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Regulation of NK1.1 Expression During Lineage Commitment of Progenitor Thymocytes

James R. Carlyle and Juan Carlos Zúñiga-Pflücker

We recently identified a stage in fetal ontogeny (NK1.1+/CD117+) that defines committed progenitors for T and NK lymphocytes. These cells are found in the fetal thymus as early as day 13 of gestation, but are absent in the fetal liver. Nonetheless, multipotent precursors derived from both the fetal thymus and fetal liver are capable of rapidly differentiating to the NK1.1+ stage upon transfer into fetal thymic organ culture (FTOC). This suggests that expression of NK1.1 marks a thymus-induced lineage commitment event. We now report that a subset of the most immature fetal thymocytes (NK1.1+/CD117+) is capable of up-regulating NK1.1 expression spontaneously upon short-term in vitro culture. Interestingly, fetal liver-derived CD117+ precursors remain NK1.1− upon similar culture. Spontaneous up-regulation of NK1.1 surface expression is minimally affected by transcriptional blockade, mitogen-induced activation, or exposure of these cells to exogenous cytokines or stromal cells. These data suggest that induction of NK1.1 expression on cultured thymocytes may be predetermined by exposure to the thymic microenvironment in vivo. Importantly, multipotent CD117+ thymocytes subdivided on the basis of NK1.1 expression after short-term in vitro culture show distinct precursor potential in lymphocyte lineage reconstitution assays. This demonstrates that even the earliest precursor thymocyte population, although phenotypically homogeneous, contains a functionally heterogeneous subset of lineage-committed progenitors. These findings characterize a thymus-induced pathway in the control of lymphocyte lineage commitment to the T and NK cell fates. The Journal of Immunology, 1998, 161: 6544–6551.

The thymus is formed during embryonic development from an involution of the third pharyngeal pouch between day 10–11 of gestation (1). In addition to cells of hematopoietic origin, the thymic anlage includes epithelial and mesenchymal tissues, both of which are required for functional thymopoiesis (2, 3). It is believed that the early thymic rudiment is first colonized by definitive hematopoietic stem cells (HSCs) by day 11–12 of gestation (4). These precursors travel via the fetal circulation and originate from early sites of primary hematopoiesis, such as the embryonic aorta-gonad-mesonephros region and the fetal liver (FL) (5–7). Later in life, the neonatal and adult bone marrow serve as the primary source of precursors seeding the thymus (8). Both fetal and adult thymopoiesis depend on a continual source of these hematopoietic progenitors (9). Yet, despite intensive investigations, the phenotype and precursor potential of these cells remain controversial.

The earliest precursor population to colonize the thymus contains multipotent hematopoietic potential for both the lymphoid and myeloid lineages (10, 11). These cells reside in the fetal thymic rudiment at day 12 of gestation and are phenotypically and functionally similar to HSCs (11, 12). However, between days 12–14 of gestation, recoverable myeloid potential within the most immature thymocyte population rapidly diminishes (10), such that after day 13 of gestation and throughout adult life, only lymphoid potential can be rescued intrathymically (13–16). This suggests that the characteristics of either the thymic microenvironment or the thymus-colonizing precursors themselves change during development. In keeping with this, the day 11–12 fetal thymic rudiment does not appear to be optimally capable of supporting αβ T lymphopoiesis (12, 17). This indicates that there may be a delayed functional maturation of the thymic microenvironment itself, which includes its ability to efficiently induce lymphoid lineage commitment of incoming precursors. Alternatively, there is also evidence in support of cell-autonomous differentiation of developing hematopoietic precursors. This includes the finding that full lymphohematopoietic potential among HSCs appears to undergo maturation from a primitive state to a definitive precursor during embryonic development (5, 6, 18). Furthermore, there is recent evidence that restricted common lymphoid progenitors exist in adult bone marrow, suggesting that similar precursors may develop during fetal ontogeny, and that these cells may be predominantly responsible for mobilization and homing to the thymus (19).

In any case, the most immature hematopoietic 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 (9, 15, 16, 20). Collectively termed thymic lymphoid progenitors (TLPs), these multipotent cells are characterized by high-level expression of CD117 (c-kit) and a lack of expression of hematopoietic lineage (Lin−) differentiation markers (9, 15, 16). Whether these cells comprise a homogeneous population of lymphoid-restricted precursors or represent a collection of phenotypically similar lineage-committed cells is unknown. However, we recently identified a stage in fetal thymic ontogeny, marked by coexpression of NK1.1 and CD117, which characterizes progenitors committed to the T and NK cell fates (16). Termed fetal TNK progenitors, cells at this stage are...
phenotypically similar to TLPs and may have been previously included among the TLP population (15). Upon reconstitution of a lymphoid fetal thymic lobes in vitro, sorted NK1.1\(^+/\)CD117\(^-\) TLPs and FL-derived hematopoietic precursors rapidly give rise to TNK progenitors (16), suggesting that the latter population is separated by a thymus-induced lineage commitment event. In an effort to further elucidate the requirements for progression to the TNK stage, we cultured fetal TLPs and FL-derived precursors with exogenous cytokine combinations in vitro. Surprisingly, fetal TLPs, but not FL-derived cells, were capable of efficiently and spontaneously up-regulating NK1.1 surface expression during short-term (48 h) in vitro culture, without a requirement for exogenous stimuli. This suggests that spontaneous progression to the TNK stage reflects a thymus-induced differentiation signal. Furthermore, this phenotypic change corresponds to a lineage commitment event to the T/NK cell fates. These findings indicate that the TLP population, although phenotypically homogeneous, contains functionally heterogeneous subsets of lineage-committed precursors. In this report, we investigate this phenomenon to gain insight into the requirements for commitment to the T/NK cell lineages from multipotent precursors.

Materials and Methods

Mice

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

Isolation of fetal cells

Fetal thymus (FT) and FL were harvested, washed three times in 5 ml complete medium (DMEM medium 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), and disrupted through 70-mM nylon mesh using a syringe plunger. Viable cells were recovered by discontinuous density gradient centrifugation over Lympholyte-Mammal (Cedar Lane, Hornby, Ontario, Canada). CD24\(^-\)/25\(^-\) fetal cells were obtained by Ab-complement-mediated lysis, as described previously (16). Briefly, 50–200 \(\mu\)l of anti-CD24 (J11d.2) and anti-CD25 (7D4) culture supernatant and a 1/10 dilution of Low-Tox rabbit complement (Cedar Lane) were added to single-cell suspensions in 2–3 ml complete 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 and washed before analysis.

Flow cytometric analysis and cell sorting

FITC-, phycoerythrin-, biotin-, and APC-conjugated mAbs, and streptavidin-APC, were obtained from PharMingen (San Diego, CA). Cell suspensions were stained in 50 \(\mu\)l staining buffer (HBSS, without phenol red, plus 1% BSA and 0.05% Na\(_3\)citrate) 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 was live-gated by forward/side light scatter and lack of propidium iodide uptake. All plots display 10,000 events; 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 Na\(_3\)citrate was added to staining buffer. Cells were sorted using a Coulter Elite cytometer (Hialeah, FL); sorted cells were \(\geq 99\%\) pure, as determined by postsort analysis.

In vitro cell culture

Sorted NK1.1\(^+/\)CD117\(^-\) cells (on ice) were washed twice and cultured in complete medium in 96-well round-bottom plates at 10\(^4\) to 5 \times 10\(^5\) cells/well at 37°C in a humidified incubator containing 5% CO\(_2\). Where indicated, the cytokines IL-3, IL-6, IL-7, and stem cell factor (SCF) were added (50 ng/ml each cytokine) to wells. Cocultures with fibroblasts or OP9 cells were performed by adding sorted precursors to wells containing confluent layers of stromal cells. RNA polymerase II transcriptional inhibition was achieved by adding \(\alpha\)-amanitin (10 \(\mu\)g/ml) (Boehringer Mannheim, Indianapolis, IN) to wells immediately before incubation at 37°C. Induction of CD25 surface expression in splenocytes following Con A activation (12 h) was completely blocked with the addition of 10 \(\mu\)g/ml of \(\alpha\)-amanitin (data not shown). PMA (10 ng/ml) (Sigma, St. Louis, MO) and ionomycin (1 ng/ml) (Sigma) were added immediately before incubation at 37°C.

RT-PCR

Single-cell suspensions prepared from total FL cells and thymocytes were depleted of CD24\(^+\)/25\(^-\) cells before sorting for NK1.1\(^+/\)CD117\(^+\) (TLP, FL) and NK1.1\(^-/\)CD117\(^+\) (NK) cells. Total RNA was isolated using the Trizol RNA isolation protocol (Life Technologies, Gaithersburg, MD). RNA was resuspended in 25 \(\mu\)l diethylpyrocarbonate (DEPC)-treated (0.1%\) DH\(_2\)O. cDNA was prepared from 300 ng of each RNA using random hexamer primers and the cDNA Cycle kit (Invitrogen, San Diego, CA). Subsequent PCR analysis was performed on an automated GeneAmp 9600 thermocycler (Perkin-Elmer, Norwalk, CT) using 20 ng of template DNA at 94°C, 30 s annealing at 60°C, and 1 min extensions at 72°C for 35 cycles, with a hot start at 94°C for 2 min and a final extension at 72°C for 6 min. PCR reactions were performed using a 20-ng equivalent of each cDNA on the same cDNA batches as shown for \(\beta\)-actin, and all PCR products correspond to the expected molecular sizes. Reverse transcriptase reactions done in the absence of avian myeloblastosis virus reverse transcriptase were included as controls. Gene-specific primers used for PCR were as follows (5’→3’): \(\beta\)-actin (5’), GAT GAC GAT ATC GCT GCG CTG; \(\beta\)-actin (3’), GTA CGA CCA GAG GCA TAC AGG; NKR-P1A/B/C (5’), AAG GTC ATT GCC AGA CAT; NKR-P1A/B/C (3’), GTA GAC ATG GCT CAG TTA TTG; NKR-P1B/C (3’), GGA CAG GGG AGA GGA AGT GAT GGA GAT GTA; NKR-P1C (3’), GAG TCA ACG AAT GGA AAG GAA. Products were separated by agarose gel electrophoresis on a 1.6% gel, and visualized by ethidium bromide staining; reverse electronic photo images are shown.

5-(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) vital dye labeling

CFSE (Molecular Probes, Eugene, OR) was a kind gift of Dr. Pamela Ohashi (Toronto, ON). Cells were labeled with CFSE, as previously described (21). Briefly, cells were washed in HBSS to remove protein, and resuspended in HBSS plus CFSE (final concentration, 0.5 \(\mu\)M; stock CFSE, 0.5 mM in DMSO). Cells were labeled for 10 min at 37°C, then washed three times in complete medium before culture. Control cultures for staining calibration and compensation were conducted in parallel by incubating cells at 4°C. After incubation, cells were prepared and analyzed by flow cytometry. Relative CFSE fluorescence was monitored in the FL1 parameter on a FACSCalibur flow cytometer.

Fetal thymic organ culture (FTOC) reconstitution

Lymphocyte-depleted thymic lobes were prepared by culturing day 15 fetal thymic lobes from timed-pregnant Sw mice in complete medium containing 1.0 mM deoxyguanosine (dG) for 5 days, as previously described (22, 23). Host dG-treated alymphoid fetal thymic lobes in Terasaki plates. After adding donor cells or complete medium alone, 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 12 days. 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. A total of 1 x 10\(^5\) donor cells were cocultured in complete medium for 7 days on confluent monolayers (6-well plates) of OP9 cells (24) in the presence of IL-3, IL-6, IL-7, and SCF (50 ng/ml each). Cells were then stimulated on a fresh OP9 monolayer in IL-7 and IL-2 for an additional 6 days before harvesting for flow cytometry.

Results

A subset of fetal progenitor thymocytes spontaneously up-regulates NK1.1 expression ex vivo

We recently identified a stage in fetal thymic ontogeny characterized by coexpression of NK1.1 and CD117, which marks the loss of precursor potential for B lymphocytes, while that for the T and

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NK cell fates is maintained (16). Early hematopoietic progenitors (NK1.1+/CD117+) in the FT and FL rapidly differentiate to the NK1.1+/CD117+ TNK stage after exposure to fetal thymic stroma in FTOC. To investigate the effects of cytokines on the survival and differentiation of early precursor thymocytes, we cultured sorted precursors under conditions shown to maintain precursor multipotency in vitro (20). We have previously demonstrated that sorted TNK (NK1.1+/CD117+) progenitors cultured under these conditions do not undergo further differentiation in vitro, unless cocultured with bone marrow-derived stromal cells to induce commitment to the NK lineage (16). However, the effects of culture on the TLP population remain unknown.

Fig. 1 shows NK1.1 vs CD117 expression on sorted NK1.1+/CD117+ fetal thymocytes (TLps) and FL precursors before and after in vitro culture in the presence of exogenous IL-3, IL-6, IL-7, and SCF, as previously described (20). Strikingly, almost half of sorted fetal thymocytes spontaneously up-regulate NK1.1 expression after 48 h under these conditions (Fig. 1b, FT). This effect is common to two NK1.1-expressing strains of mice, as revealed by comparison of Sw and C57BL/6 thymocytes (Fig. 1b, Sw vs B6, respectively). In contrast, sorted FL precursors from each of these strains remain predominantly NK1.1+ upon similar culture (Fig. 1b, FL). Nevertheless, we previously demonstrated that a subset of FL precursors does up-regulate NK1.1 expression after exposure to thymic stroma in FTOC (16). Taken together, these findings suggest that differentiation to the NK1.1+/CD117+ TNK stage is induced by either prior or continued exposure to the fetal thymic microenvironment. Thus, expression of NK1.1 in vitro may represent a delayed but passive progression to a developmental stage that reflects the receipt of a differentiation signal in vivo. Alternatively, this phenomenon might represent a default pathway of T/NK lymphocyte differentiation, which may be actively induced in the absence of an environment efficiently capable of supporting T lineage commitment and differentiation. As up-regulation of NK1.1 expression on TLPs was substantial within 48 h culture ex vivo, we investigated the kinetics further in an attempt to delineate between these possibilities.

**Spontaneous expression of NK1.1 occurs rapidly in vitro**

To examine the temporal requirements for NK1.1 expression on precursor thymocytes, we assessed their phenotype at different intervals under conditions identical to those in Fig. 1B. As shown in Fig. 1c, surface expression of NK1.1 on sorted TLPs becomes evident as early as 16 h after removal from the thymus. Furthermore, the majority of thymocytes up-regulate NK1.1 surface expression within 48 h, with only a moderate increase after this time point. Importantly, the maintenance of a subset of precursors with a TLP phenotype, even after 6 days in vitro, indicates that not all thymocytes undergo differentiation in vitro. Similarly, up-regulation of NK1.1 does not occur at any time point on FL-derived precursors, indicating that this effect is specific to only a subset of fetal thymocytes. Taken together, these rapid and specific kinetics of differentiation may indicate that only a subset of fetal thymocyte precursors have received an irreversible signal to differentiate and that the effect of this signal is manifest in phenotype over the short-term culture period. Alternatively, due to the distinct lineage potential of the precursor populations involved (16), the conditions of culture might be directly affecting the fetal thymocytes in a differential manner from FL precursors. To address this, we cultured these two precursor subsets under various culture conditions, ranging from minimal survival requirements to a bone marrow-derived stromal coculture environment strongly supportive of B and NK lymphoid differentiation (16, 25).

**Spontaneous up-regulation of NK1.1 on precursor thymocytes represents a cell fate predetermined by exposure to fetal thymic stroma**

Although the combination of IL-3, IL-6, IL-7, and SCF have been shown previously to augment proliferation of the earliest CD117+ thymic precursors and maintain their precursor potential in vitro, the effect of these cytokines on the overall phenotype was not determined (20). Nonetheless, in agreement with our observations, these studies indicated that SCF, the ligand for CD117, appears to be necessary for the survival of these progenitors when cultured in isolation (20, 26). Therefore, we tested whether up-regulation of NK1.1 after 48 h in vitro occurred in the presence of SCF alone. Fig. 2 shows that there was little difference in NK1.1 up-regulation on cultured fetal thymocytes and FL precursors whether cultured in SCF alone (Fig. 2, SCF) or in the presence of SCF plus IL-3, IL-6, and IL-7 (Fig. 2, SCF + IL-3,6,7). Thus, as previously shown (20), these cytokines do not appear to have a differentiation-inducing effect on these precursors in vitro. In keeping with this, CD117+ FL cells obtained from several different days of gestation (days 12–14) and cultured with various cytokine cocktails for ≥8 days showed no significant up-regulation of NK1.1 expression (data not shown).

We next tested the effect of coculture of precursor thymocytes with the bone marrow-derived stromal cell line, OP9 (24), which efficiently induces and supports B and NK lineage differentiation (16, 25). In particular, OP9 coculture induces the rapid (48-h) down-regulation of CD117 expression on sorted NK1.1+/CD117+ TNK precursors and results in a predominant induction of B lineage differentiation upon coculture of NK1.1+/CD117+ TLPs (16). Interestingly, OP9 had no significant effect on spontaneous NK1.1 expression, and only a minor effect on CD117 expression, when cocultured with either precursor subset (Fig. 2, SCF + stroma, SCF + IL-3,6,7 + stroma). A similar result was observed upon coculture of these precursors with fibroblasts (data not shown). Thus, it appears that spontaneous up-regulation of NK1.1 expression reflects the induction of a cell fate predetermined by exposure to fetal thymic stroma in vivo. Therefore, we examined the transcriptional regulation of NK1.1 expression on fresh and cultured thymocytes.

**Initiation of NK1.1 transcription occurs in vivo**

Spontaneous up-regulation of NK1.1 on precursor thymocytes appears to be a predetermined cell fate, which occurs within the thymic microenvironment during the course of normal lineage commitment. However, initiation of NK1.1 expression might occur in vitro simply as a consequence of removing these cells from the thymus and culturing them in isolation. In the latter case, NK1.1 expression might reflect the induction of a default pathway of differentiation. To distinguish between these possibilities, we examined NK1.1 expression at the transcriptional level using the RNA polymerase II inhibitor, α-amanitin, to block nascent transcription of mRNA in vitro. As a control, Con A-stimulated splenocytes treated with α-amanitin showed a complete block in the induction of CD25 surface expression (data not shown). Fig. 3a shows fetal thymocytes and FL precursors cultured for 24 h without (Fig. 3a, 24-h control) and with (Fig. 3a, 24 h + α-amanitin) pharmacological blockade of transcription. A 24-h time point was used due to the toxicity resulting from global transcriptional repression for longer time periods. Although α-amanitin treatment reduced both the fraction of NK1.1+ thymocytes and NK1.1 expression levels significantly (approximately 50%, Fig. 3a), it failed...
to completely block this induction, suggesting that at least a subset of thymocytes contained pre-existing transcripts for NKR-P1C (CD161), a ligand for the anti-NK1.1 mAb (27). To investigate this further, we employed RT-PCR on thymocytes fresh ex vivo.

Fig. 3b shows analysis of NKR-P1 gene expression on RNA isolated from fresh day 15 fetal thymocytes and FL cells (25). RT-PCR analysis reveals that sorted NK1.1<sup>+</sup>/CD117<sup>+</sup> TLPs (Fig. 3b, TLP) express pre-existing transcripts for members of the...
NKR-P1 family (28), albeit at reduced levels compared with the sorted NK1.1^+/CD117^- mature NK cell cohort (Fig. 3b, NK). This may be due to the fact that some fetal thymocytes, which have just started to express NKR-P1C mRNA, may not yet express sufficient levels of NK1.1 Ag on the cell surface to be detected as positive by flow cytometry. Therefore, these cells will be sorted within the NK1.1-negative fraction as part of the TLP population. Importantly, control PCR reactions done in the absence of reverse transcriptase (Fig. 3b, Con) and RT-PCR of sorted NK1.1^+ / CD117^- FL cells (Fig. 3b, FL) did not yield significant product for the NKR-P1 genes. These data suggest that surface NK1.1^+ / CD117^- fetal thymocytes and FL cells differentially regulate endogenous expression of the NKR-P1 genes, a finding that supports their phenotype change during short-term in vitro culture. Thus, although this does not rule out the possibility that NK1.1 expression in vitro may additionally reflect a delayed in vivo signal or default in vitro signal, at least some of the surface staining for NK1.1 observed upon culture of thymocytes is likely the result of direct translation of pre-existing mRNA transcripts (Fig. 3b). The presence of NKR-P1C transcripts among freshly sorted NK1.1^- thymocytes and the failure of transcriptional blockade to completely inhibit NK1.1 expression suggest that up-regulation of

**FIGURE 2.** Spontaneous up-regulation of NK1.1 on CD117^+ progenitor thymocytes is not affected by exposure to exogenous cytokines or stromal cells. Flow cytometric analysis of sorted NK1.1^+ / CD117^- fetal thymocytes and FL cells (Sw) cultured for 48 h with or without the cytokines IL-3,6,7 and/or OP9 bone marrow-derived stromal cells (or fibroblasts, not shown), as indicated. SCF was added to all cultures to maintain viability.

**FIGURE 3.** Spontaneous up-regulation of NK1.1 on CD117^+ progenitor thymocytes is minimally affected by transcriptional blockade or mitogen-induced activation. a, Flow cytometric analysis of sorted NK1.1^+ / CD117^- fetal thymocytes and FL cells (Sw) cultured with SCF + IL-3,6,7 for 24 h with or without the RNA polymerase II inhibitor, α-amanitin, as indicated. b, RT-PCR for NKR-P1 genes on RNA prepared from fresh sorted NK1.1^+ / CD117^- (TLP) and NK1.1^+ / CD117^- (NK) fetal thymocytes and FL cells from Sw mice. The control (Con) sample is as in FL without the addition of reverse transcriptase prior to PCR. c, Flow cytometric analysis of sorted NK1.1^+ / CD117^- fetal thymocytes and FL cells (Sw) cultured with SCF + IL-3,6,7 for 24 h in the presence of PMA/ionomycin. d, Sorted NK1.1^+ / CD117^- fetal thymocytes and FL cells (Sw) were cultured with SCF + IL-3,6,7 for 48 h, then resorted for NK1.1^+ / CD117^- cells, and further cultured for 72 h (with SCF + IL-3,6,7), prior to analysis by flow cytometry.
NK1.1 on precursor thymocytes is a cell fate predetermined by exposure to thymic stroma in vivo.

We next determined whether up-regulation of NK1.1 on precursor thymocytes could be mimicked by mitogenic activation of these cells. Pharmacological treatment using phorbol ester and calcium ionophore induces expression of CD25 to high levels on precursor thymocytes, mimicking progression to the pro-T cell stage (14). A similar effect is induced upon treatment of these cells with the cytokines, TNF-α and IL-1α (14). Interestingly, these treatments fail to augment CD25 expression on FL precursors (J.R.C. and J.C.Z.-P., unpublished observations). Therefore, we tested the effect of these treatments on NK1.1 expression in these cells. Treatment of sorted fetal thymocytes and FL cells with PMA and ionomycin only had a marginal effect on NK1.1 expression (Fig. 3c, 24h + PMA/iono; compare Fig. 3a, control). Furthermore, treatment with TNF-α and IL-1α had no significant effect (data not shown). Minimally, these data indicate that CD25 and NK1.1 expression are regulated by distinct pathways in precursor thymocytes, and that NK1.1 up-regulation is not due to generalized activation, at least that mediated through protein kinase C and Ca2+ flux.

The kinetic data presented in Fig. 1c suggest that the majority of thymocyte precursors up-regulate expression within 48 h after removal from the thymus and that a substantial subset remains NK1.1+ in longer culture. This suggests that a subset of fetal thymocyte precursors, possibly those which have not received a thymic signal, are analogous to FL precursors. To test this, we cultured fetal thymocytes ex vivo for 48 h, then resorted the NK1.1+ fraction and retested their propensity for NK1.1 up-regulation in vitro thereafter. As shown in Fig. 3d, over 90% of these cells remained NK1.1+ over the next 72 h (compare Fig. 3d with Fig. 1c). This lends further support to the notion that up-regulation of NK1.1 on thymocyte precursors represents a delayed phenotypic change in response to a signal received intrathymically in vivo.

**Spontaneous up-regulation of NK1.1 represents a differentiation event and is not due to outgrowth of NK1.1+ cells**

Due to the large percentage of NK1.1+ precursors that appear after culture, it is possible that they represent the outgrowth of a small number of differentiating cells. To test this, we sorted fresh thymocytes ex vivo for an NK1.1+/CD117+ phenotype and labeled them in vitro with the vital dye, CFSE, before culture. CFSE is a fluorescent membrane-localizing dye partitioned evenly into daughter cells upon division, and it allows the visualization of proliferating cells by revealing twofold stepwise decreases in green fluorescence detectable by flow cytometry (21). Fig. 4 shows fetal thymocytes and FL precursors cultured for 48 h after CFSE labeling. Cells cultured at 4°C, and thus prevented from proliferating, displayed no apparent loss in fluorescence intensity (Fig. 4, top panel). This data also indicates that both sets of progenitors were labeled with equal efficiency. However, when cultured at 37°C, FL precursors were found to divide more rapidly than the majority of fetal thymocytes, as indicated by their lower relative fluorescence (Fig. 4, middle panel). However, when cultured thymocytes were gated according to their NK1.1 phenotype, NK1.1+ precursors were found to divide at a rate similar to FL cells, while the NK1.1− subset divided approximately one cell division less overall (Fig. 4, bottom panel). This indicates that the NK1.1+ fetal thymocyte subset does not outgrow in culture, rather these cells undergo a differentiation event that appears to slow their proliferation relative to the NK1.1− fraction.

**Up-regulation of NK1.1 marks lineage commitment to the T/NK lymphocyte fates, with the subsequent loss of B lymphoid potential**

We previously demonstrated that NK1.1+/CD117+ thymocytes sorted fresh ex vivo are capable of generating T and NK cells, but lack potential for B lymphocytes in lineage reconstitution assays (16). Furthermore, NK1.1+/CD117+ TLPs are capable of giving rise to all three lineages. Therefore, to determine whether spontaneous up-regulation of NK1.1 in vitro recapitulated in vivo lineage commitment, we tested the lymphocyte potential of cultured TLPs subdivided according to their NK1.1 phenotype. Fig. 5a shows the gates used to sort purified TLPs cultured for 48 h in vitro. Reconstitution of dG-depleted FTOCs with both the NK1.1+ (Fig. 5a, R1) and NK1.1− (Fig. 5a, R2) subsets resulted in the generation of both CD3+ T cells and NK1.1+ NK cells (Fig. 5b). As previously demonstrated ex vivo (16), T lineage progeny consisted of both immature CD4/CD8 double-positive and mature single-positive conventional T cells, while NK cells lacked expression of CD3e and TCR-αβ (data not shown). DNA isolated from sorted T lineage cells also possessed D-J rearrangements at the TCR-β loci, while that from NK cells was retained in the germline TCR-β configuration (data not shown). Thus, both populations possess potential for T and NK cells in FTOC.

However, coculture of the same subsets with the bone marrow-derived stromal cell line, OP9 (24), reveals that they possess distinct reconstitution potential for the B lymphocyte lineage (Fig. 5c). Specifically, similar to their ex vivo counterparts (16), the NK1.1+ precursor fraction was capable of generating only NK lineage cells, while the remaining NK1.1− subset gave rise to both NK cells and B lymphocytes on OP9 (Fig. 5c). B lineage cells
were identified by their CD45R<sup>−</sup>/NK1.1<sup>−</sup> phenotype (Fig. 5c), expression of CD19, and detection of D-J rearrangements at the IgH loci from isolated DNA (data not shown). NK cells derived from OP9 coculture were CD45R<sup>−</sup>/NK1.1<sup>−</sup> (Fig. 5c), a subset also expressed the pan-NK cell marker, DX5 (29, 30), and DNA derived from these cells was retained in the germline IgH configuration (data not shown). Taken together, these data indicate that the up-regulation of NK1.1 on the surface of TLPs cultured in vitro corresponds to a bona fide lineage commitment event identical to the TLP to TNK transition in vivo (16). Thus, a subset of TLPs appears to receive a thymus-derived signal during residency in the thymic microenvironment, which is consistent with their delayed up-regulation of NK1.1 and commitment to the T/NK cell lineages during short-term in vitro culture.

Discussion

The most immature hematopoietic precursors in the FT, the TLPs, display multipotent lymphoid lineage potential and are characterized by high-level expression of CD117 (c-kit) and a lack of expression of lineage differentiation (Lin<sup>−</sup>) markers (31), including NK1.1 (16, 25). Purified fetal thymocytes at the TLP stage in development have been demonstrated previously to be capable of maintaining precursor multipotency for up to 7 days in vitro in the presence of exogenous cytokines (20). This technique has been used to study the cytokine requirements that are necessary for the survival and proliferation of early precursor thymocytes. However, the effect of in vitro culture on the overall phenotype and precursor potential of these cells as a population remain unknown. To investigate this further, we examined the effect of short-term in vitro culture of isolated fetal precursor cells in the presence of exogenous cytokines.

Our data indicate that even though the TLPs appear to be phenotypically homogeneous, they contain a functionally heterogeneous mixture of cells with distinct precursor potential. This functional heterogeneity is revealed phenotypically in a delayed manner upon short-term culture of these precursors under conditions that maintain their proliferation and survival in vitro (Figs. 1–3). In particular, the majority of TLPs cultured in isolation, with at least exogenous SCF, efficiently and spontaneously induces NK1.1 expression ex vivo within 48 h. The subset of cultured TLPs that remains NK1.1<sup>−</sup> in vitro retains multipotent lymphoid precursor potential for the T, NK, and B lineages, while the NK1.1<sup>−</sup> precursors are restricted to the T and NK lineages (Fig. 5).

Thus, up-regulation of NK1.1 surface expression in vitro corresponds to a bona fide lineage commitment event analogous to that observed for the phenotypically identical TNK stage in vivo.

Spontaneous up-regulation of NK1.1 in vitro appears to represent a cell fate predetermined by exposure to the thymic microenvironment. This is supported by the fact that this effect is specific to fetal thymocyte precursors and does not efficiently occur with FL cells, unless the latter are cocultured with fetal thymic stroma in FTOC, in which case the majority of FL-derived cells pass through a NK1.1<sup>−</sup> stage (16). Moreover, induction of NK1.1 expression on thymocytes is minimally affected by transcriptional blockade, mitogen-induced activation, or exposure to exogenous cytokines of stromal cells (Figs. 2 and 3). Indeed, transcripts for the NKR-P1 (CD161) family members (28), of which NKR-P1C corresponds to a ligand for the anti-NK1.1 mAb (27), were detected among sorted NK1.1<sup>−</sup> TLPs fresh ex vivo. This suggests that at least a subset of these cells was already destined to express surface NK1.1. Furthermore, expression of NK1.1 in vitro was found to correlate with reduced proliferation, compared with the remaining NK1.1<sup>−</sup> counterpart (Fig. 4). This reduced cell turnover suggests that the NK1.1<sup>−</sup> cells undergo a differentiation event during culture, which is consistent with their restricted lineage potential (Fig. 5).

Taken together, these data suggest that a subset of TLPs has already received a thymus-induced signal that results in lineage commitment, while the remaining fraction has not yet received this signal, despite their residency within the thymus. This signal, in turn, could represent either a direct commitment event to the TNK stage or a T lineage commitment event that does not preclude a default developmental pathway for NK cell potential. The difference between these two possibilities may depend on whether the
NK1.1 subset was destined to express NK1.1 in vivo, or whether this subset would have followed a different developmental pathway if not removed from the thymic microenvironment. Notably, the phenomenon of spontaneous up-regulation in vitro is specific to NK1.1 and CD16/32 (data not shown), as induction of CD25 to a similar extent does not occur (data not shown). Thus, either the duration of this normally transient NK1.1+ stage is proportionately pronounced in the absence of continued thymic influence, or this phenomenon represents a default pathway before the pro-T stage of development that occurs upon removal from an environment capable of efficiently inducing T lineage commitment. Nonetheless, the interpretation remains that the majority of multipotent TLPS, en route to becoming committed T lineage precursors, undergo lineage commitment to a stage which does not preclude differentiation to either of the T or NK lineages. Therefore, the TNK phenotype seems to represent a true cellular commitment pathway induced by thymic stroma. Interestingly, however, this stage in not thymus-dependent, as phenotypically and functionally identical precursors are found in the fetal circulation (32, 33). In addition, a small subset (1–2%) of FL precursors also spontaneously up-regulates NK1.1 surface expression (Figs. 1–3), indicating that the thymus is not strictly required for this event.

Our findings characterize a lineage commitment pathway common to T and NK lineage precursors. Whether this pathway represents the predominant course of T and NK cell precursor potential in vivo or an alternative, default pathway for T and NK cell development remains unknown. However, the ability of precursor thymocytes to spontaneously undergo delayed lineage commitment in vitro highlights a caveat in assays of precursor activity, in that even a phenotypically homogeneous population of cells may be functionally heterogeneous. Thus, single-cell assays may represent the only unambiguous means of determining lineage potential.

Acknowledgments

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References