Differential requirement for the transcription factor PU.1 in the generation of natural killer cells versus B and T cells

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PU.1 is a member of the Ets family of transcription factors required for the development of various lymphoid and myeloid cell lineages, but its role in natural killer (NK) cell development is not known. The study shows that PU.1 is expressed in NK cells and that, on cell transfer into lymphoid Rag2γ−/− mice, hematopoietic progenitors of PU.1−/− fetal liver cells could generate functional NK cells but not B or T cells. Nevertheless, the numbers of bone marrow NK cell precursors and splenic mature NK cells were reduced compared to controls. Moreover, PU.1−/− NK cells displayed reduced expression of the receptors for stem cell factor and interleukin (IL)-7, suggesting a nonredundant role for PU.1 in regulating the expression of these cytokine receptor genes during NK cell development. PU.1−/− NK cells also showed defective expression of inhibitory and activating members of the Ly49 family and failed to proliferate in response to IL-2 and IL-12. Thus, despite the less stringent requirement for PU.1 in NK cell development compared to B and T cells, PU.1 regulates NK cell differentiation and homeostasis. © 2001 by The American Society of Hematology

Introduction

Natural killer (NK) cells are a distinct subset of lymphocytes that mediate important functions in innate immunity being able to eliminate tumor cells and to produce cytokines without prior sensitization (reviewed in Trinchieri1). NK cells can distinguish cells with disparate levels of major histocompatibility complex (MHC) class I expression, killing cells that, due to viral infections or transformation, have low MHC expression, and sparing those with normal expression (reviewed in Ljunggren and Karre2). Such discrimination is mediated by self MHC-specific inhibitory receptors on NK cells that belong to one of 3 groups: killer immunoglobulin-like receptors on human NK cells, lectin-like Ly49 receptors on murine NK cells, and lectin-like NKG2/CD94 cells that are found on both human and rodent NK cells (reviewed in Lanier3). Despite our appreciation of these different NK cell functions, the developmental relationship of NK cells with other hematopoietic lineages is not clear. A rare population of common lymphoid progenitors (CLPs) that can give rise to T, B, and NK cells has been identified in mouse bone marrow (BM),4 and NK and T cells have been suggested to derive from a common progenitor during fetal life.5,6 NK and T cells also share expression of several differentiation antigens and effector functions, yet the great majority of T cells are generated in the thymus, whereas the predominant site for NK cell development is the BM.

Part of the difficulty in understanding the developmental relationship of NK cells with other hematopoietic lineages stems from our incomplete knowledge of NK cell ontogeny. Notwithstanding, it is now clear that development of NK cells is strictly dependent on cytokines that promote survival, proliferation, and differentiation. Interleukin (IL)-15 plays a pivotal role in NK cell differentiation, thus NK cells are extremely reduced or absent in mice deficient for IL-157 or for any of the IL-15 receptor subunits (IL-15Ra, IL-2RB, γc) or its downstream signaling molecules Jak38 and Stat5.9 However, IL-15 intervenes in a rather late stage of development, when the commitment to the NK cell lineage has already been made. Although “early acting” cytokines, including IL-7, stem cell factor (SCF), and Flk2L/Flt3L, are best candidates for driving the commitment to the NK cell lineage (reviewed in Williams et al10), the relative contribution of these growth factors is not fully appreciated. Therefore, the molecular mechanisms marking NK cell specification are not completely understood. It is conceivable that these cytokines activate genetic programs that use multiple transcription factors to silence or to activate lineage-specific genes. However, although the transcriptional regulation of lineage commitment during lymphopoiesis has been quite extensively studied in the context of B- and T-cell development (reviewed in Glimcher and Singh11), the transcription factors that control engagement to the NK cell lineage have only recently started to be identified. Mice deficient for interferon regulatory factor-1 fail to develop NK cells, due to impaired transcriptional activation of the Il15 locus.12 The absence of CCAAT/enhancer binding protein γ (CREB-γ) impinges selectively on NK cell development and not on B or T lymphopoiesis.13 Disruption of the Ifi2 gene results in the block of NK cell development, whereas B- and T-cell differentiation unfolds normally.14 The further understanding of the transcriptional regulation of NK cells may help to define stages in which lineage specification takes place, thereby clarifying the developmental relationship of NK cells with B- and T-cell lineages.

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Materials and methods

Mice and generation of FL hematopoietic chimeras

Mice with a null mutation in the common γ chain (γc) were from the third generation backcross to the C57Bl/6 background. Rag2⁻/⁻ mice (10th backcross to C57Bl/6) were bred with B6.129.H-2d (H-2d), and F1 progeny were intercrossed to generate Rag2⁻/--;deficient mice on the H-2d background. These mice were then bred with Rag2⁻/--; mice to generate Rag2⁻/--; mice carrying the H-2d haplotype. PU.1⁺/⁻ mice (C57Bl/6 × 129, H-2d) were screened by polymerase chain reaction (PCR), using genomic DNA and specific primers for PU.1 and Neo gene (5'-GGG ATG TGG TTC CCT TAT CAA AC-3', 5'-TGA TCT TCT TCA CGC CTC TG-3', 5'-CAG AAA GCC GAG CAA AGC TG-3'). PU.1⁺/⁻ mice were intercrossed to generate PU.1⁺/⁺ and control (PU.1⁺/⁻ or PU.1⁺/-; thereafter referred to as WT) embryos. Analysis was performed on a FACScan or FACScalibur flow cytometer, using the CellQuest software (Becton Dickinson, San Diego, CA). The purity of the sorted populations was reproducibility greater than 95%.

NK cell lytic activity

A standard ¹²⁵I release assay was used to measure NK lytic activity in vitro as described. YAC-1 cells (mouse thymoma; H-2d) were used as target cells and were maintained in complete medium (CM, RPMI-1640 with 10% fetal calf serum, 10⁻³ M β-ME, 100 μg/ml streptomycin, 100 μU/ml penicillin). Target cells were labeled with 10 μCi ¹²⁵I (ICN Pharmaceuticals, Costa Mesa, CA), and 2.5 to 5 × 10⁶ cells were incubated with graded numbers of effecter cells in 200 μl medium for 4 hours. Effector cells were either NK cells that were isolated from splenocytes by cell sorting or IL-2-activated NK cell cultures. The radioactivity released into the cell-free supernatant was measured, and the percentage of specific lysis was calculated as follows: 100 × (experimental release − spontaneous release)/ (maximum release − spontaneous release).

Reverse transcriptase–PCR and Western blotting

RNA was isolated from freshly sorted NK cell populations from RNABlde (EUROBIO, Les Ulis, France) according to the manufacturer’s instructions. Complementary DNA was synthesized, using reverse transcriptase (RT) from avian myeloblastosis virus (PROMEGA, Madison, WI), hexanucleotides, and oligo-dT (Amersham Pharmacia, Uppsala, Sweden). PCR was performed, using Taq Platinum polymerase (GIBCO BRL). Primer sequences were as follows: P1 forward 5'-GAG TGT TTC TGG AAG ACC TGC CAC-3' and reverse 5'-TCA AAC ACA AAC AGG CAA CC-3'; P2 forward 5'-TCT TTA CGA GTG AGA ACA TGC-3' and reverse 5'-CAG TGG TCG TGG AGA -3'; P3 forward 5'-TCC GGG TGC CGC CGC CGG CAC-3' and reverse 5'-GAC CAC ATT CAG TCT GCT GGC ATG-3'; P4 forward 5'-TCT TTA CTA CGA GCA TGC CTA TGC CTA-3' and reverse 5'-TCT CCG GTG TGG AGA ACA TGC-3'; P5 forward 5'-TGC TGG GCT TGG CAT TGC CTC GGC-3' and reverse 5'-CAT TGG GCT TGG GCT TGG -3'; P6 forward 5'-CAC ACC ATT CAG TGC GCT TGC CTC-3' and reverse 5'-CTC TTA CGA GCA TGC CTA TGC CTA-3'. The radioactivity released into the cell-free supernatant was measured, and the percentage of specific lysis was calculated as follows: 100 × (experimental release − spontaneous release)/ (maximum release − spontaneous release).

Flow cytometry analysis and cell sorting

Single cell suspensions were prepared from blood, thymus, BM, spleen, and liver. Erythrocytes were lysed in ammonium chloride, and cells were resuspended in phosphate-buffered saline with 1% bovine serum albumin and 0.01% sodium azide. Cell viability was evaluated by trypan blue exclusion. Monoclonal antibodies (mAbs) directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), Tricolor (TRI), allophycocyanin (APC), or biotin were used for immunofluorescence analysis, including mAbs specific for immunoglobulin M (IgM), IgD, T-cell receptor α(β) (TCRαβ), TCRγδ, CD3, CD4, CD8, CD11a (LFA-1), CD11b (Mac-1), CD19, CD45R (B220), CD90 (Thy-1.2), CD117 (c-kit), CD122 (IL-2Rβ), CD161 (NK1.1), DX5, 2B4, Ly49A, Ly49C1, Ly49D, Ly49G2, Gr-1, TER-119, and H-2 (all from Pharmingen, San Diego, CA). Biotin-conjugated mAbs were revealed by streptavidin-TRI (Caltag, Burlingame, CA). Cells (10⁴) were first incubated with anti-FcγRII/III (hybridoma 2.4G2) for 20 minutes on ice to avoid unspecific binding to low affinity FeRs. Thereafter, cells were stained with a mixture of biotinylated and fluorochrome-labeled mAbs at saturating concentrations, washed twice, and finally incubated with streptavidin-TRI.
Figure 1. NK cells express PU.1. Cell lysates were generated from thymocytes, splenocytes, pre-B cells (cell line 18.81), and purified splenic IL-2–activated NK cells. Protein extracts were resolved by SDS-PAGE and probed with Western blotting with an affinity-purified anti-PU.1 antibody. Blots were stripped and reprobed with antitubulin antibody to control for sample loading.

Cell-cycle analysis

Cell-cycle analysis was performed on in vitro IL-2–activated NK cells and on circulating CD3+ NK1.1+ cells from peripheral blood, using 7-aminoactinomycin-D (7-AAD) incorporation into saponin-permeabilized cells as described.23 Sorted splenic CD3+ NK1.1+ cells were plated at 10^4 cells/well in round-bottom microtitre plates in 200 μL CM and activated with 1000 U/mL huIL-2 (Peprotech, Rocky Hill, NJ) for 7 days. To stimulate NK cell proliferation and to promote subsequent activation-induced cell death, 2 ng/mL, IL-12 (Peprotech) was added during the final 48 hours before the cell-cycle analysis.

Statistical analysis

Data were analyzed with the Microsoft Excel software, applying the paired Student t test. The null hypothesis was rejected, and difference was assumed significant when P < .05.

Results

Expression of PU.1 in NK cells

Previous studies have assessed PU.1 expression in CLPs, immature B- and T-cell precursors, and mature B cells.21,26,27 However, little is known about PU.1 expression in NK cells and its putative role during NK cell differentiation. We, therefore, tested and found PU.1 protein in cell lysates derived from sorted NK cells expanded in IL-2 (Figure 1). As expected, PU.1 was also found in control lysates derived from total splenocytes and pre-B cells but not in thymocyte lysates (Figure 1). Thus, mature NK cells, like B cells and unlike T cells, maintain PU.1 expression throughout development.

Lymphoid cell development in the absence of PU.1

To address the role of PU.1 in NK cell development in vivo, we generated hematopoietic chimeras by injecting PU.1−/− or WT FL-HSCs (H-2b) into alymphoid Rag2−/− mice. In this system,20,24 all lymphoid cells are donor derived, and any host-derived cell can be identified by virtue of their differential H-2 expression. Because PU.1−/− FL cells are known to contain fewer hematopoietic progenitors than control cells,25 the reconstitution capacity of 3 different doses (8 × 10^5, 25 × 10^5, or 75 × 10^5) of FL-HSCs from PU.1−/− embryos was analyzed and compared to WT controls. Seven to 13 weeks after the transfer, chimeras were killed, and the lymphoid cellularity in thymus, BM, spleen, and liver was evaluated. Splenic T cells (CD3+NK1.1+) were virtually absent in PU.1−/− chimeras (Table 1), and the thymi of PU.1−/− chimeras were hypocellular, containing almost exclusively early CD4+CD8− double-negative thymocytes (Figure 2). However, in very rare PU.1−/− chimeras, we could detect the 4 populations of CD4+, CD4+CD8+, CD8+, and CD4−CD8− thymocytes (data not shown), consistent with a variable penetrance of the T-cell-deficiency phenotype.20,21 B220+IgM+ B cells were absent in the spleen (Table 1) and BM of PU.1−/− chimeras (Figure 2), confirming the essential role of PU.1 in B-cell differentiation.19,20

In contrast with the T- and B-cell deficiency, CD3+NK1.1+ NK cells were present in spleen, liver, and BM of all PU.1−/− chimeras analyzed (n = 16), although they were reduced in absolute numbers compared to controls (n = 11; Table 1). The kinetics of peripheral NK cell generation in the absence of PU.1 was somewhat slower than controls (data not shown), a phenomenon already described for the in vivo generation of T cells in the viable PU.1−/− strain20 and in the in vitro generation of lymphoid-derived PU.1−/− dendritic cells.28 The data in Table 1 demonstrate that by injecting more PU.1−/− FL-HSCs, more NK cells could be generated, but the NK cell numbers in PU.1−/− chimeras never reached those found in WT chimeras. Thus, 10-fold less PU.1−/− NK cells were present in the spleens of chimeras generated with 8 × 10^5 FL-HSCs, but only 3- to 4-fold less PU.1−/− NK cells were present in the spleens of PU.1−/− chimeras generated with 25 × 10^5 FL-HSCs. However, T- and B-cell numbers did not significantly increase with higher doses of FL-HSCs and remained 400- to 6000-fold reduced compared to controls. The numbers of NK cells generated with even higher doses of FL-HSCs (75 × 10^5) were not greater than those obtained by injecting 25 × 10^5 FL-HSCs (Table 1 and data not shown), suggesting that the reduction of NK cells in PU.1−/− chimeras is not only due to the reduced frequency of progenitors in the FL of PU.1−/− embryo donors. These results clearly demonstrate that NK cell development is permissive in the absence of PU.1.

Reduced production of early NK cell precursors in the absence of PU.1

HSCs of PU.1−/− embryos fail to express VLA-4/CD49d–VLA-5/CD49e-CD11b integrins that has been hypothesized to result in defective homing to the BM.29 To directly test whether defects in engraftment of PU.1−/− FL-HSCs in the adult BM could explain the lower numbers of NK cells in PU.1−/− chimeras, we searched for donor-derived HSCs 8 weeks post-transfer. These cells are contained in a population of BM cells that is negative for the host H-2b, does not express lineage-specific markers (including CD19, B220, CD3, CD4, CD8, Gr-1, Mac-1, TER-119, and NK1.1), and is positive for both Sca-1 and
c-kit. We found HSCs (Sca-1⁺, c-kit⁺, Lin⁻, and H-2k⁻) in \( PU.1^{-/-} \) chimeras (Figure 3A) and the presence of donor-derived CD45⁺ cells at 16 weeks post-transfer (data not shown). These results rule out a major defect in engraftment or homing of FL-HSCs in \( PU.1^{-/-} \) chimeras.

Collectively, these observations suggest that PU.1 plays an intrinsic and essential role in early NK cell differentiation. We have recently identified a cell population in murine BM that appears to represent a committed NK cell precursor (NKP), having lost any potential for B, T, or myeloid differentiation (E. Rosmaraki et al, manuscript submitted). NKP are Lin⁻ and share IL-2Rβ⁺ expression with mature NK cells, but in contrast they do not express NK1.1 or DX5. Eight to 10 weeks after the transfer, we enumerated these Lin⁻ IL-2Rβ⁺ NK1.1-DX5 NKP in WT and \( PU.1^{-/-} \) chimeras. NKP were 10- to 12-fold reduced in the BM of \( PU.1^{-/-} \) chimeras (Figure 3B,C). This observation suggests that \( PU.1^{-/-} \) hematopoietic progenitors can only poorly generate the early NK cell compartment. Accordingly, the numbers of NK cells in older \( PU.1^{-/-} \) chimeras (>30 weeks; \( n = 5 \)) decline with age to almost undetectable levels (data not shown).

**Characterization of \( PU.1^{-/-} \) NK cells**

The development of hematopoietic progenitors is associated with dynamic changes in the expression of a number of transcription factors. The transcription factors Ets-1 and Id2 have been shown to be crucial for NK cell development.

Figure 4A shows that \( PU.1^{-/-} \) NK cells express Id2 transcripts and, interestingly, up-regulate expression of Ets-1 compared to control NK cells.

We further analyzed the cell surface phenotype of the splenic NK cells that developed in the absence of PU.1. A series of differentiation antigens, including NK1.1, DX5, CD2, 2B4, Mac-1, and Thy-1, were expressed at expected frequencies in \( PU.1^{-/-} \) NK cells and at levels comparable to those of controls (Figure 4B and data not shown). The LFA-1 complex is expressed on all leukocytes and is composed of CD11a and CD18 integrins. CD11a is a putative target gene of PU.1. Nevertheless \( PU.1^{-/-} \) NK cells expressed normal levels of LFA-1 (Figure 4B). Mature NK cells express inhibitory and activating members of the Ly49 family of receptors that recognize MHC class I antigens on the surface of target cells and play a critical role in regulating NK cell cytotoxic activity (reviewed in Raulet\(^\text{33}\)).

Figure 3. Reduced numbers of donor-derived NK cell precursors in \( PU.1^{-/-} \) chimeras. (A) BM cells of WT and \( PU.1^{-/-} \) chimeras were isolated, and lineage-positive cells were eliminated by a combination of magnetic bead depletion and electronic gating. HSCs (boxed cells) were identified by c-kit and Sca-1 expression as shown. Percentages were calculated on total BM cells. Data are representative of 4 independent experiments. (B) Lin-depleted BM cells were in parallel stained with mAbs specific for DX5-FITC, NK1.1-PE, and IL-2Rβ-APC. Absolute numbers ± standard deviation of Lin-depleted BM cells are indicated on top of the dot plots. Lin-IL-2Rβ⁺ cells were electronically gated, and their percentages are indicated. (C) The percentages of gated Lin-IL-2Rβ⁺ cells that were positive or negative for NK1.1 and DX5 are indicated. Data are representative of 3 independent experiments, including 8 WT and 5 \( PU.1^{-/-} \) chimeras.
NK cells, excluding the possibility that PU.1 is an essential regulator of TCF-1 expression.

**Mature PU.1−/− NK cells fail to proliferate in response to IL-2 and IL-12**

A survival and/or proliferation defect of mature NK cells could contribute to the reduced absolute numbers of NK cells in PU.1−/− chimeras. We, therefore, measured the percentages of cycling cells and hypodiploid apoptotic cells among circulating NK cells in PU.1−/− chimeras. Although there was no obvious increase in the proportion of PU.1−/− NK cells undergoing apoptosis (data not shown), the fraction of NK cells in cycle was significantly lower in PU.1−/− NK cells, as compared with controls (Figure 5A). IL-2 mediates survival, activation, and expansion of NK cells in vitro, and addition of IL-12 to IL-2–stimulated NK cells promotes their activation-induced cell death. Purified splenic NK cells from PU.1−/− chimeras remained viable throughout the culture period in IL-2 but did not appreciably expand (Figure 5B). In addition, they proliferated little in IL-12, although a normal apoptotic response to IL-2 + IL-12 was preserved (Figure 5C). Finally, we failed to generate NK cells in vitro from PU.1−/− FL cells (data not shown), using a combination of cytokines (SCF, Flk2L/Flt3L, IL-7, and IL-2) that drives NK cell differentiation with high efficiency from WT FL cells. Together, these results indicate that PU.1−/− NK cells have defective responses to cytokines.

**Expression of growth factor receptors on PU.1−/− NK cells**

PU.1 has been implicated in the regulation of cytokine receptor genes. Developing NK cell precursors rely on Flk2L/Flt3L, SCF, IL-7, and IL-15 to survive, proliferate, and differentiate (reviewed in Williams et al). The receptor for IL-7 is expressed during the early lymphopoiesis, and transcripts for the IL-7Rα chain are also found in mature NK cells (Figure 6A). However, PU.1−/− NK cells failed to express IL-7Rα. Interactions of SCF with its c-kit receptor are essential for expansion of NK cell precursors and full maturation of NK cells. A small subset of mature NK cells express the c-kit receptor, but this subset was 6- to 7-fold reduced in PU.1−/− NK cells (Figure 6B). NK cells derived from c-kit–deficient FL-HSCs also express low levels of the activation marker B220. Consistent with the reduction in c-kit expression, the B220− NK

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Figure 4. Characterization of PU.1−/− NK cells. (A) RT-PCR for the indicated transcripts was performed, using RNA prepared from sorted splenic NK cells (CD3−NK1.1+) from WT and PU.1−/− chimeras. (B) Splenic NK cells were stained with mAbs specific for the indicated surface antigens. Histogram profiles are shown for CD3−NK1.1+ gated cells. Data are representative of 8 independent experiments.

Figure 5. Reduced proliferation in PU.1−/− NK cells. (A) Blood cells were isolated from chimeras 4 weeks after the transfer of FL-HSCs. The figure indicates the mean and standard deviation of cycling NK cells in 6 WT and 8 PU.1−/− chimeras; *P = .008. (B) Freshly sorted NK cells from spleen of chimeras were plated at 2 × 10^5 cells/well and expanded in IL-2 for 7 days, and thereafter counted. (C) The same cells were replated at 10^5 cells/well and further stimulated with IL-12 overnight. For detection of apoptotic and proliferating NK cells, cells were stained with mAbs specific for NK1.1-PE and further stained with 7-AAD to reveal the DNA content. Proliferating cells (in G2/M) contain more DNA, whereas cells dying by apoptosis are hypodiploid.

Figure 6. Expression of growth factor receptors and activation markers on PU.1−/− NK cells. (A) RT-PCR for the indicated transcripts was performed, using RNA prepared from sorted splenic NK cells (CD3−NK1.1+) from WT and PU.1−/− chimeras. (B) Splenic NK cells were stained with mAbs specific for the indicated surface antigens. Histogram profiles are shown for CD3−NK1.1+ gated cells. Data are representative of 8 independent experiments.
cell fraction was clearly underrepresented in \( PU.1^{-/-} \) chimeras (Figure 6B). Signaling through the IL-15 receptor is crucial to drive NK cell development.\(^7,9\) This tripartite receptor is composed of the IL-15Rα, IL-2Rβ, and γc chains (the latter 2 are shared with the IL-2 receptor and can signal in response to high doses of IL-2). In line with the capacity of IL-2 to promote survival of \( PU.1^{-/-} \) NK cells (Figure 5B), essentially all of these cells expressed IL-2Rβ and contained RNA transcripts for IL-15Rα and γc (Figure 6B and data not shown).

**Lytic activity of \( PU.1^{-/-} \) NK cells**

We have previously observed a correlation between reduced expression of B220 and defective lytic activity in \( c-kit^{-/-} \) NK cells.\(^{23} \) Because B220 and \( c-kit \) expression were reduced in \( PU.1^{-/-} \) NK cells, we assessed the capacity of freshly isolated splenic \( PU.1^{-/-} \) NK cells to lyse YAC-1 thymoma targets in a standard \( ^{51} \)Cr release assay. As shown in Figure 7, \( PU.1^{-/-} \) NK cells were fully competent in lysing this NK-sensitive target. In addition, IL-2–activated \( PU.1^{-/-} \) NK cells could kill both YAC-1 and P815 targets (data not shown). These results show that differentiation of the lytic machinery for natural cytotoxicity does not require \( PU.1 \).

**Discussion**

In this report, we demonstrate that NK cells like B but not T lymphocytes express \( PU.1 \), but this transcription factor is not strictly required for the generation of functional NK cells in vivo. Our studies made use of a novel alymphoid mouse strain, \( RAQ2/γc^{-/-} \) mice, which were reconstituted with \( PU.1^{-/-} \) FL hematopoietic progenitors. In this setting, B-cell, T-cell, and NK cell development is entirely donor derived, and little or no competition with endogenous early lymphoid precursors is observed.\(^{22-24} \) Moreover, the potentially lethal effects associated with defective granulocyte and macrophage development in the absence of \( PU.1 \) are avoided since these hematopoietic lineages develop normally in \( RAQ2/γc^{-/-} \) mice.\(^{30} \) This genetic approach allowed us to assess the role of \( PU.1 \) in NK differentiation. \( PU.1^{-/-} \) chimeras remain B and T cell deficient but generate BM precursors and peripheral functional NK cells, although both are reduced in numbers.

We show here that NK cells maintain expression of \( PU.1 \) throughout differentiation. Consistent with this finding, mature \( PU.1^{-/-} \) NK cells displayed phenotypical abnormalities, such as reduced expression of certain surface antigens, including the receptors for IL-7 and SCF, and defective proliferative responses to potent NK cell mitogens, such as IL-2 and IL-12. However, \( PU.1^{-/-} \) NK cells could mediate natural cytotoxicity and survive in vitro on stimulation with IL-2. Taken together, our results demonstrate a differential requirement of \( PU.1 \) for NK versus B- and T-cell lymphopoiesis.

How do we explain the partial effects of \( PU.1 \) deficiency on NK cell differentiation in the context of the known roles that this transcription factor plays during hematopoiesis? \( PU.1 \) expression appears to be regulated in a complex and dynamic fashion throughout the hematopoietic system. \( PU.1 \) is expressed in HSCs and in common myeloid progenitors and in CLPs.\(^{26} \) Although the precise effects of \( PU.1 \) deficiency on these hematopoietic subsets remain to be determined, \( PU.1 \) is not required for the generation of HSCs, since this cell subset could be detected after cell transfer of \( PU.1^{-/-} \) FL precursors into \( RAQ2/γc^{-/-} \) recipients. \( PU.1 \) is essential for myeloid differentiation,\(^{19,20} \) yet its absence causes divergent consequences in distinct cell subsets. Monocytes and macrophages strictly depend on \( PU.1 \) for development,\(^{37} \) whereas neutrophils can develop in \( PU.1^{-/-} \) mice, although they show defective effector functions.\(^{38} \) Myeloid-derived dendritic cells also fail to develop in the absence of \( PU.1 \), whereas the requirement for \( PU.1 \) in development of lymphoid-derived dendritic cells is controversial, and the expression of \( PU.1 \) in this subset has not been documented.\(^{28,39} \) \( PU.1 \) expression is maintained as CLPs differentiate toward the B-cell lineage but is turned off early in T-cell development.\(^{21,27} \)

The requirement of \( PU.1 \) for B-cell development appears absolute, as shown by the complete absence of fetal and BM-derived B lineage cells in \( PU.1^{-/-} \) mice.\(^{19,20,25} \) We confirm in this report. \( PU.1 \) regulates the expression of the IL-7Rα gene during fetal hematopoiesis.\(^{34,40} \) \( PU.1^{-/-} \) fetal hematopoietic progenitors do not express transcripts for the B lineage–specific transcription factors EBF and Pax-5.\(^{14,25} \) Thus, the profound block to B-cell development caused by the \( PU.1 \) mutation may be due to defects in both IL-7–induced proliferation as well as EBF and Pax–5–mediated differentiation of B lineage progenitors. \( PU.1 \) may directly regulate the expression of the immunoglobulin loci.\(^{41,43} \) Interestingly, low levels of \( PU.1 \) are essential for B-cell development, whereas high levels inhibit B-cell differentiation and instead promote macrophage development.\(^{44} \)

In contrast, \( PU.1 \) expression is restricted to a discrete stage of T lineage differentiation. Only the earliest intrathymic progenitors (CD44+CD25+ ) express \( PU.1 \), which is rapidly extinguished as T cells mature.\(^{31,32} \) Still, T-cell development is profoundly impaired in \( PU.1^{-/-} \) mice.\(^{19,20} \) However, studies using fetal thymic organ culture have shown that the few thymocytes in \( PU.1^{-/-} \) mice that can bypass this developmental block develop into mature T cells that have normal function,\(^{23} \) thus \( PU.1 \) expression in T-cell differentiation is essential only during early thymopoiesis. CD44+CD25+ cells of fetal thymus contain a bipotential precursor endowed with both NK and T potential,\(^{45} \) and \( PU.1 \) deficiency may block development of these 2 closely related cell lineages. However, this bipotential precursor may not represent the main pool of NK progenitors in adult life, when NK lymphopoiesis occurs in the BM. We show here a reduction of NKP in \( PU.1^{-/-} \) BM, whereas their frequency of HSCs was normal. This suggests a NK lineage–specific role for \( PU.1 \), which may be independent of its role in early T-cell development.

The mechanisms by which the absence of \( PU.1 \) disrupts hematopoietic development are being clarified. A major function of

![Figure 7. \( PU.1^{-/-} \) NK cells are able to lyse tumor cells in vitro. Splenic CD3–NK1.1+ NK cells were purified by sorting and were used as effectors in a classical \( ^{51} \)Cr release assay versus YAC-1 thymoma cells. There was no significant difference in the lytic capacity of splenic NK cells purified from \( PU.1^{-/-} \) (C) or WT (○) chimeras. Data from 2 separate experiments are shown.](image-url)
PU.1 is to control the transcription of growth factor receptor genes in developing blood cells. In the context of myeloid development, evidence supports a model in which PU.1 is required for expression of the granulocyte colony-stimulating factor, macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and c-fms receptors on early myeloid progenitors (see Held et al and references therein). However, retroviral infection with c-fms could only restore the proliferation but not differentiation deficient in PU.1-deficient myeloid precursors, suggesting additional roles for PU.1 in macrophage development. Thus, the absence of expression of certain cytokine receptors during early myeloid and B-cell development in PU.1 mutants can partly explain the developmental blocks.

In line with this, PU.1 deficiency impaired the expression of some cytokine and growth factor receptors on developing NK cells. Previous studies have identified 4 ligand/receptor systems that play an important role in the generation of NK cells from hematopoietic precursors: Flk2/Fli3, c-kit, IL-7, and IL-15 (reviewed in Williams et al ). Of these, we found that PU.1/–/– NK cells express IL-2Rβ and transcripts for IL-15Rα and yc. In contrast, IL-7Rα was absent, and c-kit expression was reduced on PU.1/–/– NK cells. The reduction in numbers of NK precursors seen in PU.1/–/– BM may well reflect a synergistic effect of the defective IL-7Rα and c-kit expression. In the absence of c-kit and IL-7R signaling, NK cell development may be sustained by Flk2L/Fli3L, IL-15, or other cytokines. A similar cumulative defect in these cytokine receptors may also explain the severe block in early T lymphopoiesis in PU.1/–/– mice. Thus, the requirement for IL-7 and c-kit in early NK lymphopoiesis may be less strict than for early thymopoiesis. Mice deficient in both IL-7 and c-kit will help to test this hypothesis.

T-cell progenitors lose the expression of PU.1 as they commit to the T lineage (CD44+/CD25+), whereas NK cells express it throughout development. In line with this, mature PU.1/–/– NK cells are less in cycle, fail to proliferate in response to mitogens, and do not express a normal pattern of surface antigens (including Ly49A, Ly49D, and B220) as they differentiate. Yet they are competent for natural cytology of lymphomas, suggesting that PU.1-independent transcriptional regulation governs this crucial NK cell effector function.

Expression of Ly49 molecules may be acquired in an ordered sequence, although expression patterns of genes within the NK cell appear to be regulated independently. However, the mechanisms that control this process remain ill defined. We found that PU.1 deficiency was associated with a selective reduction in the expression of Ly49A and Ly49D, whereas NK1.1, Ly49G2, and Ly49C/I were normally expressed in PU.1/–/– NK cells. The significance of this observation awaits further investigation but indicates that PU.1 is involved in the fine-tuning of Ly49 expression. One possibility is that PU.1 may affect the acquisition of early (Ly49A) but not late Ly49 (Ly49C/I) members. The TCF-1 transcription factor has also been implicated in the specific regulation of Ly49A. However, we could exclude, on the basis of gene expression analysis, a direct effect of PU.1 on TCF-1 expression, and it is, therefore, likely that the defective Ly49 expression seen in PU.1/–/– NK cells is independent of TCF-1, although TCF-1 and PU.1 may cooperate to activate Ly49A expression.

The Ets family of transcription factors comprises multiple members, and, although their expression is regulated in a dynamic way during development, overlapping expression patterns of distinct members is documented and may allow functional redundancy. Another Ets family member expressed in developing lymphoid cells is Ets-1. Ets-1–deficient mice demonstrate T-cell survival defects, accelerated terminal differentiation of B cells, and reduced numbers of NK cells. We found that PU.1/–/– NK cells express higher levels of Ets-1 transcripts, and it is tempting to speculate that, in the absence of PU.1, Ets-1 may compensate to some extent for the transcriptional regulation that leads to NK cell development but not to B- and T-cell development. However, a functional redundancy between PU.1 and Ets-1 is difficult to imagine, as these 2 members share only 40% homology in their DNA-binding domain and have a different panel of target genes. Alternatively, they may regulate nonoverlapping functions during NK differentiation. For example, both Ets1/–/– mice and PU.1/–/– chimeras have reduced numbers of NK cells, but natural cytotoxicity is abolished in the absence of Ets-1 and is preserved in the absence of PU.1. Therefore, PU.1 deficiency is compatible with a normal differentiation of the lytic machinery, including expression of known targets of Ets factors such as LFA-1 and perforin, whereas Ets-1 may be essential for these genes. One may argue that the NK cells accumulating in the periphery of PU.1/–/– chimeras may have undergone critical alterations due to effects of the mutation at early developmental stages. As such, the role of PU.1 in mature NK cells cannot be unambiguously assessed. Conditional gene targeting may provide a solution to this problem, although no NK-cell lineage–specific transgenes have been characterized to date. Alternatively, retroviral-mediated transfer of PU.1/2 into mature PU.1/–/– NK cells may allow for correction of their phenotypic and functional abnormalities. Such technologic improvements should allow for a finer definition of the role of PU.1 in NK cell biology. Our results suggest that there is a less restrictive requirement for PU.1 in NK cell generation, as compared to myeloid, B cells, and T cells. Nevertheless, PU.1 plays critical roles in NK cell development, during expansion of committed NK precursors, and in the homeostasis and differentiation of mature NK cells.

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Differential requirement for the transcription factor PU.1 in the generation of natural killer cells versus B and T cells

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