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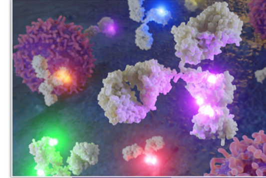

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# Clonal Characterization of a Bipotent T Cell and NK Cell Progenitor in the Mouse Fetal Thymus[1]

**Alison M. Michie, James R. Carlyle, Thomas M. Schmitt, Belma Ljutic, Sarah K. Cho, Quyen Fong, and Juan Carlos Zúñiga-Pflücker[2]**

**We recently described a population of fetal thymocytes with a CD117$^+$NK1.1$^+$CD90$^{low}$CD25$^-$ phenotype, which were shown to contain committed T cell and NK cell progenitors. However, the characterization of a single cell with a restricted T and NK cell precursor potential was lacking. Here, using an in vitro model for T and NK cell differentiation, we provide conclusive evidence demonstrating the existence of a clonal lineage-restricted T and NK cell progenitor. These results establish that fetal thymocytes with a CD117$^+$NK1.1$^+$CD90$^{low}$CD25$^-$ phenotype represent bipotent T and NK cell progenitors.** ***The Journal of Immunology,* 2000, 164: 1730–1733.**

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During mouse fetal ontogeny, the earliest precursor population to colonize the thymus contains multipotent progenitors that can give rise to both lymphoid and myeloid cells (1, 2). These cells are present in the fetal thymic rudiment by day 12 of gestation and are phenotypically similar to fetal liver-derived hemopoietic stem cells (2, 3). However, between days 12 and 14 of gestation, recoverable myeloid potential rapidly diminishes (1, 4), such that after day 14 of gestation and throughout adult life only lymphoid potential can be rescued intrathymically (5–8). In keeping with this, the most immature hemopoietic precursors common to the fetal and adult thymus appear to possess a lymphoid-restricted potential and are capable of giving rise to the B, T, NK, and lymphoid dendritic cell lineages (7–13). Collectively termed thymic lymphoid progenitors (TLPs),[3] these multipotent cells are characterized by high level surface expression of CD117 (c-*kit*) and a lack of expression of hemopoietic lineage (Lin$^-$) differentiation markers (7–9). Whether these cells comprise a homogeneous population of lymphoid-restricted precursors or represent a collection of phenotypically similar lineage-committed cells is still controversial, although recent evidence from Katsura et al. (4) supports the latter notion.

We recently identified a later stage in fetal thymic ontogeny, marked by the expression of NK1.1 (CD161) on CD117$^+$ thymocytes, which characterizes a population of progenitors committed to the T and NK cell fates (8), termed fetal TNK progenitors. These cells are present as early as day 13 of fetal thymic ontogeny and also can be generated on reconstitution of alymphoid fetal thymic lobes in vitro with sorted NK1.1$^-$CD117$^+$ TLPs or fetal liver-derived hemopoietic precursors (8). In addition, we have recently characterized a similar progenitor population in the fetal circulation (14), indicating that commitment of precursors to the T and NK cell lineages can occur before thymic entry.

To conclusively determine whether individual CD117$^+$NK1.1$^+$ TNK cells can give rise to descendants in both the T and NK cell lineages, we performed single-cell reconstitution assays in alymphoid fetal thymic organ cultures (FTOCs) with fetal TNK progenitors. Our results provide direct evidence for the existence of a common lineage-restricted T and NK cell progenitor. Thus, these findings establish that bipotent T and NK cell progenitors within the fetal thymus have a CD117$^+$NK1.1$^+$CD90$^{low}$CD25$^-$ phenotype.

## Materials and Methods

### *Mice*

Timed-pregnant Swiss.NIH and C57BL/6 mice were obtained from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD).

### *Isolation of fetal thymocytes*

Fetal thymuses were harvested at day 14 of gestation, washed three times in 5 ml FTOC medium (DMEM supplemented with 12% FCS, 2 mM glutamine, 10 U/ml penicillin, 100 $\mu$g/ml streptomycin, 100 $\mu$g/ml gentamicin, 110 $\mu$g/ml sodium pyruvate, 50 $\mu$M 2-ME, and 10 mM HEPES, pH 7.4) (GibcoBRL, Burlington, Canada), and disrupted through 70-$\mu$m pore size nylon mesh with a syringe plunger. CD24$^{low}$CD25$^-$ thymocytes were obtained by Ab/complement-mediated lysis, as described previously (8). Briefly, 50–200 $\mu$l of each anti-CD24 (J11d.2) and anti-CD25 (7D4) culture supernatant and a 1/10 dilution of Low-Tox rabbit complement (CedarLane, Hornby, ON, Canada) were added to single-cell suspensions in 2–3 ml medium, and cells were incubated at 37°C for 30 min. After incubation, viable cells were recovered by discontinuous density gradient centrifugation over Lympholyte-Mammal (CedarLane) and washed before analysis.

### *Flow cytometric analysis and cell sorting*

FITC-, PE-, biotin-, and APC-conjugated mAbs and streptavidin-APC were obtained from PharMingen (San Diego, CA). Cell suspensions were stained in 50 $\mu$l FACS buffer (HBSS without phenol red, plus 1% BSA and 0.05% $NaN_3$) for 20 min on ice and washed twice before analysis. Stained cells were analyzed with a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, Mountain View, CA); data were live-gated by forward/side light scatter and lack of propidium iodide uptake. Frequencies in each quadrant are given as percent of total in the upper right corner. For cell sorting, single-cell suspensions were prepared and stained for FACS as described above, except that no $NaN_3$ was added to the FACS buffer. Cells were sorted using a Coulter Elite cytometer (Hialeah, FL); sorted cells were ≥99% pure, as determined by post sort analysis. Cells were sorted into

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[3] Abbreviations used in this paper: TLP, thymic lymphoid progenitors; FTOC, fetal thymus organ culture; SCF, stem cell factor; TNK, T and NK progenitors.

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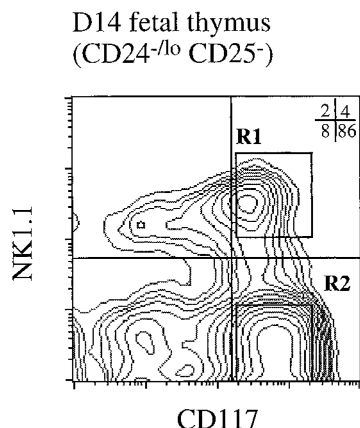


**FIGURE 1.** Flow cytometric analysis of TLP and TNK populations within the fetal thymus. Day 14 fetal thymocytes, depleted of CD24$^{high}$ and CD25$^{+}$ cells by Ab complement-mediated cell lysis, were analyzed for surface expression of NK1.1 and CD117. Gates used for the isolation of TNKs (R1) and TLPs (R2) cells are shown.

FTOC media supplemented with 10 ng/ml stem cell factor (SCF) (R&D Systems, Minneapolis, MN).

*Single-cell reconstitution of FTOCs*

Sorted donor cells (CD117$^{+}$NK1.1$^{+}$Ly-9.1$^{+}$) were resuspended in media containing 10 ng/ml SCF at 0.3 cell/25 $\mu$l and then transferred to a Terasaki plate (Nalge Nunc, Naperville, IL) at 25 $\mu$l/well. Each well was checked by examination under an inverted microscope to verify that only a single cell was present. Day 15 host fetal thymic lobes from timed-pregnant C57BL/6 mice (Ly-9.2$^{+}$) were irradiated with 30 Gy (Gammacell-1000, MDS Nordion, Kanata, ON, Canada), and one lobe was then transferred to a well of a Terasaki plate containing a single donor cell. Terasaki plates were inverted ("hanging drop") and cultures were incubated at 37°C in a humidified incubator containing 5% $CO_2$ for 24 h. Lobes were then transferred to standard FTOC for 14 days, in the absence of exogenously added cytokines. A similar procedure was performed for bulk reconstitutions of FTOCs, in which 2000 sorted donor cells were added to each well before transferring the irradiated fetal thymic lobe. Cell suspensions from reconstituted thymic lobes were analyzed by flow cytometry.

*OP9 stromal cell line coculture*

Sorted cell populations were prepared as described above and used in parallel with FTOC reconstitution assays; 1, 3, 10, 30, or 100 donor cells were cocultured in medium for 10 days on confluent monolayers (96-well flat-bottom plates) of OP9 cells (8) in the presence of IL-3, IL-6, IL-7, and SCF (50 ng/ml each). Cells were then harvested for flow cytometric analysis.

## Results and Discussion

Day 14 fetal thymocytes, depleted of CD24$^{high}$ and CD25$^{+}$ cells, were analyzed for surface expression of CD117 and NK1.1 (Fig. 1). This analysis revealed the presence of two distinct CD117$^{+}$ populations that differed in their surface expression of NK1.1. We previously described that the CD117$^{+}$NK1.1$^{-}$ population (TLPs) corresponds to progenitor thymocytes with B, T, and NK cell precursor potential (8), whereas the CD117$^{+}$NK1.1$^{+}$ population (TNKs) is restricted to the T and NK cell lineages (8). Thus, characterization of this latter population supported the notion that a common T and NK cell progenitor is present within the fetal thymus. However, the identification of a single bipotent cell with T and NK cell lineage potential within the fetal TNK cell population has not been demonstrated. To this end, we used flow cytometry to isolate TNK cells (R1) and TLP cells (R2) (Fig. 1) and then tested the ability of each to reconstitute irradiated FTOCs.

Fig. 2 shows host irradiated FTOCs that have been reconstituted with TLPs (R2 gate; Fig. 1), in which donor cells can be identified by the surface expression of Ly-9.1 (donor Swiss.NIH thymocytes), whereas host cells express the Ly-9.2 allele (host C57BL/6 FTOCs). Flow cytometric analysis of donor-derived cells (Ly-9.1$^{+}$, R3-gated), revealed that both T and NK cells can be recovered from FTOCs reconstituted with 2000 TLPs, as determined by surface expression of $\alpha\beta$-TCR and NK1.1 on distinct populations of cells (Fig. 2). Unreconstituted host irradiated FTOCs remained devoid of lymphocytes and did not contain Ly-9.1$^{+}$ cells (Fig. 2). As previously described (8, 15), donor-derived NK1.1$^{+}$ cells lacked expression of $\alpha\beta$-TCR and therefore corresponded to mature and functional NK cells, rather than the unique subset of T cells expressing NK1.1 (16). Moreover, further analysis showed that donor-derived NK1.1$^{+}$ cells also lacked surface expression of CD3$\epsilon$, indicating these cells do not express TCR/CD3 complex on the cell surface (data not shown) (8, 15). Together with our previous findings, these results indicate that both T and NK cells can be generated in FTOCs and that this assay serves to identify the lineage potential of progenitor thymocytes.

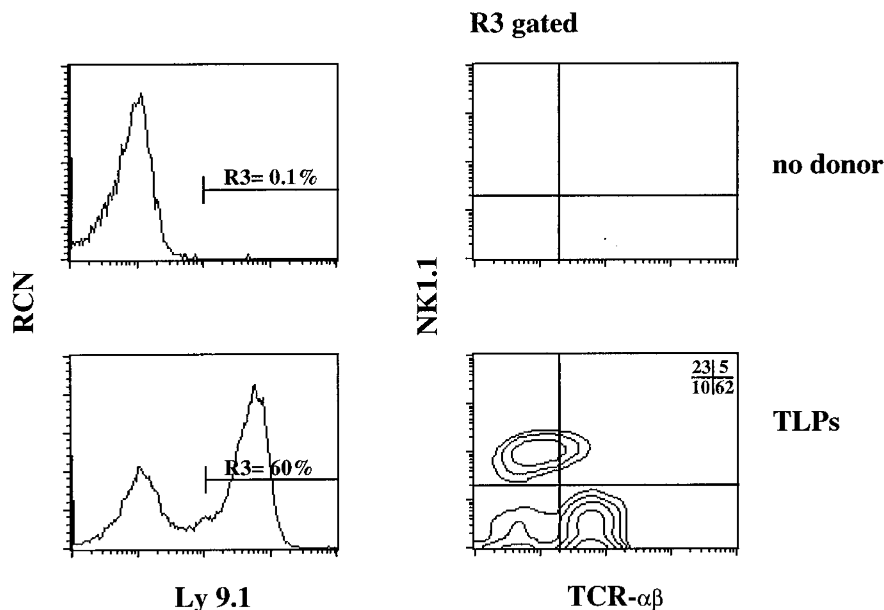


**FIGURE 2.** Analysis of host irradiated FTOCs reconstituted with fetal TLPs. Host irradiated (Ly-9.1$^{-}$) FTOCs were reconstituted with freshly sorted (Ly-9.1$^{+}$) TLP cells (2000 cells/thymic lobe). After 14 days, FTOCs were analyzed by flow cytometry. *Left,* analysis for Ly-9.1 surface expression (*top,* no donor; *bottom,* TLPs). *Right,* analysis for surface expression of TCR-$\alpha\beta$ and NK1.1 of donor-derived Ly-9.1$^{+}$ cells (R3 gated).

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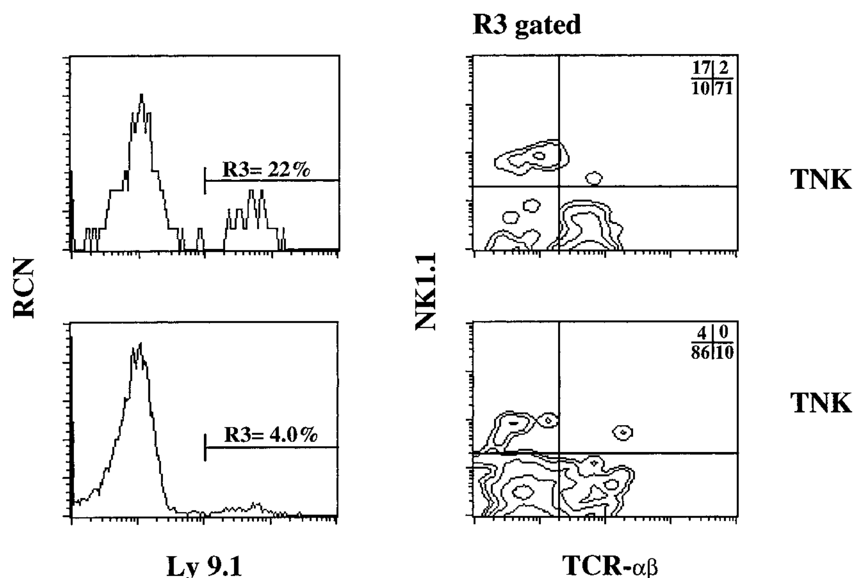


**FIGURE 3.** Analysis of host irradiated FTOCs reconstituted with a single fetal TNK cell. Host irradiated (Ly-9.1$^-$) FTOCs were reconstituted with a single cell from a freshly sorted (Ly-9.1$^+$) TNK population (1 cell/thymic lobe). After 14 days, each FTOC was analyzed by flow cytometry. *Left,* analysis for Ly-9.1 surface expression. *Right,* analysis for surface expression of TCR-$\alpha\beta$ and NK1.1 of donor-derived Ly-9.1$^+$ cells (R3 gated).

To address whether the TNK progenitor cells comprise a homogeneous population of T- and NK-restricted precursors or represent a collection of phenotypically similar T or NK lineage-committed cells, we performed a clonal assay in which a single cell was used to reconstitute host irradiated FTOCs. CD117$^+$NK1.1$^+$CD90$^{low}$CD25$^-$ TNK cells were micromanipulated to ensure that a single cell was used to reconstitute host FTOCs (see *Materials and Methods*). Fig. 3 shows that a single TNK progenitor cell possesses the ability to differentiate and give rise to both $\alpha\beta$-TCR$^+$NK1.1$^-$ T cells and $\alpha\beta$-TCR$^-$NK1.1$^+$NK cells. Two representative FTOCs are shown to indicate the varying levels of reconstitution observed in this assay (Fig. 3; 1–22% Ly-9.1$^+$ donor cells).

In addition, our analyses identified TNK progenitor cells that gave rise only to either T cells or NK cells (Table I). Thus, it appears that the TNK population contains T and NK bipotent cells (Fig. 3), as well as cells that are precommitted to the T or NK cell lineages. However, it is difficult to resolve whether precommitted cells were contained within the TNK cell phenotypic subset, or whether these cells were derived from a bipotent cell that stochastically committed to either cell lineage at the start of the assay. In this regard, the existence of committed T cell progenitors within an early subset of fetal thymocytes has been previously described by Katsura et al. (4). However, this report did not address whether the cells that were shown to be restricted to the T cell lineage were also capable of giving rise to NK cells. Therefore, our findings represent the first evidence for the existence of a precommitted T cell subset within the TNK progenitor population.

Table I. *Cell lineage potential of a single TNK progenitor cell in fetal thymic organ cultures*[a]

| Donor-Derived Cell Lineages (Ly-9.1$^+$) | No. of FTOCs Reconstituted ($n$ = 44) |
|---|---|
| T and NK | 8 (18%) |
| T only | 2 (5%) |
| NK only | 8 (18%) |
| Nonreconstituted | 26 (59%) |

[a] Host irradiated FTOCs received a single TNK progenitor cell (CD117$^+$NK1.1$^+$CD90$^{low}$CD25$^-$) and were cultured for 14 days. After this time, the FTOCs were analyzed by flow cytometry for TCR-$\alpha\beta$, NK1.1, and Ly9.1 surface expression to determine the level of reconstitution. FTOCs showing $<$1% Ly9.1$^+$ cells were scored as nonreconstituted.

The data summarized in Table I indicates that several irradiated FTOCs receiving a single cell contained donor-derived T and NK cells (8 of 44 lobes), although the majority of the FTOCs failed to reconstitute (26 of 44 lobes showed no donor cells). We also observed lobes in which only T or NK cells were generated (Table I). Notably, the majority of these lobes contained only NK cells. This observation provides support for the notion that the NK cell fate may represent a default pathway for TNK progenitors. Alternatively, it is conceivable that TNK cells may represent progenitors that have already begun to differentiate exclusively toward the NK cell lineage. Both circumstances are consistent with the possibility that manipulation of TNK cells and time spent outside the thymic microenvironment may contribute to NK cell lineage bias, which would account for the observed prevalence of NK cell-only reconstituted lobes.

To determine whether TNK progenitors were potentially biased toward the NK cell lineage, we performed in vitro clonogenic assays, in which progenitors were cocultured with the bone marrow-derived stromal cell line, OP9. This stromal cell line has been shown to support the differentiation of TLPs (Fig. 1; R2) toward the B and NK cell lineages (8). In particular, TNK progenitors cocultured on OP9 cells failed to generate B cells (up to 300 cells/well), while even at the clonal level mature functional NK cells were routinely recovered (data not shown) (8). Thus, taken together with the data shown in Table I, these results further establish that TNKs cells in the absence of continued thymic influence proceed down an NK cell differentiation pathway. Indeed, these results are consistent with the fact that NK cell differentiation generally occurs extrathymically (17, 18), thus supporting our notion that in vitro manipulation of TNK progenitors promotes the default pathway of NK cell differentiation.

Although the existence of a common precursor for both T and NK cells present within the mouse thymus was suggested many years ago (8, 19, 20), its identification at the clonal level has never been demonstrated. Nevertheless, a common T and NK cell progenitor has been characterized within a subset of human fetal thymocytes (21, 22). In this regard, our results close the unresolved dilemma pertaining to the existence of a similar bipotent progenitor within the mouse fetal thymus.

This report uses a previously characterized population of T and NK cell-restricted progenitor thymocytes (8) to determine the existence of a common T and NK cell progenitor. Indeed, our

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findings conclusively demonstrate that cells within this population contain a true common bipotent progenitor for both lineages. Therefore, this report sheds light on the events that occur during lymphocyte lineage commitment by finally identifying, at the clonal level, a mouse progenitor thymocyte capable of giving rise to T cells and NK cells. This new understanding should facilitate the further molecular characterization of key genetic events responsible for T and NK cell lineage commitment.

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