Human NK cell development in NOD/SCID mice engrafted with cord blood CD34+ cells

Christian P. Kalberer, Uwe Siegler, and Aleksandra Wodnar-Filipowicz
From the Experimental Hematology, Department of Research, University Hospital Basel, Basel, Switzerland

Short title: Human NK cell development in NOD/SCID mice
Scientific heading: Hematopoiesis
Word counts: Manuscript text: 5443
Abstract: 234

Correspondence: Aleksandra Wodnar-Filipowicz, Experimental Hematology, Department of Research, University Hospital Basel, Hebelstrasse 20, CH-4031 Basel, Switzerland; phone: ++41-61-2652333, FAX: ++41-61-2652350, e-mail: Aleksandra.Wodnar-Filipowicz@unibas.ch

Supported by grants from the Swiss National Science Foundation (31-67072.01 and 4046-058689), Stiftung zur Krebsbekämpfung and the Foundation Cord Stem Cell Therapy, Switzerland.
Abstract

Definition of the cytokine environment which regulates the maturation of human natural killer (NK) cells has been largely based on in vitro assays due to lack of suitable animal models. Here we describe conditions leading to the development of human NK cells in NOD/SCID mice engrafted with hematopoietic CD34+ precursor cells from cord blood. Following 1 week-long in vivo treatment with various combinations of interleukin (IL)-15, flt3 ligand, stem cell factor, IL-2, IL-12 and megakaryocyte growth and differentiation factor, CD56+CD3- cells were detected in bone marrow (BM), spleen and peripheral blood (PB), comprising 5% - 15% of human CD45+ cells. Human NK cells of NOD/SCID mouse origin closely resembled NK cells from human PB with respect to phenotypic characteristics, interferon (IFN)-γ production and cytotoxicity against HLA class I-deficient K562 targets in vitro and anti-tumor activity against K562 erythroleukemia in vivo. In the absence of growth factor treatment, CD56+ cells were present only at background levels but CD34+CD7+ and CD34-CD7+ lymphoid precursors with NK cell differentiation potential were detected in BM and spleen of chimeric NOD/SCID mice for up to 5 months after transplantation. Our results demonstrate that limitations in human NK cell development in the murine microenvironment can be overcome by treatment with NK cell growth-promoting human cytokines, resulting in maturation of IFN-γ producing cytotoxic NK cells. These studies establish conditions to explore human NK cell development and function in vivo in the NOD/SCID mouse model.

Corresponding author’s e-mail address: Aleksandra.Wodnar-Filipowicz@unibas.ch
Introduction

Human natural killer (NK) cells comprise approximately 10% of peripheral blood (PB) lymphocytes and are characterized phenotypically by expression of CD56 and lack of CD3 cell surface antigens. They are important effectors of the innate immune system and contribute to the first line of defense against infections and malignancy. In contrast to T lymphocytes, NK cells are able to kill cancer and virus-infected target cells without the need for prior antigen stimulation. NK cell precursors have been identified within the CD34+ hematopoietic cell population in adult bone marrow (BM) and umbilical cord blood (CB). These precursors can efficiently generate mature NK cells in vitro in the presence of interleukin (IL)-15 and early acting cytokines such as flt3 ligand (FL) or stem cell factor (SCF) which increase the frequency of NK cell precursors responding to IL-15. IL-15 is a key cytokine in NK cell development. Both targeted disruption of the IL-15 or IL-15 receptor genes in mice and spontaneously occurring mutations in the signaling components of the receptor in humans cause a block in early NK cell development. FL and SCF play an important role in early differentiation steps of NK cells and in their subsequent expansion and functional maturation as revealed in mice rendered FL deficient or carrying mutations in the c-kit receptor. Other important regulatory cytokines are IL-12 and IL-18 which enhance cytotoxicity and trigger cytokine release by mature NK cells.

Biological responses of NK cells are controlled by a balance of signals from inhibitory and activatory receptors. The inhibitory receptors recognize epitopes shared by different alleles of HLA class I molecules and deliver negative signals, thereby suppressing NK cell function and blocking lysis of normal cells. In the absence of inhibitory signals, the ability of NK cells to lyse cells altered by virus infection and tumor transformation is mediated by the activatory receptors, including the Fcγ receptor III (CD16) and natural cytotoxicity receptors (NCRs) which were recently identified as unique NK-specific cell surface molecules. In human leukemia, expression of NCRs is downregulated and correlates with low NK cell cytotoxicity while, conversely, anti-leukemic responses are enhanced by NK cell alloreactivity in mismatched stem cell transplants. Therefore, NK cells appear important for tumor surveillance and might be exploited in immunotherapy of diseases.

However, understanding the development and function of human NK cells is largely based on in vitro analyses and models to study human NK cells in vivo are lacking. Results of murine NK cell studies are not always applicable to the human NK cell system due to the lack of a murine homologue of CD56 and to differences in the inhibitory and activatory receptors. Furthermore, it has not been possible to use nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice repopulated with human hematopoietic progenitors for studies on human NK cells since the lymphoid differentiation in these mice is restricted to the B cell lineage, whereas T and NK cells are produced at a minimum level or not at all.
this study, we show that NOD/SCID mice repopulated with CB CD34+ cells contain human NK cell precursors and that administration of human recombinant IL-15, together with other NK cell growth-promoting cytokines, leads to the in vivo development of NK cells in BM, spleen and blood circulation. The combination of IL-15 and FL is sufficient to generate NK cells at levels comparable to the NK cell content in human PB. NK cells generated in NOD/SCID mice are CD56+CD3+, express CD16 and NKp46 and are functional with respect to IFN-γ production in response to IL-12 and IL-18. Furthermore, NK cells of NOD/SCID origin show cytotoxic activity against K562 cells in vitro and reduce growth of K562 erythroleukemia in vivo. Establishment of human NK cell development in NOD/SCID mice provides an in vivo system to investigate NK cells in immunotherapeutic strategies against infectious diseases and cancer.
Materials and methods

Cord blood cell preparation

CB was kindly provided by the Department of Obstetrics and Gynecology, University Hospital Basel and the Department of Obstetrics and Gynecology, Kantonsspital Bruderholz, Switzerland with informed consent of the mothers. The investigations were approved by the Ethical Committee of the University Hospital Basel. CB mononuclear cells were separated by Histopaque (<1.077 g/cm³; Sigma, St. Louis, MO) density-gradient centrifugation and subsequent red blood cell lysis. Frozen samples were pooled after thawing and CD34⁺ cells were isolated with superparamagnetic MACS (magnetic cell sorting) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. The purity of the CD34⁺ cell population ranged from 80 to 95%.

Transplantation of CD34⁺ cells into NOD/SCID mice

NOD/LtSz-scid/scid (NOD/SCID) mice (The Jackson Laboratory, Bar Harbor, ME) were maintained under specific pathogen-free conditions in the animal facility of the Research Department, University Hospital Basel. 1-2 x 10⁵ CB CD34⁺ cells together with 1 x 10⁶ irradiated (1500 cGy) human PB mononuclear carrier cells were injected intravenously (i.v.) into the tail vein of 8 week-old NOD/SCID mice previously given 375 cGy (Co-60 source, 2 cGy/min). Mice were kept on acidified drinking water supplemented with Bactrim (32/160 mg/L; Roche Pharma AG, Reinach, Switzerland) for the duration of the experiment.

In vivo treatment with human growth factors

8-10 weeks after transplantation, NOD/SCID mice were injected intraperitoneally with phosphate-buffered saline (PBS) or the following recombinant human growth factors: IL-15, FL (both from Immunex, Seattle, WA), SCF (Amgen, Thousand Oaks, CA), PEGylated megakaryocyte growth and development factor (MGDF; Amgen and Kirin Brewery, Tokyo, Japan), IL-2 (Novartis, Basel, Switzerland) and IL-12 (Roche, Nutley, NJ). IL-15, FL, SCF, MGDF and IL-2 were administered for 7 consecutive days, each at 10 µg daily, and IL-12 was administered at 1 µg per day for the last 2 days of the treatment. 24 hours after the last injection, spleen and BM cells were harvested and single-cell suspensions of spleen and BM were prepared. To measure circulating human NK cells, PB was drawn weekly up to 3 weeks after growth factor injections. Statistical analyses for comparison of treatment groups were performed with the Mann-Whitney U-test.
Flow cytometry and cell sorting

Three-color fluorescence-activated cell sorter (FACS) analysis was used to characterize human engraftment of transplanted NOD/SCID mice. Single-cell suspensions from BM and spleen were resuspended in FACS buffer containing PBS, 2% fetal calf serum (FCS; both from Invitrogen, Carlsbad, CA) and 0.02% NaN₃ (Fluka, Buchs, Switzerland) and stained on ice for 20 minutes with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or allophycocyanin (APC)-conjugated monoclonal antibodies (mAbs) against human CD2, CD3, CD7, CD16, CD19, CD33, CD34, CD38, CD45, CD56, CD62L, CD69, CD158a, CD158b, NKB1 and HLA-DR, or isotype control antibodies (all from BD Pharmingen, San Jose, CA). Staining with unlabelled mAb anti-CD94 (clone 39B10), anti-NKp46 (clone 9E2; both a generous gift from M. Colonna and A. Bouchon, Basel Institute for Immunology, Basel, Switzerland) and anti-IL-2Rγ chain (clone CP.B8; kindly provided by D. Baker, Biogen Inc., Cambridge, MA) was revealed with secondary PE- or FITC-conjugated goat anti-mouse antibodies (Southern Biotechnology Associates, Birmingham, AL). Normal mouse serum was used to saturate free binding sites of secondary antibodies before cells were subsequently incubated with directly labeled mAbs. Propidium iodide (PI; Sigma) staining was used to exclude dead cells from the analysis. FACS analysis of circulating NK cells was performed by incubating whole blood with labeled antibodies at RT for 20 min, followed by erythrocytes lysis with FACS Lysing Solution (BD Pharmingen) and subsequent washing with FACS buffer. FACS analysis was performed on a FACS Calibur (Becton Dickinson) and data analyzed using CellQuest Pro software (Becton Dickinson). For cell-sorting experiments, BM cells from transplanted NOD/SCID mice were resuspended in FACS buffer without NaN₃ and stained with the appropriate antibodies. Cells were washed, incubated with PI and sorted on a FACS Vantage SE (Becton Dickinson).

NK cell differentiation in vitro

Cell suspensions from NOD/SCID mice BM or spleen containing 20% - 90% human CD45⁺ cells were seeded at 1-2 x 10⁶/mL cells in 24-well plates in IMDM containing 5% FCS, 5% human AB⁺ serum (Blutspendezentrum Basel), 380 µg/mL iron-saturated human transferrin, and 1% bovine serum albumin and supplemented with IL-15, FL and SCF (each at 100 ng/mL). After 1 week, cells were transferred to 6-well plates and half the medium was replaced once a week for further 2-5 weeks as specified in the Results. The development of CD56⁺ NK cells was determined at the indicated time points by FACS.

NK cell lines were cultured by restimulation with irradiated mononuclear cells from human PB and phytohaemagglutinin HA 16 (2 µg/mL; Murex Biotech Ltd., Dartford, England) in the presence of 100 U IL-2 every 3 - 4 weeks.
NK cell cytotoxicity and IFN-γ production

After 4-5 weeks of differentiation in culture, CD56+ cells were purified by positive selection with MACS CD56-microbeads (Miltenyi Biotec). Cells were washed, resuspended in IMDM containing 2% FCS, and cytotoxicity against the NK-sensitive target K562 was determined in a 4-hour lactate dehydrogenase (LDH) release assay (CytoTox 96; Promega, Madison, WI) according to the manufacturer’s instructions. The effector:target ratio ranged from 10:1 to 0.6:1.

IFN-γ production was measured by intracellular flow cytometry. MACS-purified NK cells from differentiation cultures (purity 80% - 95%) or FACS sorter-purified NK cells from BM of growth factor-treated NOD/SCID mice were washed and 1 x 10^6 cells/mL were plated in 96-well plates for 36 hours in IMDM containing 5% FCS, 5% AB + human serum, 10 U/mL IL-12 and 100 ng/mL IL-18 (PeproTech Inc, Rocky Hill, NJ). Brefeldin A (Sigma) was added at 5 µg/mL for the final 8 hours of the culture. Cells were washed with FACS buffer, stained with anti-CD56-PE mAb for 20 minutes on ice and fixed in 2% paraformaldehyde for 15 minutes at RT. Cells were washed twice and permeabilized in saponin-containing FACS buffer. Anti-IFN-γ-FITC and isotype control mAb (BD Pharmingen) were added for 30 minutes at RT. Cells were washed twice in permeabilization buffer, once in FACS buffer and analyzed with FACS Calibur.

K562 tumor formation

K562 erythroleukemia cells were resuspended in 100µl PBS and injected subcutaneously (s.c.) into the dorsal lateral thorax of NOD/SCID mice. NK cells, resuspended in 200µl PBS, were injected i.v. one day after the tumor cell inoculation. In mice engrafted with CB CD34+ cells, K562 cells were inoculated one day after 7 day-long treatment with IL-15 and FL. The effect of NK cells on tumor growth was determined in groups of 2-4 animals. The tumor growth was monitored weekly and the tumor surface area was calculated using the formula (a/2 x b/2) x π, where a and b are the long and short diameters (mm). Statistical analyses for comparison of treatment groups were performed with the unpaired Student’s T-test.
**Results**

**Growth factors induce maturation of human NK cells in NOD/SCID mice**

To investigate the requirements permissive for human NK cell development in vivo, the NOD/SCID xenotransplantation system was chosen to test the effect of human growth factors known to be involved in human NK cell development in vitro. Groups of NOD/SCID mice transplanted 2 months earlier with highly enriched CB CD34+ cells were injected for 7 consecutive days with one of the following 4 combinations of cytokines: IL-15/FL, IL-15/FL/SCF, IL-15/FL/IL-12 and IL-15/FL/SCF/MGDF/IL-2. On day 8, BM and spleens were examined for the presence of human NK cells (Table 1). Among the CD45+ human lymphocytes, only background levels of 0.3% ± 0.1% of human CD56+ NK cells were detected in untreated control animals (Table 1 and Figure 1A). Upon administration of growth factors, the frequency of CD56+ cells increased approximately 15-fold and was similar among mice tested with the four growth factor combinations (range: 3.9% ± 0.9% to 4.9% ± 2.0%). The content of NK cells generated in the BM of NOD/SCID mice with human growth factor treatment was comparable to that in human BM and CB (1% - 10% and 4% - 12% CD56+ cells, respectively, as determined using 6-8 samples from both blood sources; Figure 1C and results not shown). Human NK cells were also found in spleens of NOD/SCID mice where they constituted 3.2% ± 1.4% to 5.0% ± 1.4% of human CD45+ cells depending on the growth factors used; IL-15/FL/IL-12 led to NK cell development mainly in the BM but only marginally in the spleen (Table 1). Also in the blood circulation of NOD/SCID mice, 12.2% ± 0.8% (n=8) of CD45+ human cells were CD56+ NK cells, which is corresponding to the number of NK cells in human PB (7% - 15%, Figure 1D, E), and they were detectable for at least 3 weeks after growth factor-treatment.

Human NK cells which developed in NOD/SCID mice were examined for expression of a panel of cell surface markers. The staining patterns of NK cells residing in BM and spleen were indistinguishable, and there were no marked differences in NK cell phenotype with respect to the growth factor combination used to generate them. NK cells from BM of IL-15/FL-treated mice are shown in Figure 1B. The CD56+ cells were always CD3-, indicating that these cytokine combinations promoted only development of bona fide NK cells and not the CD56-CD3+ NKT cell population. CD94 and the IL-2Rγ chain were expressed on all CD56+ cells from NOD/SCID mice. The early activation marker CD69 was expressed on virtually all NK cells, likely as a response to IL-15, while CD25, the low-affinity IL-2 receptor α-chain, was not present. Of the other tested markers, CD2, CD7 and CD62L selectin were expressed by 30% - 45%, 65% - 80% and up to 60% of NK cells, respectively (Figure 1B). Intracellular perforin was expressed by all NK cells, HLA-DR was expressed only at low levels or not at all, and c-kit was absent (not shown). Interestingly, staining of CD16 and...
CD56 surface markers revealed that the prevalence of the CD56\textsuperscript{dim}CD16\textsuperscript{high} population which is characteristic of human CB and PB NK cells (Figure 1C, D) was not apparent in NK cells generated in NOD/SCID mice. Instead, CD16 was expressed at various cell surface densities on 50% - 70% of all CD56\textsuperscript{+} NK cells (Figure 1B, E). NKp46, the major NCR selectively expressed by NK cells, showed a heterogeneous expression pattern of 5% to 19% (Figure 1B), which was lower than in human NK cells from PB and CB (Figure 1C, D). This lower NKp46 expression was not due to a limiting availability of the CD3 chain, the coreceptor for NKp46, since FACS staining revealed CD3\textsuperscript{+} expression in all human NK cells generated in transplanted mice (data not shown). Based on these results we conclude that human recombinant growth factors, including IL-15 and FL, specifically promoted the maturation of human NK cells in the BM and spleen microenvironments of NOD/SCID mice. The phenotype of these cells closely resembled but was not identical to NK cells from human PB.

**Effect of growth factor treatment on human cell engraftment and lineage composition**

The effect of growth factor treatment of transplanted NOD/SCID mice on the cellularity of BM and spleen and on engraftment with human cells was also examined (Figure 2). The BM of 4 long bones of untreated control NOD/SCID mice contained 22 ± 4 x10\textsuperscript{6} cells of which 64% ± 5% were of human origin. The growth factor injections reduced the cellularity of BM but not spleens. In particular, a fast and dramatic 10-fold cell loss was observed with the administration of IL-15/FL/IL-12 which involved 2 days treatment with IL-12 (Figure 2A). This is in accord with the reported hypoplasia in BM but not spleen upon IL-12 administration in both humans and mice.\textsuperscript{25-27} The overall level of human CD45\textsuperscript{+} cells was reduced by 30% - 50% in BM and spleens in all groups of growth factor-treated mice except the IL-15/FL/IL-12 group (Figure 2B). This reduction was mainly associated with a decrease in CD19\textsuperscript{+} B lymphocytes which comprised the majority of human cells in both untreated and treated mice and which were reduced by 15% - 30% after growth factor injections (Table 1). Concomitantly, the proportion of myeloid cells expressing CD33 was increased up to 3-fold. The lower engraftment and changes in the proportions between B cells and myeloid lineages upon growth factor treatment are reminiscent of the effects of FL alone and together with IL-7 and SCF, which were reported to have an even greater effect when injected over several weeks.\textsuperscript{28}

**Lymphoid precursors with NK cell differentiation potential are present in BM and spleen of transplanted NOD/SCID mice**
The finding that human NK cells developed within 7 days of growth factor administration prompted us to characterize the NK precursor cells in NOD/SCID mice grafted with human hematopoietic cells. In a first series of experiments, we investigated whether CD56+ NK cells could also be generated in vitro from BM and spleen cells obtained from NOD/SCID mice not treated with growth factors (Figure 3). In cultures containing IL-15, FL and SCF, human CD56+ cells developed within the first week and reached 68% - 96% by 3 weeks (Figure 3A). This was faster and more efficient than the development of NK cells from CB CD34+ progenitors cultured under the same conditions (17% ± 10% CD56+ cells at week 3), in particular considering the fact that the purity of CB CD34+ cells at the start of culture was at least 80%, while CD34+ human cells constituted only 19.0% ± 1.1% and 6.4% ± 0.9% of all cells in NOD/SCID BM and spleen, respectively (see Table 2). The phenotypes of NK cells of NOD/SCID mice origin and those generated from CB CD34+ cells were similar (Figure 3B). CD16 was only expressed at low levels by a minor population of 2% - 15% of all NK cells. Remarkably, NKp46 was expressed by up to 60% of NK cells generated in vitro (Figure 3C), indicating that NKp46 is present on CD16- and CD16+ NK cells. The surface markers CD7, CD62L, CD69 and CD161 were expressed at various levels on 5% - 30% of in vitro-generated NK cells from NOD/SCID BM. The killer immunoglobulinlike receptors CD158a, CD158b and NKB1 were not detected (data not shown), in agreement with reports that NK cells differentiated in vitro from CD34+ cells are mainly negative for these receptors. CD56+CD3+ NKT cells or CD3+ T cells were never found in these cultures (data not shown).

To define the precursor cell populations able to give rise to NK cells in vivo and in vitro, we performed FACS sorting of human cells from BM of growth factor-untreated NOD/SCID mice (Figure 4A). CD34+CD38- and CD34+CD38+ hematopoietic progenitor cells as well as CD34+CD7+ and CD34+CD7+ cells, which have been reported to contain NK precursor cells, were cultured in the presence of IL-15, FL and SCF and NK cell development was examined. The fastest differentiation toward CD56+ cells, apparent after 3 days, was obtained with CD34+CD7+ cells, suggesting the highest frequency of NK precursors in this cell fraction (Figure 4B). CD34+CD7+ cells also gave rapidly rise to 75% CD56+ cells after 14 days. In contrast, in cultures initiated with primitive hematopoietic CD34+CD38- progenitors, only 20% of all cells expressed CD56 by day 14. These results indicate that even in the absence of growth factor treatment, human NK cell precursors had progressed in the NOD/SCID microenvironment to an advanced differentiation stage, providing an explanation for the faster response of unseparated NOD/SCID-derived cells compared to CB CD34+ cells cultured under the same conditions (Figure 3A). Despite different kinetics, NK cells generated in vitro from sorted cell populations (R1 to R4) of NOD/SCID mouse BM acquired the same phenotypic characteristics as those from bulk cultures described in Figure 3 (results not shown).
Treatment of transplanted NOD/SCID mice with IL-15/FL, IL-15/FL/SCF, and IL-15/FL/IL-12 combinations in vivo specifically increased the population of human CD34^+ CD7^+ NK cell precursors up to 5 fold in both BM and spleens (Table 2). This increase is in accord with the increase in CD56^+ cells observed following growth factor injections (see Table 1), some of which were CD56^+CD7^+ (data not shown). The proportion of total CD34^+ cells was significantly decreased in BM, primarily due to a reduction in CD34^+CD19^+ pre-B cell levels. Neither the CD34^+CD19^- nor the CD34^+CD7^- cell populations were affected by the cytokine treatment in either organ. We conclude that the tested growth factors acted on a CD7^+ NK precursor at the transition from the CD34^+ to CD56^+ stage.

IFN-γ production by human NK cells generated in NOD/SCID mice

A hallmark of NK cell function is their ability to produce IFN-γ. To assess this feature, CD56^+ NK cells from BM of growth factor-treated NOD/SCID mice were isolated by cell sorting and stimulated in vitro for 36 hours with IL-12 and IL-18. Additionally, NK cells were isolated after 4 week-long culture of BM from NOD/SCID mice, both growth factor-treated and untreated. The proportion of IFN-γ-producing NK cells was measured by intracellular FACS analysis. NK cells differentiated in vivo and in vitro responded equally well to the cytokine stimulus (Figure 5). Of the three types of tested NK cells, production of IFN-γ was found in 14% ± 6% and 15% ± 5% of cells purified either directly or following culture of growth factor-treated mouse BM, and in 6.4% ± 1.7% of NK cells generated in vitro from BM of untreated NOD/SCID mice. For comparison, purified NK cells from human PB were included in the assay and responded more readily to IL-12 and IL-18 (33% ± 10%). The differences in IFN-γ production may be related to the maturation stage of the different NK cell populations generated under these different experimental conditions.

Cytotoxic activity of NOD/SCID mouse-derived human NK cells

Another characteristic of NK cells is the capacity to kill target cells without prior sensitization. To study the cytotoxic activity of NOD/SCID mouse-derived human NK cells, we used HLA class I-deficient human K562 erythroleukemia cells which represent a sensitive NK cell target in vitro and, upon s.c. inoculation in vivo, form a solid tumor whose growth can be easily followed over time.32

For the analysis of K562 lysis in vitro, NK cells generated in growth factor-treated mice were not directly examined due to the limited number of cells that could be isolated by FACS sorting. Instead, we tested the cytotoxicity of human NK cells obtained in higher numbers in differentiation cultures of BM and spleen cells of growth factor-untreated NOD/SCID (Figure 6A). These NK cells were highly cytotoxic; in particular, the killing
potential of BM-derived cells was even higher than that of human PB NK cells. The well-pronounced cytotoxic properties of in vitro generated human NK cells of NOD/SCID origin are in accord with high levels of the activatory receptor NKp46 expressed by these cells (Figure 3C).\textsuperscript{33} To study the cytotoxic effect of human NK cells in vivo, NOD/SCID mice were inoculated s.c. with K562 cells and challenged i.v. one day later with NOD/SCID BM-derived NK cells which showed strong cytotoxicity in vitro. Tumor growth, apparent after 3 and 4 weeks following K562 inoculation, was significantly suppressed (60-70\%) by adoptively transferred NK cells (Figure 6B). This retardation of tumor growth was comparable to the effect of IL-2 stimulated NK cells obtained from human PB. We have also inoculated K562 cells into NOD/SCID mice which had been transplanted with CB CD34\(^+\) cells and injected with IL-15 and FL for 7 days. Under these conditions, which lead to generation of endogenous human NK cells, a marked reduction (up to 50\%) of tumor growth was observed (Figure 6B). A possibly lower yield of in vivo generated NK cells may account for a lesser anti-tumor effect than using a controlled number of cotransplanted NK cells. Based on these results, we conclude that NOD/SCID mouse-derived human NK cells are functional with respect to cytotoxicity against HLA class I-deficient targets both in vitro and in vivo.
Discussion

The NOD/SCID mouse transplantation model has been widely used to study the biology of human hematopoietic stem cells of different ontological origins.\textsuperscript{34-36} These studies indicated that the BM microenvironment of NOD/SCID mice supports the survival and multilineage development of human hematopoietic progenitors, but can be selective with regard to the maturation of individual blood cell lineages. Within the lymphoid compartment, the B cell lineage develops the most efficiently.\textsuperscript{20,37} On the other hand, human T and NK cells have never been reproducibly detected, and only the transplantation of human thymic tissue or the use of IL-2R-blocking antibodies allowed T lymphopoiesis in vivo.\textsuperscript{38,39} In this study, we identified CD34$^+$CD7$^+$ and CD34$^-$CD7$^+$ NK progenitor cells in BM and spleen of NOD/SCID mice transplanted with CB CD34$^+$ cells and we achieved NK cell differentiation after in vivo administration of human cytokines. The establishment of human NK cell development in NOD/SCID mice provides for the first time an in vivo system to study the mechanisms governing human NK cell differentiation and function.

Several studies have demonstrated that lineage development of the human graft in NOD/SCID mice can be modulated by administration of human cytokines. The effects seen in vivo often did not reproduce those predicted from in vitro cultures, such as a decrease in B cell lineage development in mice receiving FL and IL-7\textsuperscript{28} or inhibition of platelet production by a growth factor combination known to support megakaryopoiesis from cultured progenitors.\textsuperscript{40} In contrast, all the cytokine combinations used in our study, namely IL-15/FL, IL-15/FL/SCF, IL-15/FL/IL-12 and IL-15/FL/SCF/MGDF/IL-2, enabled in vivo maturation of NK cells, thus recapitulating the previously reported effects of these cytokines on the generation of NK cells from cultured CD34$^+$ progenitors. Previous studies identified IL-15 as the crucial factor for NK cell development, along with FL and SCF as cytokines which increase the frequency of NK cell precursors through upregulation of expression of the IL-15R complex.\textsuperscript{3,5} With human NK cells differentiated in vitro from CD34$^+$ progenitors, a functional redundancy of FL and SCF on IL-15 responsiveness has been reported.\textsuperscript{3} Accordingly, the combination of IL-15 and FL was found to be sufficient to induce NK cells in NOD/SCID mice and the number and phenotype of the NK cells did not differ depending on the cytokines used to generate them although subtle differences of additional parameters cannot be excluded. Administration of cytokines was associated with a moderate reduction in the cellularity and the level of human cell engraftment in BM and spleens. This is in agreement with previous studies,\textsuperscript{28,40,41} although the opposite effect was also seen in mice receiving only
limiting doses of stem cells. A rapid and nearly total disappearance of both murine and human cells in NOD/SCID BM was observed when IL-12 was combined with IL-15 and FL. This is possibly associated with the activation of newly formed NK cells, since an IL-12-dependent release of IFN-γ by NK cells resulting in suppression of hematopoiesis in the BM has been postulated in humans and mice. In growth factor-untreated mice we detected human CD34+CD38−, CD34+CD38+, CD34+CD7+ and CD34−CD7+ cell populations, all of which responded to IL-15, FL and SCF in vitro and gave rise to CD56+ NK cells. Judging from the kinetics of CD56+ cell generation in vitro, these different populations may represent early, intermediate and late stages in NK cell development. These NK cell precursors were found as late as 5 months post-transplantation, indicating that they can persist for a long time in the NOD/SCID microenvironment. Similarly, undifferentiated common lymphomyeloid CD34+CD38−CD19− progenitors capable of generating human B, T, NK and granulomonocytic cells under appropriate culture conditions in vitro were maintained over the long-term in transplanted NOD/SCID mice. Altogether, these results indicate that the microenvironment of chimeric NOD/SCID mice harbors human NK progenitors, but an insufficient concentration or lack of cross-reactivity of endogenous murine NK cell-promoting cytokines, in particular IL-15, prevents progression to mature CD56+ NK cells above the background levels observed in untreated control NOD/SCID mice.

Human PB NK cells can be divided into two subsets according to the expression of cell surface markers and functional characteristics. About 10% of all NK cells are CD56highCD16low and produce abundant cytokines following activation. The prominent CD56dimCD16high subset, representing at least 90% of NK cells, secretes less IFN-γ but is more naturally cytotoxic. This phenotypic distinction was not apparent in the NK cell population developing in NOD/SCID mice after growth factor injections. CD16 was expressed at various surface densities with a large proportion of NK cells being CD16−/low. The fact that CD56dimCD16high cells were not present as a predominant NK cell population in growth factor-treated NOD/SCID mice suggests that the development of this subset requires the presence of other, possibly yet unknown, cytokines or that cellular selection is important and cannot be mimicked in the NOD/SCID mouse microenvironment. Despite this difference in phenotypic characteristic, human NK cells of NOD/SCID origin produce IFN-γ, and are strongly cytotoxic against HLA class I-deficient K562 targets in vitro and also capable to reduce K562 erythroleukemia tumor formation in vivo, thus functionally resembling human PB NK cells.
While inhibitory and activatory receptors have a defined function in mature PB NK cells, their role in developing immature NK cells is not clear. In growth factor-treated NOD/SCID mice, we consistently found fewer human NK cells expressing the major triggering receptor NKp46 than CD16+ NK cells. This is different from NK cells generated in vitro from CD34+ cells, where NKp46 is expressed by the majority of CD56+ cells and CD16 only by a small proportion. Based on data from in vitro-differentiated NK cells, two other groups also observed NKp46 preceding CD16 expression.\textsuperscript{47,48} The NOD/SCID-derived NK cells acquired the NKp46\textsuperscript{bright} phenotype only following isolation of the progenitors and further culture. The mechanism that controls the level of NKp46 expression during NK cell development is not known. Noteworthy, however, is that the surface density of NKp46 on mature NK cells from human PB is heterogeneous, with different donors harboring different proportions of NKp46\textsuperscript{bright} and NKp46\textsuperscript{dim} cells which display high and low cytotoxic activity, respectively.\textsuperscript{33} In contrary to the NKp46 activatory receptor, the inhibitory receptors CD158a, CD158b and NKB1 were not detected on NOD/SCID-derived human NK cells following culture. This may result in the absence of inhibitory signals and enhance the killing capacity of NK cells against neoplastic targets, as shown by the important graft-versus-leukemia effect in the haploididentical transplantation setting.\textsuperscript{17}

NK cells, through production of immunoregulatory cytokines and cytotoxic effects, are candidates for immunotherapy of transformed or infected tissues. It remains to be seen whether adoptively transferred NK cells of NOD/SCID origin which recognized and killed HLA-deficient targets can also suppress growth of primary human tumors. Notably, cytokines which boost maturation of NK cells in chimeric NOD/SCID mice have been shown to generate potent anti-tumor responses. Treatment of tumor-bearing mice with FL leads to tumor regression through activation of dendritic and NK cells.\textsuperscript{49} IL-15 activates both T and NK cells, and preclinical studies showed enhanced protection against viral challenge and a potential of IL-15 as a tumor vaccine adjuvant.\textsuperscript{50,51} Interestingly, we recently found that IL-15 upregulates FL expression and that following stem cell transplantation, FL levels are strongly increased\textsuperscript{52,53} suggesting that these two growth factors contribute to fast recovery of NK cells in transplanted patients.\textsuperscript{17} The ongoing discoveries of NK-specific receptors and increasing knowledge about their respective ligands\textsuperscript{54} provide means to modulate the responses of mature NK cells, either using specific antibodies\textsuperscript{55} or by genetic modification. NOD/SCID mice transplanted with hematopoietic progenitors represent an important model for evaluating the immunotherapeutic efficacy of human NK cells and also for investigation of factors and signals orchestrating formation of the NK cell compartment in humans.
Acknowledgment

We thank Elena Chklovskiaia for help with the NK cytotoxicity assay and Verena Jäggin for cell sorting; S. D. Lyman for human recombinant cytokines IL-15 and FL; U. Gubler for human recombinant IL-12; W. Holzgreve, D. Surbek and S. Heinzl for providing cord blood samples; M. Colonna, A. Bouchon and D. Baker for antibodies; G. De Libero, A. Gratwohl, A. Rolink and S. Bridenbaugh for critically reading the manuscript.
References


32. Weichold FF, Jiang YZ, Dunn DE, et al. Regulation of a graft-versus-leukemia effect by major histocompatibility complex class II molecules on leukemia cells: HLA-DR1 expression renders K562 cell...


35 Wang JC, Doedens M, Dick JE. Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. Blood. 1997;89:3919-3924.

36 van der Loo JC, Hanenberg H, Cooper RJ, Luo FY, Lazaridis EN, Williams DA. Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse as a model system to study the engraftment and mobilization of human peripheral blood stem cells. Blood. 1998;92:2556-2570.


42 Bonnet D, Bhatia M, Wang JC, Kapp U, Dick JE. Cytokine treatment or accessory cells are required to initiate engraftment of purified primitive human hematopoietic cells transplanted at limiting doses into NOD/SCID mice. Bone Marrow Transplant. 1999;23:203-209.


Table 1. Effect of growth factor administration on human hematopoietic lineages in BM and spleen of NOD/SCID mice transplanted with CD34⁺ cord blood cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bone marrow*</th>
<th>Spleen*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD56⁺</td>
<td>CD19⁺</td>
</tr>
<tr>
<td>no treatment</td>
<td>0.3±0.1</td>
<td>87.0±1.5</td>
</tr>
<tr>
<td>(20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-15/FL</td>
<td>3.9±0.9‡</td>
<td>62.0±4.0‡</td>
</tr>
<tr>
<td>(7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-15/FL/SCF</td>
<td>4.1±1.1‡</td>
<td>64.0±4.5‡</td>
</tr>
<tr>
<td>(6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-15/FL/IL-12</td>
<td>4.9±2.0‡</td>
<td>86.0±4.0</td>
</tr>
<tr>
<td>(5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-15/FL/SCF/MGDF/IL-2</td>
<td>4.1±1.3†</td>
<td>70.0±3.5†</td>
</tr>
<tr>
<td>(3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* mean % ± SEM of human CD45⁺ cells.
† p-value <0.05
‡ p-value <0.005
Table 2. Effect of growth factor administration on hematopoietic progenitor and lymphoid precursor cells in BM and spleen of NOD/SCID mice transplanted with CD34+ cord blood cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bone marrow*</th>
<th>Spleen*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD34*</td>
<td>CD34* CD19* CD7*</td>
</tr>
<tr>
<td>(number of mice)</td>
<td>CD34* CD34* CD34* CD34* CD34*</td>
<td>CD34* CD19* CD7* CD7*</td>
</tr>
<tr>
<td>no treatment</td>
<td>19.0±1.1</td>
<td>13.2±1.2</td>
</tr>
<tr>
<td>(20)</td>
<td>7.7±1.8‡</td>
<td>2.1±0.4‡</td>
</tr>
<tr>
<td>IL-15/FL</td>
<td>9.7±1.8†</td>
<td>2.7±0.7‡</td>
</tr>
<tr>
<td>(7)</td>
<td>8.3±0.6†</td>
<td>4.2±1.1‡</td>
</tr>
<tr>
<td>IL-15/FL/SCF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-15/FL/IL-12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* mean % ± SEM of human CD45+ cells.
† p-value <0.05
‡ p-value <0.005
Figure legends

Figure 1. Phenotype of human NK cells in BM of growth factor-treated NOD/SCID mice. Two months after transplantation of CB CD34+ cells, NOD/SCID mice were treated with combinations of growth factors (see text) for 7 consecutive days and BM, spleen and PB cells were analyzed 1 day later by flow cytometry. (A) Human NK cells in untreated control (no growth factors, GF) and growth factor-treated mice were detected by staining with the human leukocyte marker CD45 and the NK cell specific marker CD56. The analysis was performed with total mononuclear cells; the numbers indicate the percentage of total BM cells. (B) Human NK cells were characterized by staining with antibodies against CD56 and the indicated NK surface marker. The numbers indicate the percentage of human CD45+ cells. For comparison, human NK cells from (C) CB and (D) PB were stained with antibodies against CD56 and CD16 (gated on lymphocytes) and NKp46 (gated on CD56+ cells). The thin line indicates staining with isotype-matched control antibody. (E) NK cells in PB of NOD/SCID mice treated with IL-15 and FL. The numbers indicate the percentage of human CD45+ cells.

Figure 2. Cellularity and level of human engraftment in BM and spleen of NOD/SCID mice after growth factor treatment. Two months after transplantation of CB CD34+ cells, NOD/SCID mice were treated with the indicated human growth factor combinations. (A) Total cell number was determined from the BM (4 long bones; open bars) and from spleen (closed bars). (B) Human engraftment was defined by staining BM and spleen cells with human anti-CD45 antibody. The mean percentage values ± SEM are shown. Significant differences between untreated and treated groups are marked (p-values *<0.05, **<0.005).

Figure 3. In vitro differentiation of human NK cells from NOD/SCID BM and spleen and from human CB. (A) Single-cell suspensions of BM (I: n=13, white bars) and spleen of transplanted NOD/SCID mice (II: n=5, stripped bars) and purified CB CD34+ cells (III: n=7, black bars) were cultured for 4 weeks in NK differentiation medium containing human recombinant IL-15, FL and SCF. The appearance of CD56+ NK cells was determined by FACS analysis. Shown is the mean percentage ± SEM of total cells in the cultures, nd: not determined. (B) Expression of CD16 and CD56 and (C) of NKp46 on CD56+ NK cells generated in cultures I, II and III at 4 weeks. The thin line indicates staining with isotype-matched control antibody.

Figure 4. In vitro differentiation of human NK cells derived from FACS sorter-purified progenitor cells of NOD/SCID mouse BM. (A) Hematopoietic progenitor cells and NK precursor cells were identified in the BM of untreated NOD/SCID mice by staining with
CD34/CD38 (left panel) and CD34/CD7 (right panel). Cells falling into the gates R1 (CD34⁺CD38⁻: 1.5% of total BM cells), R2 (CD34⁺CD38⁺: 12%), R3 (CD34⁺CD7⁺: 0.5%) and R4 (CD34⁻CD7⁺: 1.5%) were sorted and cultured for 14 days in NK differentiation medium (see Materials and methods). (B) The development of CD56⁺ NK cells in vitro was followed by FACS analysis at the indicated time points.

Figure 5. IFN-γ production by NK cells from NOD/SCID mice. (A) FACS sorter-purified CD56⁺ NK cells from BM of growth factor (GF)-treated NOD/SCID mice (I: n=3), NK cells generated in vitro within 4 weeks from BM of GF-treated (II: n=3) and untreated control (III: n=5) NOD/SCID mice, and purified NK cells isolated from human PB mononuclear cells (IV: n=2) were stimulated with medium containing IL-2 (white bars) or IL-12 and IL-18 (black bars) for 36 hours. Synthesis of IFN-γ was measured by intracellular FACS analysis and the percentages ± SEM of IFN-γ⁺ NK cells are shown. (B) Representative FACS pictures of IFN-γ⁺-producing NK cells generated in cultures II, III and IV are shown. The numbers represent the percentage of total cells in culture.

Figure 6. Cytotoxicity of NK cells from NOD/SCID mice. (A) NK cells were harvested after 28 days culture of NOD/SCID BM (n=2, stripped bars) or spleen cells (n=3, dotted bars) in the presence of IL-15, FL and SCF and, for comparison, NK cells were purified from human PB mononuclear cells (n=2, white bars). Their cytotoxic activity was tested against the NK-sensitive K562 target cell line for 4 hours with an enzymatic LDH release assay. (B) NOD/SCID mice were injected s.c. with K562 cells (1x10⁷) alone (black bars) or followed one day later by NK cells (2-5x10⁶) from human PB (white bars) or generated in vitro from NOD/SCID BM (stripped bars). NOD/SCID mice which had been transplanted with CB CD34⁺ cells were injected with human IL-15 and FL for 7 days and inoculated with K562 cells one day later (grey bars). The tumor size is expressed as mean area ± SEM of 2-4 animals per treatment group. Significant differences compared to the group without NK cells are marked (p-values *<0.05, **<0.005).
Kalberer et al. Fig. 2

A

Number of cells x 10^6

B

% human CD45+ cells

BM

spleen

no factors

IL-15

IL-15

IL-15

IL-15

IL-15

FL

FL

FL

FL

SCF

SCF

IL-12

IL-2

MGDF
Kalberer et al. Fig.4

A

B

Days in culture

% CD56+ cells
A

I NK cells sorted from GF-treated mice
II NK cells from BM cultures of GF-treated mice
III NK cells from BM cultures of untreated mice
IV hu PB NK cells

% IFN-γ+ NK cells

B

IL-2

II IL-12 + IL-18

CD56

IFN-γ

80 0.1

62 18

92 7

45 44

Kalberer et al. Fig. 5
Human NK cell development in NOD/SCID mice engrafted with cord blood CD34+ cells

Christian P Kalberer, Uwe Siegler and Aleksandra Wodnar-Filipowicz