Role of Interleukin-2 (IL-2), IL-7, and IL-15 in Natural Killer Cell Differentiation From Cord Blood Hematopoietic Progenitor Cells and From \( \gamma c \) Transduced Severe Combined Immunodeficiency X1 Bone Marrow Cells

By M. Cavazzana-Calvo, S. Hacein-Bey, G. de Saint Basile, C. De Coene, F. Selz, F. Le Deist, and A. Fischer

Natural killer (NK) cells are characterized by their ability to mediate spontaneous cytotoxicity against susceptible tumor cells and infected cells. They differentiate from hematopoietic progenitor cells. Patients with X-linked severe combined immunodeficiency (SCID X1) carry mutations in the \( \gamma c \) cytokine receptor gene that result in lack of both T and NK cells. To assess the role of interleukin-2 (IL-2), IL-7, and IL-15 cytokines, which share \( \gamma c \) receptor subunit, in NK cell differentiation, we have studied NK cell differentiation from cord blood CD34(+) cells in the presence of either stem cell factor (SCF), IL-2, and IL-7 or SCF and IL-15. The former cytokine combination efficiently induced CD34(+) CD7(+) cord blood cells to proliferate and mature into NK cells, while the latter was also able to induce NK cell differentiation from more immature CD34(+) CD7(-) cord blood cells. NK cells expressed CD56 and efficiently killed K562 target cells. These results show that IL-15 could play an important role in the maturation of NK cell from cord blood progenitors. Following retroviral-mediated gene transfer of \( \gamma c \) into SCID X1 bone marrow progenitors, it was possible to reproduce a similar pattern of NK cell differentiation in two SCID-X1 patients with SCF + IL-2 + IL-7 and more efficiently in one of them with SCF + IL-15. These results strongly suggest that the \( \gamma c \) chain transduces major signal(s) involved in NK cell differentiation from hematopoietic progenitor cells and that IL-15 interaction with \( \gamma c \) is involved in this process at an earlier step than IL-2/IL-7 interactions of \( \gamma c \). It also shows that gene transfer into hematopoietic progenitor cells could potentially restore NK cell differentiation in SCID X1 patients.

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MATERIALS AND METHODS

Cell preparation. Control cells were obtained from human umbilical cord blood following delivery. SCID-X1 bone marrow cells were obtained from two patients (P1 and P2). Mutations of the \( \gamma c \)-encoding gene were characterized in both SCID-X1 patients. In P1, a missense mutation changed a cysteine to a glycine at position 62 in the extracellular region of the molecule. This mutation did not modify \( \gamma c \) chain transcription. In patient P2, a deletion of the \( \gamma c \) gene led to an absence of \( \gamma c \) transcription. \( \gamma c \) chain expression was undetectable at the surface of both patients’ B lymphocytes.15 In patient P1 peripheral blood, the lymphocyte count was 1,980/\( \mu \)L, CD3(+) cells 2%, CD19(+) 99%, and CD16(+) 0%. In patient P2, the lymphocyte count was 800/\( \mu \)L, CD3(+) cells 2%, CD19(+) 64%, and CD16(+) 2%.

Patients’ bone marrow samples were obtained at the ages of 7 and 13 months, respectively. They were collected under general anesthesia indicated for central line insertion for parenteral nutrition. Informed consent from parents was obtained.

Mononuclear cells from umbilical cord blood, isolated by density

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The presence of SCF, 11-7, and IL-2 (A) or SCF and 11-15 (C) were studied for the respective cytotoxic activities against antibody coated P815 cell line 1-1. Cytotoxicity against uncoated P815 cells was all- 1-1 or the K562 cell line (ΔΔΔ). Cells were eluted by gently squeezing the avidin column. Cell purity was assessed by flow cytometric analysis. CD34 immunoselected cells were, in some experiments, sorted on the basis of their CD7 surface antigen expression. Enriched CD34+ cells were incubated simultaneously with phycoerythrin (R-PE)-labeled anti-CD34 (PE-HCPA2; Becton Dickinson [BD], Mountain View, CA) and fluorescein isothiocyanate (FITC)-labeled anti-CD7 (Leu 9; BD). After washing, cells were suspended in Iscove's modified Dulbecco medium (IMDM; GIBCOBRL, Gaithersburg, MD) at a concentration of 5 × 10^6 cells/mL and separated by cell sorting. Cells were sorted on a FACS-Star Plus cell sorter (BD) at a flow rate of 1,000 to 1,500 cells/second. Residual erythrocytes and dead cells were gated out using forward scatter and side scatter channels. Patients' bone marrow samples were exclusively separated on Ficoll-hypaque before manipulation to avoid any additional cellular loss.

### CD34+ CD7+ Immunophenotype Determination

CD34+ CD7+ immunophenotype determination at day 0 was limited as the number of available sorted double positive cells ranged between 2 × 10^2 and 3 × 10^4. Results are given as the mean of six experiments ± SD.

**Abbreviation:** ND, not done.

### Table 1. Differentiation of CD34+ CD7+/− Human γc+ Cord Blood Cells in the Presence of SCF, IL-2, and IL-7

<table>
<thead>
<tr>
<th>CD34+ CD7+</th>
<th>Day 0 %</th>
<th>Day 21 % (± SD)</th>
<th>CD34+ CD7−</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>CD7</td>
<td>100</td>
<td>30 (± 20)</td>
<td>0</td>
</tr>
<tr>
<td>CD16</td>
<td>0</td>
<td>34.5 (± 12)</td>
<td>0</td>
</tr>
<tr>
<td>CD2</td>
<td>ND</td>
<td>42 (± 11)</td>
<td>0</td>
</tr>
<tr>
<td>CD16/CD2</td>
<td>ND</td>
<td>25 (± 11)</td>
<td>0</td>
</tr>
<tr>
<td>CD56</td>
<td>0</td>
<td>25 (± 15)</td>
<td>0</td>
</tr>
<tr>
<td>CD56/CD16</td>
<td>ND</td>
<td>5 (± 1.8)</td>
<td>ND</td>
</tr>
<tr>
<td>CD56/CD2</td>
<td>ND</td>
<td>2.3 (± 1.2)</td>
<td>0</td>
</tr>
<tr>
<td>CD8</td>
<td>0</td>
<td>1 (± 0)</td>
<td>0</td>
</tr>
</tbody>
</table>

Results are given as the mean ± SD. Day 0-1. Cytotoxicity against uncoated P815 cells was all-1-1 or the K562 cell line (ΔΔΔ). Cells were eluted by gently squeezing the avidin column. Cell purity was assessed by flow cytometric analysis. CD34 immunoselected cells were, in some experiments, sorted on the basis of their CD7 surface antigen expression. Enriched CD34+ cells were incubated simultaneously with phycoerythrin (R-PE)-labeled anti-CD34 (PE-HCPA2; Becton Dickinson [BD], Mountain View, CA) and fluorescein isothiocyanate (FITC)-labeled anti-CD7 (Leu 9; BD). After washing, cells were suspended in Iscove's modified Dulbecco medium (IMDM; GIBCOBRL, Gaithersburg, MD) at a concentration of 5 × 10^6 cells/mL and separated by cell sorting. Cells were sorted on a FACS-Star Plus cell sorter (BD) at a flow rate of 1,000 to 1,500 cells/second. Residual erythrocytes and dead cells were gated out using forward scatter and side scatter channels. Patients' bone marrow samples were exclusively separated on Ficoll-hypaque before manipulation to avoid any additional cellular loss.

### Table 2. Differentiation of CD34+ CD7+/− Human γc+ Cord Blood Cells in the Presence of SCF and IL-15

<table>
<thead>
<tr>
<th>CD34+ CD7+</th>
<th>Day 0 %</th>
<th>Day 21 % (± SD)</th>
<th>CD34+ CD7−</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>CD7</td>
<td>100</td>
<td>72.5 (± 15)</td>
<td>0</td>
</tr>
<tr>
<td>CD16</td>
<td>ND</td>
<td>26 (± 12)</td>
<td>0</td>
</tr>
<tr>
<td>CD2</td>
<td>ND</td>
<td>4 (± 1.5)</td>
<td>0</td>
</tr>
<tr>
<td>CD16/CD2</td>
<td>ND</td>
<td>14.5 (± 10)</td>
<td>0</td>
</tr>
<tr>
<td>CD66</td>
<td>0</td>
<td>94 (± 10)</td>
<td>0</td>
</tr>
<tr>
<td>CD66/CD16</td>
<td>0</td>
<td>16 (± 10)</td>
<td>0</td>
</tr>
<tr>
<td>CD66/CD2</td>
<td>0</td>
<td>5 (± 2)</td>
<td>0</td>
</tr>
<tr>
<td>CD8</td>
<td>0</td>
<td>2 (± 0.3)</td>
<td>0</td>
</tr>
</tbody>
</table>

Results are given as the mean ± SD. Day 0-1. Cytotoxicity against uncoated P815 cells was all-1-1 or the K562 cell line (ΔΔΔ). Cells were eluted by gently squeezing the avidin column. Cell purity was assessed by flow cytometric analysis. CD34 immunoselected cells were, in some experiments, sorted on the basis of their CD7 surface antigen expression. Enriched CD34+ cells were incubated simultaneously with phycoerythrin (R-PE)-labeled anti-CD34 (PE-HCPA2; Becton Dickinson [BD], Mountain View, CA) and fluorescein isothiocyanate (FITC)-labeled anti-CD7 (Leu 9; BD). After washing, cells were suspended in Iscove's modified Dulbecco medium (IMDM; GIBCOBRL, Gaithersburg, MD) at a concentration of 5 × 10^6 cells/mL and separated by cell sorting. Cells were sorted on a FACS-Star Plus cell sorter (BD) at a flow rate of 1,000 to 1,500 cells/second. Residual erythrocytes and dead cells were gated out using forward scatter and side scatter channels. Patients' bone marrow samples were exclusively separated on Ficoll-hypaque before manipulation to avoid any additional cellular loss.

### Retroviral vector and virus-producing cell line

Human γc chain cDNA extending from the initiation codon ATG (nucleotides numbered 1 to 1114) was generated by reverse transcription polymerase chain reaction (RT-PCR) from mRNA of control B-cell lines. For-ward primer 5'-GCAAGCGACATGTTGAAGCC-3', and reverse primer: 5'-GAGGATCCGGGTTCAGGC-3', respectively for γc chain insertion in the retroviral vector. Correct γc sequence was assessed by direct sequencing of the entire PCR amplified fragment. Human γc chain was then inserted at the Moloney murine leukemia virus (MO-MLV) LTRs for transcription of the viral genome and contains the B2 mutation corresponding to a single G to A transition at position +160 of the MO-MLV sequence.19 This vector, named MFG (B2)-γc, does not contain a selection marker.

Retroviral producer cell lines were generated by cotransfecting the MFG (B2)-γc plasmid with the plasmid pSV2-neo into the amphotropic packaging cell line ψCRIP as previously described.20 Two days later, the transduced cells were diluted 10 times and placed under G418 (Geneticin; GIBCOBRL) selection at 0.8 to 1 mg/mL active metabolite until individual resistant colonies formed. Titration was performed by infecting 5 × 10^6 NIH3T3 cells with 0.5 mL of a 24-hour supernatant from the virus-producing clones in the presence of 8 mg/mL of Polybrene (Sigma Chemical Co, St Louis, MO). Thirty clones were screened for high-titer virus production by Southern blot analysis. Southern blot analysis confirmed the presence of an unrearranged proviral genome from these producer clones and
were used to determine the number of proviral copies integrated in the target population. The clone that presented the highest transmission efficiency of virus for transduction of NIH3T3 cells was in the range of 0.5 copy of provirus per cell. This clone was thus used in the experiments described below. Supernatants from producer clones were also tested for the presence of replication-competent virus by using a virus mobilization assay and were found negative.

Retroviral transduction protocol of SCID-X1 mononuclear bone marrow cells (SCID-X1 BMC). SCID-X1 BMC were prestimulated with SCF (100 ng/mL; kindly provided by Amgen, Thousand Oaks, CA), IL-3 (20 ng/mL; kindly provided by Sandoz, Basel, Switzerland), and IL-6 (20 ng/mL) at 37°C, 5% CO₂ for 24 to 36 hours. These cells were then cocultured with MFG (B2)-yc adherent producer cells previously irradiated at 30 Gy in the presence of the same cytokines and Polybrene (8 mg/mL) for 3 days.

Culture conditions of hematopoietic cells in NK cell differentiation assay. Aliquots of 200 μL of the myelocult medium (Stem-Cell Technologies, Vancouver, Canada) containing 50 ng/mL recombinant human (rhu) SCF (Amgen) 500 U/mL rhuIL-2 (kindly provided by Chiron, France) and/or 20 ng/mL rhuIL-7 (Genzyme, Cambridge, MA) were placed in 96-well flat-bottomed microplates. One to three thousand control CD34⁺ CD7⁺ or transduced SCID-X1 BMC were plated to each well and cultured for 3 to 5 weeks. The medium was replaced with fresh medium with identical concentrations of cytokines every 4 days. In other experiments IL-15 (20 ng/mL; a kind gift from Dr T. Troutt, Immunex, Seattle WA) was used in combination with SCF. After 3 to 5 weeks, cultures were harvested, washed, counted, and assessed for viability by trypan blue exclusion.

Antibodies. Cell suspensions were stained for 20 minutes with the following phycoerythrin (PE) or FITC-conjugated monoclonal antibodies (MoAbs): anti-CD7 (Leu 9; BD), anti-CD2 (leu 5b; BD) anti-CD34 (HPCA-2; BD), anti-CD19 (Leu 12; BD), anti-CD38 (Leu 17; BD), anti-CD14 (Leu M3; BD), anti-CD16 (Leu 11b; BD), and anti-CD56 (Leu 19; BD). Control isotype antibodies (IgG1, IgG2, FITC-IgG1, and PE-IgG) were obtained from Pharmingen (San Diego, CA). Three unconjugated MoAbs specific for the IL-2 receptor subunits and two unconjugated MoAbs specific for the p58 NK cell receptor were also used; ie, anti-IL-2-Rα (CD25; BD), anti-IL-2-Rβ (341, Immunotech, Marseilles, France), the rat anti-IL-2-Rγ (TuGb4; kindly obtained from Dr K. Sugamura), GL183 and EB6 (kindly obtained from Dr L. Moretta). m

Immunofluorescence analysis. For direct conjugated antibodies, cell staining was performed according to the manufacturer’s instructions. For the unconjugated antibodies (ie, IL-2-R MoAb), cells were first incubated with human serum (dilution 1/5) for 15 minutes and stained with the appropriate MoAb, washed twice and stained by the biotin-conjugated rat antimouse Ig or mouse antirat (γ chain) Ig preadsorbed with human immunoglobulins (Jackson, Westgrove, PA). Both secondary antibodies were shown with PE-conjugated avidin (Caltag, San Francisco, CA). Cells were suspended in phosphate-buffered saline (PBS), kept at 4°C, and analyzed on a FACS (BD) with the Lysis II software. A total of 5 × 10⁶ events were acquired from low-density marrow and isolated CD34⁺ cells and stored in list mode files.

Natural killer activity and antibody dependent cellular cytotoxicity (ADCC) assessment. After culture, SCID-X1 and control sorted cells were suspended in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS). Cytotoxic activity against NK cell sensitive erythroleukemic cell line K562 and against rabbit IgG antibody-coated mastocytoma cell line P815 (ADCC) were assessed as previously described.

Assessment of clonogenic hematopoietic progenitor cell assay (CFU). Erythroid (colony-forming unit-erythroid [CFU-E], mature and immature burst-forming unit-erythroid [BFU-E]) and granulocytic (colony-forming unit granulocyte-macrophage [CFU-GM]) progenitors were analyzed using previously described methylcellulose assays. Each cellular sample was plated at a concentration of 0.5 to 2 × 10⁴ cells/mL of complete methylcellulose medium (Stem Cell Technologies, Vancouver, Canada) with 7% 5637 conditioned medium and human erythropoietin (h-epo; 1 U/mL; Stem Cell...
Technologies) as colony-stimulating factors. Plates were incubated at 37°C in an air atmosphere supplemented with 5% CO₂. Hematopoietic progenitors were scored at days 16 to 18 according to the previously described criteria.20

Provirus integration study in CFU. The percentage of positively \( \gamma \)c transduced hematopoietic precursor cells derived from P1 bone marrow was evaluated in CFU methylcellulose assay. Immediately after the transduction, cells were cultured on murine stromal cells MS-5 as previously described in IMDM with 12.5% FCS, 12.5% horse serum (Stem Cell Technologies). Cells were maintained at 33°C, 5% CO₂ and fed weekly by half medium change. Weekly, a cellular sample was plated at a concentration of 0.5 to 2 \( \times \) 10³ cells/mL of complete methylcellulose medium (Stem Cell Technologies) as described above. At day 14, individual clones were collected and lysed. DNA was analyzed by PCR using different PCR primers to assess the \( \gamma \)c retroviral integration and to determine among the same clones the possibility of a contamination (due to the coculture) by the neomycin gene, which is contained in the virus producing cell line. The percentage of specific \( \gamma \)c-positive clones was calculated by subtracting the neomycin-positive clones from the total number of analyzed clones.

For \( \gamma \)c amplification, two primers were used, one mapping into the retrovirus sequence and the other mapping into the \( \gamma \)c chain sequence, respectively: PM-R 5'-GACCACTGATATCCTGTC-3' and \( \gamma \)c-F 5'-CCAGCCTACCAACCTCACT-3'. For neomycin amplification by PCR the following two primers were used: neo-F 5'-CCATCGGCTGCTCTGATGCC-3'; neo-R 5'-GTAGAACCGAGGAAAGCGGT-3'. DNA was amplified by PCR using 30 cycles at an annealing temperature of 60°C. PCR was performed in a 50-mL reaction mixture, and a 20-mL portion of each amplified product was examined by 1% agarose gel electrophoresis and stained with ethidium bromide.

RESULTS

In vitro maturation of CD34⁺ cord blood cells into NK cells. We first investigated conditions enabling CD34⁺ hematopoietic progenitors to proliferate and differentiate into NK cells. Cord blood was chosen as a hematopoietic progenitor cell source as the best available age-matched cell popula-

![A](image1)

![B](image2)

**Table 3.** Differentiation Study of SCID X1 Bone Marrow Cells After \( \gamma \)c Transduction and Culture With SCF, IL-2, and IL-7 for 3 Weeks

<table>
<thead>
<tr>
<th>CD</th>
<th>Patient 1 Day 0 (%)</th>
<th>Day 21 (%)</th>
<th>Patient 2 Day 0 (%)</th>
<th>Day 21 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>20</td>
<td>0</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>CD38</td>
<td>ND</td>
<td>100</td>
<td>ND</td>
<td>100</td>
</tr>
<tr>
<td>CD7</td>
<td>ND</td>
<td>9</td>
<td>ND</td>
<td>8</td>
</tr>
<tr>
<td>CD56</td>
<td>0</td>
<td>20</td>
<td>1*</td>
<td>13</td>
</tr>
<tr>
<td>CD16</td>
<td>0</td>
<td>20</td>
<td>0.5</td>
<td>40</td>
</tr>
<tr>
<td>CD2</td>
<td>0</td>
<td>20</td>
<td>ND</td>
<td>40</td>
</tr>
<tr>
<td>CD16/CD2</td>
<td>0</td>
<td>5</td>
<td>ND</td>
<td>8</td>
</tr>
<tr>
<td>( \gamma )c</td>
<td>0</td>
<td>60</td>
<td>0</td>
<td>50</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

* CD56⁺⁻⁻⁻.

3' HRT-F 5'-TTCCCTTTCTTCAATCACAC-3' and HRT-R 5'-GACTACCTGGCTGTTCAT-3'.

To test the \( \gamma \)c transcription after the retrovirus integration, the same two primers PM-R and \( \gamma \)c-F were used. cDNA was amplified for 30 cycles of PCR at 94°C for 1 minute; at an annealing temperature of 63°C, 56°C, 56°C, 53°C, and 60°C, respectively, for 1 minute; and 72°C for 1 minute. PCR was performed in a 50-µL reaction mixture, and a 20-µL portion of each amplified product was examined by 1% agarose gel electrophoresis and stained with ethidium bromide.

![Fig 3](image3)

Expression of \( \gamma \)c chain after 4 weeks of culture of transduced P1 and P2 bone marrow cells as assessed by RT-PCR analysis (A) and immunofluorescence staining (B). (A) RT PCR analysis. RNA was isolated from the retroviral producer cell lines (lane 1: positive control), transduced P1 and P2 bone marrow cells after culture with SCF, IL-2 and IL-7 (lanes 3 and 4, respectively) and transduced P1 bone marrow cells after culture with SCF and IL-15 (lane 5). cDNA was generated and amplified by PCR using the primers as described in the Materials and Methods. Omission of template DNA (lane 2: water) served as PCR negative control. (B) Immunofluorescence staining: Surface expression of \( \gamma \)c chain on NK cells derived from \( \gamma \)c transduced P1 bone marrow cells was evaluated using an anti-\( \gamma \)c monoclonal antibody. Cells were cultured for 28 days in the presence of SCF and IL-15 x axis: log scale fluorescence intensity; y axis: relative cell number.

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NK CELL DIFFERENTIATION FROM γC-TRANSUCED SCID-X1 CELLS

Fig 4. Kinetics of CD56 (upper histograms) and CD16 (lower histograms) expression on γc (-1 P1) bone marrow cells following γC gene transfer and culture for 3 weeks in presence of SCF, IL-2 and IL-7. x axis: log scale fluorescence intensity, y axis: relative cell number. The transduced bone marrow cells from patient P2 showed exactly the same CD16 and CD56 expression kinetics.

tion for SCID-X1 patients. After double positive selection of CD34+ cells (by immunoaffinity column and cell sorting) CD3+, CD16+, or CD56+ cells became undetectable by immunofluorescence study (Table 1). At day 0, after the double positive immunoselection, 70% of CD34+ cells expressed γc chain on their surface, while only 15% of them expressed the β chain and less than 1% the α chain of IL-2 receptor (data not shown). γc transcripts were also detected in the purified CD34+ cells (data not shown). As shown in Table 1, CD34+ 7+ cells from cord blood could differentiate into CD56+ cells in the continuous presence of SCF + IL-2 and IL-7 for 3 weeks. These CD56+ cells coexpressed in a very limited percentage CD2 or CD16 antigens (range, 1% to 5%). In all instances, SCF was necessary to induce cell proliferation because cord blood hematopoietic precursor cells did not proliferate in the presence of IL-7 and IL-2 alone (data not shown). The best results were always obtained by the association of IL-2 with IL-7, although SCF plus IL-2, could induce NK cell differentiation to a lesser magnitude (data not shown). Active proliferation occurred, as a 164-fold (±30-fold) cell number increase was observed at the end of the culture period. Cells harvested at day 21 exhibited anti-K562 and ADCC cell cytotoxicity (Fig 1A) as tested on K562 and antibody-coated P815 target cells, respectively. CD34+ 7+ cells isolated from cord blood had a poorer ability to mature into CD56 cells in the same culture conditions and no cytotoxicity against K562 could be detected (Table 1 and Fig 1B). Their proliferation index was intense, but more variable (ie, 223-fold ± 180-fold).

In the presence of SCF and IL-15, culture of CD34+ CD7− cord blood cells differentiated into NK cells (Table 2) exhibiting both anti-K562 and ADCC cell cytotoxicity (Fig 1C and D). As shown in Table 2 and in Fig 1C, the SCF and IL-15 cytokine combination was a more potent inducer for cord blood CD34+ cell differentiation into functional NK cells than the SCF + IL-2 and IL-7 combination. Ninety-five percent of CD34+ CD7− cultured cells expressed CD56 after 3 weeks and a 600-fold (±173-fold) cell number increase was observed after a 3-week culture period. The vast majority of differentiated CD56+ cells, strongly coexpressed CD7 (72.5% ± 15%), while 16% (±10%) and only 5% (±2%) of them coexpressed CD16 and CD2, respectively (Fig 2). In addition, a fraction of these cells expressed p58 (20% ± 10%). SCF and IL-15 also enabled less differentiated CD34+ 7− cells to give rise to functional NK cells (Fig 1D). No cells expressed CD57 whatever the cytokine combination used.

The CD34+ CD7− colony forming unit-granulocyte and macrophage cloning efficiency (CFU-GM) in methylcellulose was tested after a 3-week culture period in the presence of either SCF + IL-2, SCF + IL-7 or SCF + IL-15. The percentage of CFU-GM was respectively 12% for cells cultured with SCF + IL-2, 5% with SCF + IL-7 and only 1% with SCF + IL-15, suggesting that SCF and IL-15 preferentially induced differentiation of immature cells towards the NK cell lineage. Similarly CD34+ CD7−, were unable to differentiate into CFU-GM after a 3-week culture with SCF + IL-15 (data not shown). Whatever the cytokine combination used, a fraction of CD34+ precursor cells differentiated into a CD16+ CD2− CD56− population. In an effort to understand the lineage commitment of this subset, these cells were purified by cell sorting and assessed for their potential toward myeloid differentiation (CFU-GM), for ADCC activity, and for expression of CD3c transcripts. These cells were unable to form (CFU-GM) colonies in vitro, while they exhibited an ADCC activity similar to that of an unselected population, CD3c transcripts could also be detected in this subset (data not shown).

γc gene transfer into γc (-) marrow cells from two SCID-X1 patients. Unseparated patients' mononuclear bone marrow cells were used for transduction experiments, because the harvest sample size was limited, to minimize hematopo-
etic cell loss. Mononucleated cells previously cryopreserved were thawed the day of the experiment and immediately cultured in the presence of SCF, IL-3 and IL-6. After a 3-day coculture with irradiated ψ CRIP cells producing the MFG-B2-γc vector, γc(−), marrow cells from the two patients were further cultured in the presence of either SCF, IL-2, and IL-7 or SCF plus IL-15 to assess the possible restoration of γc expression, cell proliferation, and maturation into functional NK cells.

Immediately after γc gene transduction, 8% to 10% of bone marrow cells expressed γc at the cell surface (data not shown). As shown in Fig 3A, γc specific transcript could be detected by RT-PCR in transduced marrow cell populations tested after 21 to 28 days of culture in the presence of SCF + IL-2 + IL-7 or SCF + IL-15. Immunofluorescence analysis showed that a majority of cultured cells expressed γc at their cell surface (60% and 80% for PI marrow cells cultured with IL-2 + IL-7 or IL-15, respectively; Fig 3B). The magnitude of expression was similar between transduced cells and control cells (ie, cord blood from a normal newborn). After mock-infection by coculture with ψ CRIP cells, no cell proliferation of CD34+ γc (−) cells could be achieved in the presence of these cytokines. Cells died within 8 days of culture. In contrast, in both patients following γc gene transfer, a sevenfold cell number increase occurred after 3 weeks in the presence of both SCF + IL-2 and IL-7 or SCF + IL-15. Although before γc gene transfer virtually none of the bone marrow mononuclear cells expressed either NK cell membrane marker (Table 3) or T-cell markers (data not shown), a significant proportion of proliferating γc(+) cells from both patients’ marrow samples were found to express NK cells membrane markers, ie, CD7, CD8, CD2, CD16, and CD56 following γc gene transfer (Table 3 and Fig 4). As soon as 2 weeks after cell culture initiation, cells expressing CD16 or CD56 could be detected (Fig 4), the frequency of which increased by day 21. Of note, following culture in the presence of SCF and IL-15 of transduced marrow cells from patient 1, the proportion of CD56 increased slowly as a high percentage of CD56+ cells was only noticed at day 42 (Table 4 and Fig 5). Remarkably, significant NK and ADCC activities could be detected as tested on K562 and antibody-coated P815 cell lines, respectively (Fig 6). Culture of bone marrow mononuclear cells in SCF and IL-15 was more potent in inducing maturation into fully functional NK cells (Fig 6B) as previously found with normal cord blood cells (Fig 1).

CD3 ε, δ, and γ chain transcripts expression was studied on these in vitro differentiated NK cells as a marker of cell differentiation30 using RT-PCR. It was compared with NK cells derived from control cord blood CD34 cells. As shown in Fig 7, whatever the cytokine combination used to induce NK cell differentiation, the cytoplasmic expression of CD3 ε and δ, but not γ could be detected in NK cells derived from patients’ and cord blood hematopoietic precursors. These results are similar to what has been previously described in fetal NK cells.30

Integration of γc gene following gene transfer was investigated in myeloid cell differentiation assays. Individual CFU-GM and BFU-E colonies derived from γc (−) transduced marrow cells were analyzed by PCR for γc gene integration after γc gene transfer. As shown in Fig 8, 40% of colonies derived from PI marrow cells integrated the γc gene-containing provirus when tested after 3 weeks of culture.

DISCUSSION

In this study, we first assessed differentiation of cord blood CD34 hematopoietic progenitor cells into NK cells as a prerequisite for studying NK cell differentiation of γc (−) marrow cells from SCID X1 patients following γc gene transfer.

| Table 4. Differentiation Study of SCID X1 Bone Marrow Cells From Patient 1 After γc Transduction and Culture With SCF and IL-15 |
|-----------------|-----------------|-----------------|
| CD34 | Day 0 (%) | Day 28 (%) | Day 42 (%) |
| CD34 | 20 | 0 | 0 |
| CD38 | ND | 100 | ND |
| CD7 | ND | 2 | 15 |
| CD56 | 0 | 5 | 35 |
| CD16 | 0 | 48 | 20 |
| CD2 | 0 | 28 | ND |
| CD16/CD2 | 0 | 3 | ND |
| γc | 0 | 80 | 80 |

Abbreviation: ND, not done.
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Fig 6. Anti-K562 and ADCC cytotoxic activity of γc transduced mononucleated bone marrow cells. After γc gene transfer, P1 bone marrow mononucleated cells cultured for 3 weeks in the continuous presence of SCF + IL-2 and IL-7 (A) or SCF + IL-15 (B) were assessed for their respective cytotoxic activities against antibody-coated P815 cell line (○) or the K562 cell line (□). CD34+ γc+ cord blood cells were cultured in the same conditions and tested for their cytotoxic activity against uncoated P815 was always less than 4% at any effector:target ratio studied. Cytotoxicity index is defined in the Materials and Methods section. P2 γc-transduced bone marrow mononucleated cells showed a similar cytotoxic activity after a 3-week culture in the continuous presence of SCF, IL-2, and IL-7. Experiments in the presence of SCF and IL-7 could not be performed because of cell unavailability.

It was found that cord blood-derived CD34+ cells depleted of committed cells that could be engaged into the NK cell differentiation pathway (CD2+, CD16+, CD8+, CD56+), could mature into functional NK cells after a 3-week culture period with SCF + IL-2 and IL-7 in the absence of stromal cells. Previous studies have determined that in vitro NK cell differentiation could be achieved from CD34+ lin− adult bone marrow cells in the presence of stromal cells and IL-2. It was later demonstrated that stromal cells could be replaced by SCF, a result we confirmed here for cord blood cells, although IL-7 was also required. As previously shown, NK cell differentiation was more efficiently achieved from more mature CD34+ CD7+ than CD34+ CD7− cells as a very low number of cells expressed CD56 after a 3-week period of culture in the presence of IL-2, IL-7, and SCF of the latter population.

Because IL-15 induces proliferation and increases cytotoxicity activity of mature NK cells, we assessed the ability of IL-15 to induce cord blood CD34+ cells to differentiate into mature NK cells. As reported herein, the SCF + IL-15 cytokine cocktail enabled committed CD34+ CD7+, as well as immature CD34+ CD7− cells from cord blood, to proliferate, to express CD56 and other NK cell-associated markers, and to exert NK and ADCC activity. IL-15 appears as a driving force towards NK cell differentiation since cord blood CD34+ cells cultured for 4 weeks in the presence of SCF and IL-15 have lost their myeloid potential as shown by the absence of maturation into CFU-GM colonies. In contrast, a proportion of the same cells cultured for 3 to 4 weeks in the presence of SCF, IL-2, and IL-7 retained the ability to grow into CFU-GM colonies. These results demonstrate that IL-15 is able to induce NK cell differentiation from cord blood hematopoietic progenitor cells when combined with SCF. Of note, Mrozek et al. have recently reported that adult CD34+ lineage-marrow cells can differentiate into CD56 (+) NK cells in the presence of IL-15. At slight difference with their findings, we found that some CD16 (+) cells exhibiting ADCC activity could also differentiate in the presence of IL-15.

The CD16+ CD2+ cell population appears to be committed towards the natural killer lineage, as it has no progenitor potential for the myeloid lineage, because it contains CD3ε transcripts and kills specifically an antibody-coated target cell line. It could represent an incomplete maturation step without expression of NK cell receptors. Alternatively, it could represent a particular subpopulation of killer cells only present during early ontogeny.

γc gene transfer into SCID XL γc− marrow cells re-
store NK cell differentiation. SCID X1 is characterized by faulty differentiation into T and NK lymphocytes due to γc gene mutations impairing signalling through γc containing cytokine receptors.\textsuperscript{5,32,33} To further assess the role of γc (+) cytokine receptors into NK cell differentiation and as a preclinical step towards SCID X1 gene therapy, SCID X1 γc (−) marrow cell differentiation into NK cells was evaluated following γc gene transfer. γc gene transfer following γc (−) marrow cells cocultured with irradiated MFG B2 γc producing & CRIP cells was efficient, as approximately 8% to 10% CD34 cells expressed γc immediately after the procedure in both cases studied. Further γc gene expression was also demonstrated by CFU-GM and BFU-E colony assays. In the presence of SCF, IL-2, IL-7, or SCF and IL-15, 60% to 80% of cultured cells were found to express γc after a 3 to 6 week culture period. Before gene transfer, marrow cells from both SCID X1 patients tested did not contain NK cells as judged by CD56 and other marker expression. γc gene expression by SCID X1 marrow cells enabled marrow cells to proliferate and to mature into CD56\textsuperscript{+} cells that display NK and ADCC activity. Amplification by gene transfer and culture with cytokines of a small pool of NK cells is highly unlikely given CD56\textsuperscript{−} cells were hardly detectable in patients’ marrow samples. Although the experiment could be performed with the marrow from only one patient, it was striking to notice that marrow cell culture in the presence of SCF and IL-15 resulted in predominantly NK cell maturation. These results are overall similar to what was observed with cord blood CD34\textsuperscript{+} cells cultured in the presence of the same cytokine combinations. SCID X1 patients, as well as cord blood-derived NK cells expressed CD36 and ε transcripts, as previously described for adult natural killer cells.\textsuperscript{30,34} These results suggest that hematopoietic progenitor cells from γc (−) patients have a similar potential for NK cell differentiation as age-matched control cells, once γc gene is expressed. The role of γc in proliferation of early NK cell progenitors is thus demonstrated. However, whether commitment to the NK cell lineage precedes or follows γε-dependent cell proliferation remains to be determined.

That γc gene transfer results in efficient NK cell differentiation from SCID X1 marrow cells further demonstrates the functional ability of transduced γc as previously shown after γc gene transfer into B-cell lines from the same patients.\textsuperscript{37} In the latter cells, stable γc expression led to high-affinity IL-2 binding and JAK-3 activation.\textsuperscript{37} Altogether, these data provide further support that retroviral-mediated γc gene transfer could partially correct the SCID X1 phenotype. However, it will be necessary to confirm in other patients these results and to show that γc gene transfer into patients’ CD34\textsuperscript{+} cells can also restore T-cell differentiation. This is presently being assessed in a fetal thymic organ culture assay. It will also be necessary to show that a sufficient, albeit still low, number of transduced pluripotent stem cells will lead to long-term in vivo differentiation of NK and T cells given the possible selective advantage conferred to transduced cells. This hypothesis, which is supported by the in vitro selective advantage of γc (+) transduced Epstein-Barr virus (EBV)-B cells and by preliminary data in patients affected by a closely related disease ADA (−) SCID treated by gene transfer into CD34\textsuperscript{+} cord blood or marrow cells\textsuperscript{35-37} needs to also be tested in vivo in animal models.\textsuperscript{6}

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Role of interleukin-2 (IL-2), IL-7, and IL-15 in natural killer cell differentiation from cord blood hematopoietic progenitor cells and from gamma c transduced severe combined immunodeficiency X1 bone marrow cells

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