biosciences) and analyzed by FACS.

Flow cytometry (FACS) analysis

Surface and intracellular stainings were performed as previously described (19). Cells were labeled with the following fluorescent mAbs: anti–TCRγδ-PE-Cy7 (HI100), anti–IFN-γ-PE (H129.19), anti–CD27-PE-Cy5.5 (LG.7F9), anti–CD3-PerCP-Cy5.5 (UCHT1), anti–CD69-PE-Cy7 (MBH1), anti–CD107a-Pacific Blue (H4A3), anti–CD45RA-allophycocyanin (HI100) from BioLegend; and anti–CD8a-PE (HIT8a), anti–IL-2-PE (MQ1-17H12), and anti–CD27-PE-Cy7 (LG.7P9), anti–IFN-γ–allophycocyanin (4S.B3), anti–CD4-eFluor 450 (RPA-T4), and anti–CD1a-eFluor 450 (H49) from eBioscience. Cell proliferation was measured by following a standard CFSE staining protocol (19) (CellTrace CFSE cell proliferation kit from Invitrogen; final concentration, 0.5 μM), whereas apoptosis was assessed by annexin V–FITC (BD Pharmingen) staining. Cells were analyzed on a FACSCalibur (BD Biosciences) and using FlowJo software (Tree Star).

Immunoblotting

Cells were lysed in lysis buffer (50 mM Tris [pH 7.6], 150 mM EDTA, 1% Nonidet P-40 in PBS) enriched with a protease inhibitor mixture (Roche) and a phosphatase inhibitor mixture (Roche). The membranes were blocked with 5% BSA and 0.5% Tween 20 in a 10% SDS-PAGE. After electrophoretic separation, the proteins were transferred to nitrocellulose blotting paper (Amersham Biosciences). These were detected using an appropriate HRP-conjugated secondary Abs and developed by chemiluminescence.

Real-time quantitative PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. Concentration and purity were determined by spectrophotometry, and integrity was confirmed using an Agilent 2100 bioanalyzer with an RNA 6000 Nano assay (Agilent Technologies). Total RNA was reverse-transcribed into cDNA using random hexamers and SuperScript II first-strand synthesis reagents (Invitrogen). Real-time quantitative PCR was performed on an ABI Prism 7500 Fast sequence detection system (Applied Biosystems). For each transcript, quantification was done using the calibration curve method. The following primers were used: B2m, forward, 5'-CTATCCAGCGT-ACTCCAAGATGC-3'; reverse, 5'-CTTGGCTGAAAGACAAGTCTGA-3'; Tbx21, forward, 5'-CACCTGTGTTGGTCCAGATTT-3'; reverse, 5'-AACATCTGTTAGGCTGGTG-3'; Pfn, forward, 5'-GCAATGTGCTATGTTGTGTG-3'; reverse, 5'-GGAGGTGTGTTACCATGGA-3'.

Statistical analysis

Differences between populations were assessed using the Student t test and are indicated in the figures when significant.

**FIGURE 1.** Human γδ thymocytes are devoid of IFN-γ production and cytotoxic functions. TCRγδCD3⁺ cells were isolated from pediatric thymic biopsies (Thymus/T) or from the peripheral blood of healthy donors (Blood/B) and analyzed ex vivo by flow cytometry. Dead cells were excluded from the analysis using LIVE/DEAD Fixable Dead Cell Stain Kits (Molecular Probes). (A) Surface expression of CD27, CD45RA, and CD1a. (B and C) Intracellular staining for IFN-γ and TNF-α (B) or the degranulation marker CD107a (C) following 4 h of stimulation with PMA and ionomycin. (D) Cytotoxic activity against MOLT-4 leukemia cells. Percentage of apoptotic γδ T cells after 3 h of coinoculation with γδ T cells at a 10:1 E:T ratio. Results in this figure are representative of 3–12 independent experiments; each dot represents an individual sample. **p < 0.005.
Results

Human γδ thymocytes are devoid of cytotoxicity and IFN-γ production

Inspired by the recent identification of fully differentiated effector γδ T cell subsets in the murine thymus (19–21, 26), we started this study by analyzing the surface phenotype and functional potential of γδ T cells isolated from human pediatric thymic samples. Based on the differentiation markers CD1a (27), CD27, and CD45RA (15), the vast majority of γδ thymocytes showed an immature and naive phenotype, which contrasted with the dominant effector/memory phenotype (15) of γδ PBLs (Fig. 1A). Also unlike these, γδ thymocytes produced negligible proinflammatory cytokines, particularly IFN-γ and TNF-α (Fig. 1B). Moreover, γδ thymocytes lacked cytolytic activity (Fig. 1C), namely against leukemia target cells, which were promptly killed by their PBL counterparts (Fig. 1D). These data clearly demonstrate that, unlike murine γδ thymocytes (19–21, 26), human γδ T cells do not complete their functional differentiation in the thymus, and they thus lack the cytotoxic type 1 characteristics of γδ PBLs.

IL-2 and IL-15 signals drive human γδ cytotoxic type 1 cell differentiation

The functional immaturity of human γδ thymocytes made them an ideal system to investigate the molecular cues required for acquisition of antitumor effector properties. Focusing first on IFN-γ

**FIGURE 2.** IL-2 and IL-15 signals differentiate γδ thymocytes into cytotoxic type 1 effector T cells. MACS-purified γδ thymocytes were cultured for 7 d in the presence of 10 ng/ml of the indicated cytokines; anti-CD3 mAb (1 μg/ml) was added when noted, and as part of the Th1 mix also contained anti-CD28 mAb (5 μg/ml), IL-2, and IL-12. (A) Intracellular staining for IFN-γ following 4 h of stimulation with PMA and ionomycin. (B) Surface expression of the activation marker CD69. (C) Surface staining for CD27 and CD45RA. (D) Intracellular staining for CD107a. (E) Cytotoxic activity against MOLT-4 leukemia cells (as in Fig. 1D). (F) Correlations between fractions of cells expressing IFN-γ, TNF-α, or CD107a. Each dot represents a specific culture condition from the experiments in Fig. (A) and (D). (G) Real-time PCR data for the expression of T-bet (Tbx21, upper panel), eomesodermin (Eomes, middle panel), and perforin (Pfn, lower panel), normalized to housekeeping β2-microglobulin (B2m), on γδ thymocytes either freshly isolated or cultured for 7 d with the indicated cytokines. Results in this figure are representative of 3–18 independent experiments; each dot represents an individual sample. *p < 0.05, **p < 0.005.
production, we considered that naive CD4+ T cells typically require TCR/CD3 and CD28 ligation in the presence of IL-2 and IL-12 for differentiation along the “Th1” pathway (28). Although such a “Th1 mix” was indeed capable of generating IFN-γ γδ T cells, we surprisingly found that IL-2 alone was also sufficient (Fig. 2A, upper panel). This effect was potentiated by IL-12, whereas TGF-β abrogated the process. Unexpectedly, the addition of TCR stimulation via anti-CD3 mAb did not enhance, but rather reduced, the IL-2–mediated differentiation of human IFN-γ γδ T cells (Fig. 2A, upper panel).

We next tested a large panel of individual cytokines and observed that besides IL-2, only IL-15 (but notably not IL-4 or IL-7) was able to induce IFN-γ production in γδ thymocyte cultures (Fig. 2A, middle panel). Both IL-2 and IL-15 treatments also promoted TNF-α expression (Fig. 2A, lower panel), upregulated the activation marker CD69 (Fig. 2B), and drove thymocytes along the effector/memory differentiation pathway, toward a CD45RA+CD27− T effector memory stage (Fig. 2C).

Concerning γδ T cell cytotoxicity, IL-2 and IL-15 (but not IL-7) induced the expression of the degranulation marker CD107a on γδ thymocytes (Fig. 2D) and endowed them with potent killing capacity against leukemia target cells (Fig. 2E). Of note, exogenous IL-2 and IL-15 also enhanced the effector functions of γδ PBLs, especially their degranulation/cytotoxic potential (Supplemental Fig. 1).

The acquisition of IFN-γ/TNF-α production and cytolytic capacity by γδ thymocytes were positively correlated, suggesting a common pathway of cytotoxic type 1 differentiation (Fig. 2F). Moreover, IL-2/IL-15 signals induced de novo expression of the type 1 master transcription factors T-bet and eomesodermin, as well as the cytolytic molecule perforin (Fig. 2G). These data firmly demonstrate that IL-2 and IL-15 are key functional differentiation factors for human γδ T cells. Importantly, they also show that IL-2 and IL-15 signals are sufficient, in the absence of TCR activation, to generate fully functional γδ T cells from immature thymocytes.

Vδ1 and Vδ2 T cell subsets follow similar rules of functional differentiation

Given that human γδ T cells comprise two major subsets, Vδ1+ cells (5–30% of γδ PBLs but more abundant in tissues) and Vδ2+ cells (60–95% of γδ PBLs), both strongly biased toward cytotoxic type 1 functions (3, 29), we next assessed whether they followed similar rules of differentiation. Consistent with the literature (25), the thymic γδ repertoire was largely biased for Vδ1+ thymocytes, whereas the Vδ2+ population was more balanced between type 1 and type 2 functions (Fig. 3A). This was reflected in the development of Vδ1+ and Vδ2+ thymocytes in vitro on OP9-DL1 monolayers cultured with IL-7 and IL-2 (Fig. 3B–D). Importantly, IL-2/IL-15 signals also enabled the acquisition of IFN-γ/TNF-α production and cytotoxic potential by Vδ1+ and Vδ2+ thymocytes, confirming the importance of these cytokines in the functional differentiation of γδ T cells.
with an average V61/V82 ratio of 25, which was maintained in vitro upon IL-7, IL-2, or IL-15 treatment (Supplemental Fig. 2A). In all thymic samples analyzed, V61+ T cells behaved as expected: they were functionally immature ex vivo and differentiated into type 1 effectors in response to IL-2 or IL-15 stimuli (Supplemental Fig. 2B). However, the results obtained by gating on the V62+ population were affected by an important intersample variation (Supplemental Fig. 2B). We considered that this could be due to blood contamination or recirculation (back to the thymus) of mature Vγ9Vδ2 cells, which are much more abundant in the blood than in the thymus (<3% of total γδ thymocytes). To overcome these problems, we purified CD11α+ γδ T cells, which are exclusive to the thymus (Fig. 1A, lower panel) and cultured them for 7 d with IL-7, IL-2, or IL-15. We found that both V61+ and V62+ cells similarly acquired type 1 effector properties in response to IL-2 and IL-15, but not IL-7 (Fig. 3A).

As an alternative developmental strategy, we differentiated γδ T cells from sorted CD3−TCRγδ−CD4−CD8− thymic precursors cultured on OP9-DL1 monolayers, as previously described (24). In the presence of IL-7 alone, V61 and V62 T cells developed normally (Fig. 3B) to a V61/V82 ratio similar to that observed ex vivo (Supplemental Fig. 2A). We therefore think this is an elegant model to characterize γδ T cell differentiation from very early developmental stages. Most importantly, the further addition of IL-2 was necessary to generate IFN-γ (Fig. 3C) and TNF-α (Fig. 3D) producing γδ T cells, and this occurred similarly for V61 and V62 T cell subsets (Fig. 3C, 3D). These data demonstrate that IL-2 (or IL-15) signals drive the functional differentiation of both major subsets of human γδ T cells.

**IL-2/IL-15 signals induce γδ type 1 cell differentiation via the MAPK/ERK pathway**

To gain further mechanistic insight into the type 1 differentiation pathway of human γδ T cells, we probed the three major signaling pathways downstream of common γ-chain cytokine receptors and observed that IL-2 stimulation hyperphosphorylated ERK1/2 (MAPK pathway), STAT5 (JAK/STAT pathway), and AKT (PI3K pathway) (Fig. 4A). To determine which of these signaling pathways was critical for functional differentiation of γδ T cells, we analyzed the effect of specific chemical inhibitors on γδ thymocyte cultures. When added at the start of the cultures, all of these drugs interfered with γδ T cell proliferation and prevented their functional differentiation (Supplemental Fig. 3A). Of note, although proliferation was necessary for IFN-γ induction, this specifically required IL-2 or IL-15 signals, as IL-7 failed to do so even after five cell divisions (Fig. 4B). To dissociate proliferation from differentiation, we added the inhibitors at day 4 of culture, when cells had already undergone five rounds of division (Fig. 4B). Whereas blocking STAT5 or PI3K/AKT had no detectable effect, the MAPK/ERK inhibitor UO126 (30, 31) completely abrogated the differentiation of IFN-γ+ or TNF-α+ γδ T cells (under IL-2 or IL-15 treatment) (Fig. 4B, Supplemental Fig. 3B).

Interestingly, IL-2 or IL-15 signals also enhanced IL-2 production by γδ T cells, thus providing an autocrine mechanism to sustain their functional differentiation (Supplemental Fig. 3B). The production of IL-2 (as well as TNF-α) by γδ T cells was also completely abolished by the addition of UO126, but not LY294002 or Ssi (Supplemental Fig. 3B). These data demonstrate that γδ T cell cytotoxic type 1 differentiation can be manipulated by drugs specifically targeting the MAPK/ERK pathway (32).

Collectively, our results identify MAPK-mediated IL-2/IL-15 signaling as the major functional differentiation pathway of human γδ T cells toward antitumor (cytotoxic type 1) effector lymphocytes.

**Discussion**

Seminal studies in murine models have shown that γδ T cells can complete their functional differentiation in the thymus (19–21, 26). In contrast, the data presented in the present study show that human γδ thymocytes (obtained from young children subjected to cardiac surgery) are functionally immature. Given that circulating γδ PBLs display type 1 effector properties (13, 18) and express memory markers (15), human γδ T cells must thus complete their differentiation in the periphery, as reported for αβ T cells (33, 34).

In healthy individuals, γδ PBLs are strongly biased toward IFN-γ production (type 1 effectors), and throughout our study we failed to identify any significant production of type 2 or type 17.
cytokines. Namely, IL-17, which is constitutively expressed by a subset of murine γδ T cells (19, 26), is rarely expressed in human γδ T cells from healthy donors (16–18). In contrast, IL-17-producing γδ T cells seem to accumulate to high numbers in clinical cases of bacterial meningitis, and their rules of differentiation have been previously dissected (17, 35).

In our study, the naive and immature phenotype of human γδ thymocytes provided an ideal system to study the molecular cues required for acquisition of type 1 effectors properties. Our work demonstrated that IL-2 or IL-15 signals are sufficient to drive the differentiation of human γδ T cells into IFN-γ/TNF-α producers and also endowed with potent cytotoxicity against tumor targets.

The redundant functions of IL-2 and IL-15 can be explained by the structure of their respective receptors, which share not only the common γ-chain but also their second signaling subunit, IL-2Rβ. A third subunit, IL-2Rα or IL-15Rα, is cytokine specific and stabilizes binding but apparently lacks signaling activity. Structural comparisons of IL-2/IL-2Rα and IL-15/IL-15Rα interactions have emphasized their similarities (36, 37), and it has been recently demonstrated that they induce similar downstream transcriptional effects (38).

On the other hand, IL-7 clearly failed to trigger differentiation of cytotoxic type 1 γδ T cells. This is in stark contrast with the major role described for IL-7 in the functional differentiation of human NKT cells (39) and IL-17-producing γδ T cells (40). These lines of evidence establish an interesting cytokine dichotomy for human γδ T cells: whereas IL-7 promotes type 17 effector functions, IL-2 and IL-15 stimulation induces γδ T cells to produce IL-2 (Supplemental Fig. 3B), this cytokine could also be the direct (autocrine) mediator of the two-step model proposed by Lio and Hsieh (42) and Farrar and colleagues (43) for murine Foxp3+ regulatory T cell development.

Importantly, whereas for regulatory T cells this two-step process is completed in the thymus, unlike murine γδ T cells, are likely additional players in the functional differentiation of human γδ T cells. This is in stark contrast with the major dysregulation of T cell differentiation: their differentiation program is not completed in the thymus, whereas murine γδ T cells (19, 20, 26), and it does not require TCR/co-receptor activation in the periphery, in contrast with naive CD4+ T cells (28). Instead, IL-2/IL-15 signals are sufficient for functional differentiation of human γδ T cells, which clearly aligns them with NK cells (45) and some naive CD8+ T cell populations (46). Thus, the three main cytotoxic type 1 lymphocyte subsets share a common, IL-2/IL-15-dependent differentiation program with key implications in cancer immunotherapy.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figure 1. Exogenous IL-2 and IL-15 enhance cytotoxic type 1 effector functions of human γδ peripheral blood lymphocytes. MACS-purified γδ PBLs were analyzed ex vivo or cultured for 7 days in the presence of 10 ng/mL of recombinant IL-2 or IL-15. Flow cytometry analysis for intracellular IFN-γ and TNF-α (upper panels); or the degranulation marker CD107a (lower panels).
Supplemental Figure 2. IL-2 and IL-15, but not IL-7, promote type 1 differentiation of human Vδ1+ and Vδ2+ thymocytes. MACS-purified γδ thymocytes were cultured for 7 days in the presence of 10 ng/mL of recombinant IL-2, IL-7 or IL-15. (A) TCRVδ1 and TCRVδ2 stainings (above) and ratios (below) in ex vivo (day 0) or cytokine-stimulated (day 7) γδ thymocytes. (B) Percentage of IFNγ+ cells upon intracellular staining in the thymocyte populations of (A). Data are representative (A) or collected (B) from 8-10 independent experiments.
Supplemental Figure 3. Effect of signaling inhibitors on the proliferation and cytokine production of γδ T cells. MACS-purified human γδ thymocytes were cultured in the presence of 10 ng/mL of IL-2, IL-7 or IL-15. Specific inhibitors of STAT5 (Ssi), PI3K (LY294002) or MEK phosphorylation (UO126) were added (20 mM each) either on day 0 (A) or day 4 (B) (after 5 cell divisions, based on CFSE dilution). At day 7 cells were restimulated for 4 hours with PMA and ionomycin and stained intracellularly for IFN-γ (A) or TNF-α and IL-2 (B). Dead cells were excluded from the analysis using LiveDead Fixable Viability Dye. Data are representative of 3-5 independent experiments with similar results.