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Long-Term Human CD34+ Stem Cell-Engrafted Nonobese Diabetic/SCID/IL-2Rγnull Mice Show Impaired CD8+ T Cell Maintenance and a Functional Arrest of Immature NK Cells

Maya C. André,* Annika Erbacher,* Christian Gille,† Vanessa Schmauke,* Barbara Goecke,* Alexander Hohberger,‡ Philippa Mang,* Aylene Wilhelm,* Ingo Mueller,* Wolfgang Herr,‡ Peter Lang,* Rupert Handgretinger,*† and Udo F. Hartwig‡,1

Allogeneic hematopoietic stem cell transplantation represents the most effective form of immunotherapy for chemorefractory diseases. However, animal models have been missing that allow evaluation of donor-patient–specific graft-versus-leukemia effects. Thus, we sought to establish a patient-tailored humanized mouse model that would result in long-term engraftment of various lymphocytic lineages and would serve as a donor-specific surrogate. Following transfer of donor-derived peripheral blood stem cells into NOD/SCID/IL-2Rγnull (NSG) mice with supplementation of human IL-7, we could demonstrate robust engraftment and multilineage differentiation comparable to earlier studies using cord blood stem cells. Phenotypical and functional analyses of lymphoid lineages revealed that >20 wk posthematopoietic stem cell transplantation, the majority of T lymphocytes consisted of memory-type CD4+ T cells capable of inducing specific immune functions, whereas CD8+ T cells were only present in low numbers. Analysis of NSG-derived NK cells revealed the expression of constitutively activated CD56brightCD16+ killer Ig-like receptor negative NK cells that exhibited functional impairments. Thus, the data presented in this study demonstrate that humanized NSG mice can be successfully used to develop a xenotransplantation model that might allow patient-tailored treatment strategies in the future, but also highlight the need to improve this model, for example, by coadministration of differentiation-promoting cytokines and induction of human MHC molecules to complement existing deficiencies in NK and CD8+ T cell development. The Journal of Immunology, 2010, 185: 2710–2720.

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Abbreviations used in this paper: BM, bone marrow; DC, dendritic cell; GvL, graft-versus-leukemia; HSC, hematopoietic stem cell; HSCT, HSC transplantation; KIR, killer Ig-like receptor; NCR, natural cytotoxicity receptor; NOD, NOD/scid/IL-2Rγnull; PBSC, peripheral blood stem cell; PMA/ionomycin; UCB, umbilical cord blood.

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addition, most of the previous studies have been performed with umbilical cord blood (UCB)-derived HSCs, an approach that does not allow patient-related studies in a haploidalidentical setting.

We report a comprehensive study to examine the use of adult PBSCs for establishing a xenotransplantation model that would allow studies on human GvL reactivity in long-term engrafted recipients following transfer of leukemia in a donor-patient–specific situation. As the treatment of CD34+ PBSC-engrafted NSG mice with human IL-7 was shown to improve the generation and long-term survival of human CD3+ T lymphocytes in humanized mice (16) (U.F.H., unpublished results), we included human IL-7 in all of our in vivo studies. Following transplantation of CD34+ selected PBSCs into adult NSG recipients and additional weekly supplementation with Fe–IL-7, we could demonstrate a robust engraftment efficiency and multilineage development, although faster engraftment at week 12 post-HSCT was observed in comparative studies using CD34+ UCB-HSC–transplanted mice. Phenotypic analyses performed at 12–30 wk posttransplantation demonstrated the presence of early developing monocytes and naive B cells, followed by T cells and NK cells. Surprisingly, the majority of T lymphocytes consisted of memory-type CD4+ T cells, whereas only small numbers of CD8+ T cells were detected in mice >20 wk after transplantation, suggesting that IL-7 alone might be insufficient to maintain peripheral CD8+ T cell homeostasis. Whereas NSG-derived T cells showed Ag-specific immune responses to human alloantigens, NK cells displayed an aberrant phenotype and were functionally inert.

Thus, the data presented in this study demonstrate that long-term PBSC-engrafted NSG mice can be successfully used to generate a small animal xenotransplant model for testing feasibility and potential of individualized patient-tailored treatment strategies, but also highlight the need for improvement of this xenotransplantation model, for example, by supporting differentiation-promoting cytokines and implementing the expression of human MHC molecules to complement currently existing deficiencies on NK and CD8+ T cell developmental and peripheral homeostatic requirements.

Materials and Methods

Mice

NOD.Cg-Pkdcre/d Il2rgm1Wjl/Sv (also termed NSG), C57BL/6, and BALB/c mice were purchased at The Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions in the research animal facilities of the Universities Tuebingen and Mainz, Germany. All approved experimental animal procedures were conducted according to German federal and state regulations.

Mobilization, harvest, and isolation of PBSCs

PBSCs were mobilized from parents of children diagnosed with various malignancies prior to planned HSCT. Five days prior to PBSC collection, the donor received 10 μg/kg/day G-CSF (Neupogen; Amgen, Thousand Oaks, CA) s.c., and leukaphereses were performed on days 5 and 6. Following approval by the local ethics committee, parents gave informed consent to donate <5% of the PBSCs to be transplanted into NSG mice and to use PBSCs for research purposes in case the patient deceased prior to HSCT. UCB-HSCs from full-term deliveries were collected with in-and to use PBSCs for research purposes in case the patient deceased prior to complement currently existing deficiencies on NK and CD8+ T cell developmental and peripheral homeostatic requirements.

Transplantation of adult PBSCs into adult NSG recipients. Eight- to 12-wk-old recipients were sublethally irradiated using a 137Cs irradiator (Gammacell 1000 Elite; MDS Nordion, Fleurs, Belgium) (female NSG mice, 200 cGy; male mice, 250 cGy). Four hours later, recipients were i.v. injected into the tail vain with 1 × 106 donor-derived CD34+ or CD133+ PBSCs. Reconstituted mice received weekly i.v. injections of 20 μg Fe–IL-7 fusion protein per mouse generously provided by Merck (Darmstadt, Germany) until sacrificed. Transplantation of neonatal cord blood-derived PBSCs into adult NSG recipients. Cord blood was collected into heparinized tubes. CD34+ HSCs were isolated using CD34 MicroBeads and MACS technology (Miltenyi Biotec) and stored at −80°C until use. For transplantation, 1 × 106 HSCs were injected i.v. into irradiated mice, as described above, followed by weekly i.v. injections of Fe–IL-7 until mice were sacrificed.

Flow cytometry

After transplantation, peripheral blood, spleen, and BM were collected at different time points, as outlined in results for flow cytometric analyses of human lymphocytes after staining using the following, nonmurine cross-reactive, mAbs: CD62L (clone Dreg56) FITC, CD25 (2A3) allophtocycin, CD8 (SU1) PerCP, CD4 (SU3) PerCP, CCR7 (3D12) PEcy7, CD27 (2B8.2) FITC, CD4 (SK3) FITC, CD3 (SK7) PerCP, CD38 (HT2) AF700, CD45RO (UCHL1) PE, CD33 (67.6) PE, CD38 (HB-7) PE, CD34 (8G12) allophtocycin, IgD (IA6-2) FITC, IgM (G20-127) PE, CD56 (NCAM16.2) FITC, CD56 (My31) PE, CD56 (B15a) allophtocycin, CD158a (HP-3E4) FITC, CD94 (HP-3D9) FITC, CD45 (HJ30) PEcy7, and the corresponding isotype controls were all purchased from BD Pharmingen (Heidelberg, Germany). CD19 (HB19) allophtocycin, CD19 (2H7) PerCP, CD45RA (H100) allophtocycin, CD45 (H5E) PE, CD4 (RPA-T4) allophtocycin, and matching isotype controls were from Biologend (Munich, Germany). Nkpo3 (Z25) PE, Nkpp4 (Z231) PE, Nkp46 (BAB281) PE, CD158b (GL183) PE, and NK2a (Z199) PE, including corresponding isotype control, were from Beckman Coulter (Krefeld, Germany); CD16 (LNK16) AF700 and the corresponding isotype control were from ExBio Praha (Vestec, Czech Republic), CD14 (MEM15) FITC and the corresponding isotype control were purchased from Immuno- toolsot (Friesoythe, Germany), CD158e (DX9) allophtocycin and isotype control were purchased from R&D Systems (Wiesbaden, Germany). Flow cytometric analyses were conducted on an LSRII (BD Biosciences) using Diva software following gating of human CD45+ cells.

Isolation of NSG-derived NK cells, CD3+ T cells, and human PBMCs

CD56+ NK cells and CD3+ T cells were isolated from freshly extracted spleens and BM of engrafted NSG mice by two-step immunomagnetic cell sorting using whole blood CD56 MicroBeads and CD3 MicroBeads, respectively (Miltenyi Biotec). Cell purity was determined by FACS analysis to be 90–98%. NKT cell isolation was excluded by flow cytometric analysis of CD56+CD3+ cells. In the majority of cases, the content of CD56+CD3+ NK cells was <1%. In selected cases, in which the NKT content was higher than 5%, an additional round of CD3+ positive selection was performed. The remaining CD56+CD3+ cell fraction was used to isolate the RNA for the subsequent killer Ig-like receptor (KIR) typing procedure. Human PBMCs were isolated from whole blood using Ficoll-Paque (Bichrom, Berlin, Germany) density gradient centrifugation to be used as positive control in various assays. Washed cells were resuspended in VEL RPMI 1640 (Biochrom), containing 10% FCS (Biochrom), and cultured overnight for next day use.

KIR typing

Total RNA was extracted from NK cells isolated as described above using the RNeasy Mini Kit from Qiagen (Hilden, Germany). KIR typing was performed by primer-specific detection of KIR gene-transcribed miRNA using the KIR typing kit from Miltenyi Biotec and PCR technology. An amplified product of a housekeeping gene served as an internal control.

Intracellular IFN-γ staining

For in vivo detection of intracellular IFN-γ synthesis, splenocytes or human PBMCs were cultured for 4 h in the presence of Golgi Plug (BD Biosciences), according to the manufacturer’s instructions. During these 4 h, a leukocyte activation mixture (BD Pharmingen) containing PMA and ionomycin was used either without or with addition of 100 U/ml IL-2 (Proleukin; Novartis, Basel, Switzerland). PBMCs and NSG-derived splenocytes were included as positive and negative controls, respectively. Cells were stained for 20 min with CD56 FITC and CD3 PerCP-Cy5.5 permeabilized (Fix & Perm Kit; ABD Bio Research, Hamburg, Germany), and incubated for 45 min with PE anti-IFN-γ (BD Pharmingen) to finally be analyzed by flow cytometry.
Allogeneic MLCs

CD3+ T cells were isolated from spleens and BM of NSG mice transplanted with HSCs from one donor or from human PBMCs, as described above. NSG- or C57BL/6 × BALB/c F1 mice (H-2Kb/- derived DCs were generated from murine BM, as described previously (17). Briefly, femur-derived BM cells were maintained in culture using RPMI 1640 supplemented with 10% FCS, 1% penicillin/streptomycin, and 10% of supernatant obtained from murine GM-CSF producing X63/10 cell transfectants for 8 d with 50% (v/v) culture medium change every second day. Accordingly, human HLA-matched and mismatched DCs were generated from PBMCs after enrichment of monocytes by adherence to plastic and culture in serum-free X-vivo 15 medium (Cambrex Bioscience, Brussels, Belgium) containing 1000 U/ml GM-CSF (Leukomax, Sandoz, Munich, Germany) and 1000 U/ml IL-4 (R&D Systems), as described previously (18). MLC of CD3+ responder cells (1 × 10^5/well) and 1 × 10^5 irradiated (30 Gy) DCs were maintained in RPMI 1640 containing 10% FCS, 1% penicillin/streptomycin, and 1% nutrition additives (NCTC-135 medium; Invitrogen, Karlsruhe, Germany) in 96-well triplicates for 5 d. PHA (Sigma-Aldrich, Holzkirchen, Germany) was added to some cultures at 10 and 2.5 μg/ml, respectively, to measure alloantigen-independent stimulation. Proliferation of CD3+ T cells was determined by thymidine uptake (0.5 μCi/well) for the last 15 h of culture and quantified using a β-plate liquid scintillation counter (Wallac, Turku, Finland). Results represent means of triplicate wells ± SD.

ELISPOT analysis

IFN-γ ELISPOT assays were performed, as recently described (18). Briefly, NSG-derived CD3+ T cells at 5 × 10^4/well and DCs at 1 × 10^5/well were seeded in ELISPOT plates in serum-free X-vivo 20 medium. T cells without APCs or stimulated in the presence of PHA (2.5 μg/ml) served as controls. After 20h incubation at 37°C, IFN-γ spots were visualized and counted using an Axisplan 2 microscope combined with the computer-assisted image analysis system KS ELISPOT 4.1 (Carl Zeiss Vision, Hallbergmoos, Germany). Results represent means of triplicate wells ± SD.

Chromium release assay

Splenocytes and BM of up to three humanized NSG mice engrafted with PBSCs of one individual donor were pooled and cultivated overnight. The erythroleukemia cell line K562 was used as target cells and labeled with 50 μCi ^51Cr (Amersham Biosciences, Freiburg, Germany) for 1.5 h. E:Tratios varied between 5:1 and 1:1 and were calculated based on the flow cytometric determination of the percentage of CD56+ human NK cells (varying between 1.4 and 3%). The presence of relevant numbers of NKT cells was excluded by experiments with the addition of IL-2 (Proleukin) at a concentration of 100 U/ml. Spontaneous release of chromium was <10% of the maximum release taken from the target cells lysed in 1% Triton X-100 (Roche, Mannheim, Germany). Percentage of lysis was calculated as follows: 100 × (experimental release – spontaneous release)/maximum release – spontaneous release. Data represent means of quadruplicates.

Pneumococcal immunization (Supplemental Data)

For determination of B cell functionality, humanized NSG mice (week 12–16) were immunized with Pneumovax (Merck) with a dosage adjusted to the murine body weight (10 μl vaccine diluted in 200 μl PBS s.c.) corresponding to 0.5 μg each of the 23 different polysaccharide types and boosted with the same dosage 4 wk later. As analysis of pneumococcal serum IgM often results in unreliable data not adequately reflecting the success of a pneumococcal immunization, we determined serum levels of whole IgM in NSG mice before immunization, prior to the boost and 2 wk after the boost using the ELISA for human IgM (Mabtech, Hamburg, Germany), including a human serum probe as internal control.

Statistics

Nonparametric methods were used to compare groups when the distribution of the dependent variable was not normal (Mann-Whitney U test). Results are shown as the means ± SD.

Results

Transplantation of donor-derived CD34+ PBSCs into NSG mice results in efficient engraftment and multilineage differentiation

Because previous experiments were mainly performed with CD34+ UCB-HSCs, we first compared engraftment efficacy and the extent of lineage differentiation following transplantation of mobilized CD34+ PBSCs and CD34+ UCB-HSCs into sublethally irradiated adult NSG recipients. As previous reports (16) and our early studies (data not shown) demonstrated a beneficial effect of human IL-7 particularly on the generation and peripheral survival of human T lymphocytes in CD34+ PBSC-engrafted mice, we chose to administer Fe–IL-7 to all experimental groups. Our grafting strategy was based on the initial garenting on all mononuclear cells (including monocytes and granulocytes) according to their characteristic forward and sideward scatter and their expression of human CD45+ cells, followed by staining with lineage-specific surface markers (Fig. 1A). This strategy underestimates engraftment rates when compared with published data often exclusively gating on individual lymphohcytic cell populations. However, because HSCT into NSG also resulted in the generation of a substantial proportion of monocytes, this gating strategy appeared to be more precise in estimating the percentage of chimerism. Comparison of engraftment rates as determined by the percentage of CD45+ cells at week 11-15 posttransplantation demonstrated significantly higher numbers of CD45+ cells in mice engrafted with UCB-HSCs than in mice that had received PBSCs, although the overall absolute leukocyte count was comparable in both experimental groups (32.7 ± 10% versus 7.2 ± 3.2%; p ≤ 0.001) (Fig. 1B). In addition, we found a lower amount of early developing CD19+ B cells and higher numbers of late appearing CD3+ T cells in UCB-HSC–transplanted mice (Fig. 1C), suggesting that transfer of UCB-HSCs results in faster engraftment and lineage differentiation. However, analyses performed at later time points (week 16–24) showed comparable levels of CD45+ cells and differentiated cells in both PBSC- and UCB-HSC-engrafted mice, indicating that the quality of HSC engraftment ultimately was the same (data not shown). Frequencies of CD14+ and CD56+ expressing monocytes or NK cells, respectively, as well as CD33+ and CD38+ cell lineage progenitors were comparable in PBSCs and UCB-HSC–transplanted NSG mice (Fig. 1C, 1D).

We conclude from these data that transplantation of PBSCs from adult donors into NSG mice with additional administration of Fe–IL-7 results in sufficient engraftment and lineage-specific cell differentiation to allow further studies on lymphocyte subpopulations.

Transplantation of CD34+-selected HSCs results in superior engraftment when compared with CD133+-selected PBSCs

Because we sought to use this xenotransplantation model as a surrogate for haploidentical HSCCT and our patients are routinely transplanted both with CD34+- and CD133+-selected PBSCs, we also evaluated the engraftment efficiencies of CD34+ and CD133+ positively selected PBSCs. Engraftment rates obtained in NSG mice transplanted with CD34+ PBSCs were significantly higher than in mice that received CD133+ PBSCs (CD34+ HSCCT, 21.5 ± 22% (n = 37); CD133+ HSCCT, 7.4 ± 6.3% (n = 32); p ≤ 0.01) (Fig. 2A). Analysis on development of lineage-specific cells demonstrated that mice transplanted with CD133+ PBSCs had significantly higher proportions of the early arising CD19+ B cells, but reduced numbers of later evolving CD3+ T cells (Fig. 2B, 2D). Whereas CD14+ cells were detected at comparable percentages in both groups, CD56+ NK cells were only present in low numbers, indicating that the microenvironment in humanized NSG mice might be suboptimal to support growth and differentiation of NK cells (Fig. 2C, 2E).

Thus, HSCCT with both CD34+- and CD133+-selected PBSCs is feasible; however, transplantation with CD34+-selected PBSCs results in higher engraftment rates and faster lymphoid-lineage differentiation than HSCCT of CD133+-selected PBSCs.
Long-term (>25 wk) engrafted NSG mice harbor predominantly naive B cells and functionally competent CD4+ T cells, but show strongly reduced numbers of CD8+ T lymphocytes

Having determined that transplantation of CD34+ PBSCs allows sufficiently high levels of human CD45+ engraftment, we next sought to investigate the properties of NSG-derived lymphocyte subpopulations upon weekly injections of 20 μg Fc–IL-7/mouse (16). Flow cytometric analyses on B cells performed at weeks 12, 16, and 25–30 following engraftment of PBSCs demonstrated high numbers of peripheral and splenic B cells predominantly expressing a naive CD20+CD27− phenotype (data not shown). These cells were mature naive, functionally competent B cells as a large number of these cells coexpressed IgM and IgD on their cell surface (30 ± 5%) and were able to secrete IgM upon immunization with a human pneumococcal vaccine (Supplemental Fig. 1). Subpopulations of activated (CD20+CD25+CD69) or memory (CD20+CD27−) B cells were not detectable in spleen or BM by means of flow cytometric analyses, suggesting a partial differentiation blockade. As T cells arise late in HSCT, we phenotypically and functionally analyzed peripheral T lymphocytes in humanized NSG mice not earlier than 20 wk posttransplantation. At that time, ≥50% of the CD45+ cells of all analyzed organs consisted of CD4+ T cells, whereas CD8+ T cells were only found in small numbers (Fig. 3A, 3B). Surprisingly, the majority of CD4+ T cells displayed a memory phenotype with high CD45RO+ expression (Fig. 3C, 3D), and ~30% of CD4+ T cells expressed CD62L and CCR7, indicative of central memory activated cells (Fig. 3E). In contrast to T cell homing in immunocompetent mice, T cells in NSG mice did not show preferential homing to the spleen, but were also present to a large extent in the BM. Flow cytometric analyses of thymi showed that the characteristic double-positive CD4+CD8+ T cells were clearly present at week 17 post-HSCT, confirming earlier reports (14–16), but disappeared thereafter (Fig. 3F). Moreover, CD8+ T cells were absent in spleens of week 17, 21, and 32 transplanted mice. Because purified CD34+ PBSCs transplants contained very small numbers of mature human CD3+ T cells (Supplemental Table I) and human 8Rec-ψJα TCR excision circles could be PCR amplified in thymic DNA from CD34+-reconstituted NSG recipients (data not shown), preferential survival of CD4+ T cells was very unlikely to reflect homeostatic proliferation of graft-immanent mature T lymphocytes. These results suggest that the administration of Fc–IL-7 supports development and survival of CD4+ T cells, but appears insufficient to promote long-term CD8+ T cell maintenance.

**FIGURE 1.** Transplantation of CD34+ PBSCs into NSG mice results in efficient engraftment and multilineage differentiation. Following sublethal irradiation, NSG mice were transplanted with either CD34+-mobilized PBSCs or CD34+ UCB-HSCs, as described in Materials and Methods. Engraftment in all recipients was supported by weekly i.v. injections of Fc–IL-7. Eleven to 15 wk posttransplantation, PBMCs were subjected to flow cytometric analysis to describe the extent and diversity of hematopoietic engraftment. A. Gating strategy: PBMCs were initially gated according to their characteristic forward and sideward scatter, followed by determination of the percentage of the human CD45+ cells. The various cell lineage-specific populations were determined using staining with human CD45 and the respective lineage-specific surface markers. B. Transplantation of UCB-HSCs into NSG mice results in superior engraftment compared with PBSCs 11–15 wk posttransfer (●, PBSCs; □, UCB-HSCs). C and D. The engraftment of hematopoietic precursors in PBSC- and UCB-HSC–transplanted mice is comparable, the lineage differentiation in UCB-HSC–transplanted mice is faster than in PBSC-engrafted mice. PBMCs of PBSC-transplanted mice predominantly consist of early arising CD19+ and CD14+ cells, whereas UCB-HSC–transplanted mice already show the later appearing hematopoietic CD3+ cell lineage (A–D, ●, HSCT from PBSCs [n = 20, from 5 donors]; □, HSCT from UCB-HSCs [n = 9, from 5 donors]).
NK cells generated in humanized NSG mice show a highly activated phenotype, lack KIR expression, and are functionally inert

We next investigated whether NSG-derived NK cells resemble human NK cells in phenotype and function. We analyzed surface expression of important natural cytotoxicity receptors and selected inhibitory KIRs in the PBSC donating parent, the transplanted pediatric patient, and humanized NSG mice between weeks 25 and 30 post-HSCT. Flow cytometric analysis of natural cytotoxicity receptors (NCRs) demonstrated that the Nkp44 receptor, which is only expressed on activated NK cells, was highly upregulated in NSG-derived NK cells (Fig. 5A, 5C). As all Nkp44+ or Nkp46+ NK cells were simultaneously CD56- receptor-positive cells did not include all NK cells. In contrast to NCR expression, the KIR expression of the most important KIRs, 2DL1, 2DL2/3, and 3DL1, was absent in NK cells from humanized NSG mice (Fig. 5B, 5C), whereas the patient exhibited a pattern comparable to the stem cell donor. Two other inhibitory receptors, NKG2A and CD94, were expressed in NSG-derived CD56- NK cells, however, at subnormal expression levels (Fig. 5C). Because KIR expression is predominantly controlled by genetically determined programs and only partially influenced by the presence of cognate MHC class I ligands, we also analyzed KIR mRNA expression in these NK cells (Fig. 5D). Despite absent surface expression, KIR-specific mRNA was present for all three important inhibitory KIRs.

The phenotype of highly activated CD56- NK cells lacking inhibitory KIR surface expression would suggest a high level of cytotoxic activity toward MHC class I-deficient target cells. However, direct ex vivo testing for cytotoxicity revealed no NK cell reactivity toward the K562 erythroleukemic cell line, and NSG-derived CD56- NK cells were not stimulated by addition of exogenous IL-2 (Fig. 6A). As we were primarily interested to establish a mouse model that would allow in vivo studies on the interaction of donor-specific NK cells with patient-specific leukemic blasts, we did not attempt to stimulate NSG-derived NK cells in vitro for a longer period of time in the presence of various differentiation-promoting cytokines. Phenotypical comparison of patient and mouse-derived NK cells showed that NSG-derived NK cells predominantly belonged to the cytokine-producing CD56int/CD16- subpopulation, whereas the majority of patient-derived NK cells belonged to the cytokine CD56dim/CD16+ subset (Fig. 6B). Nevertheless, NSG-derived CD56int/CD16- NK cells did not synthesize IFN-γ upon unspecific stimulation with PMA and ionomycin being indicative of severely impaired intracellular signal transduction mechanisms (Fig. 6C).

Collectively, these observations suggest that the generation of human CD56- NK cells in PBSC-transplanted NSG mice in the presence of Fc–IL-7 is feasible, but suboptimal, as these cells are highly activated, lack important cytotoxic subsets, and are functionally inert.

Discussion

Immunodeficient BALB/cRag2−/−IL2Rγ−/−, NODshic/SCID/IL-2Rγ−/− (NOG), and NSG mouse strains lacking B, T, and NK cells due to a deficient common cytokine receptor γ-chain have been used to demonstrate a highly efficient engraftment of human HSCs with the induction of a human immune system (14–16, 19–22). In addition, NSG mice have been shown to be excellent recipients for xenotransplantation of human acute leukemias (23, 24). Thus, we sought to establish a humanized mouse model that could resemble a donor-specific situation to study human graft-versus-host
and GvL immunobiology in vivo and potentially serve as a preclinical model to test new individualized concepts of immunotherapeutic strategies upon cotransfer of patient-specific leukemia in the future.

Most humanized mouse models reported to date have been engrafted with UCB-HSCs, an approach that is not well suitable for HLA-defined in vivo studies in a donor-patient–specific xenotransplantation model. In addition, because adoptive transfer of human PBMCs results in xenogeneic graft-versus-host disease in these mice (25), studying GvL-specific responses using this approach will be difficult. We, therefore, transplanted donor-derived CD34+ PBSCs into NSG mice to develop a xenograft model that would allow studies on long-term T and NK cell biology as a surrogate to haploidentical HSCTs in pediatric patients performed with CD34+ HSC from the same donor.

Previous work on human lymphocytes generated in NSG or NOG mice has largely been conducted without supplementing human cytokines (15, 20, 26–29). However, addition of exogenous human IL-7 to CD34+ PBSC-engrafted NSG mice was shown to improve development of human T lymphocytes (16). Moreover, because IL-7 is instrumental in T, B, and DC development (30–32) and plays a pivotal role in homeostatic proliferation of T cells (33), we did not compare engraftment efficiency and lineage differentiation on human HSC-engrafted mice in the presence or absence of human IL-7, but administered Fc–IL-7 at weekly intervals following HSCT and examined the phenotype and functionality of lymphocytes from NSG mice over 20–30 wk.

Comparative analyses on engraftment rates and efficiency upon transplantation of PBSCs or UCB-HSCs under these conditions revealed that transfer of UCB-HSCs results in faster engraftment and generation of human CD45+ cells when compared with PBSC-engrafted NSG mice 12 wk after HSCT. Because early developing CD19+ B cells were found to be decreased, but late appearing CD3+ T cells increased, this might indicate a faster differentiation potential of UCB-HSCs. These differences were not due to differential leukocyte counts that were found to be comparable in both experimental groups. Moreover, lineage-specific CD33+ and CD38+ progenitors as well as monocytes and NK cells were present in comparable numbers, and the amount of lymphocytes in PBSC- or UCB-HSC–engrafted mice reached comparable levels at week 16 posttransfer. Thus, transplantation of donor-derived CD34+ PBSCs into NSG mice proved to be sufficient to allow detailed phenotypical and functional analyses of human lymphocytes in long-term transplanted mice.

Because we perform pediatric HSCTs using both CD34+- and CD133+–enriched HSCs, we next examined engraftment efficiencies of these stem cell subsets. Interestingly, transplantation of CD34+ HSCs in NSG mice resulted in significantly higher engraftment rates and faster lineage differentiation than transplantation of CD133+ HSCs.
Collectively, our data demonstrate that transplantation of CD34+ PBSCs into adult NSG mice with additional Fc–IL-7 administration can be efficiently used to generate myeloid and lymphoid lineages.

Surprisingly, phenotypic analyses of these lymphoid populations 25–30 wk following HSCT revealed that the majority of T cells found in PBSC-engrafted NSG mice consisted of CD4+ T cells, whereas only small numbers of CD8+ T cells were found. Moreover, most CD4+ T cells expressed a CD45RO+ memory phenotype. Although we cannot completely exclude any engraftment of mature T cells persisting in the graft in such highly permissive mice, preferential expansion and survival of mature CD4+ T lymphocytes reflecting this phenotype appear very unlikely given the small numbers of CD3+ T lymphocytes cotransplanted and the presence of 8 Rec-Q/Jc TCR excision circles detected in the thymus of CD3+ HSC-engrafted NSG recipients, as shown before (16). As IL-7 mediates its effect primarily on naive and memory T cells (35), the repetitive Fc–IL-7 injections may at least partially account for the high percentage of memory T cells in our mice. Because CD4+CD8+ double-positive thymocytes and CD8+ T cells were detected in thymi of humanized NSG mice up to 17 wk post-HSCT, but were absent at later stages of HSCT, we assume that administration of IL-7 alone is in fact sufficient to maintain a pool of polyclonal CD4+, but not of CD8+ T cells. Along this line, results from our group on better engraftment of purified donor CD8+ T cells or leukemia-reactive donor CD8+ T cell lines in the presence of IL-15 support these data (M. Nonn, J. Knapstein, S.A. Khan, M. Hörz, E. Distler, A. Brunk, M.C. Ande`, R. Handgretinger, M. Theobald, W. Herr, and U.F. Hartwig, submitted for publication). In addition to IL-7, survival and homeostatic proliferation of CD8+ T cells are promoted by IL-15 (15, 33), and recent results show improved CD8+ T cell development in Rag2−/− IL-2Rγ−/− mice when IL-15 is presented in trans (36). Moreover, a lack of T cell maintenance (37) and increased CD95 (Fas)-mediated susceptibility of CD8+ T cells to undergo apoptosis have been described in humanized mice (20), possibly reflecting anti-apoptotic effects of IL-7 and IL-15 (33). Because TCR-driven signaling provided by human MHC class I-restricted recognition of Ags might further promote survival and homeostasis of CD8+ T cells in humanized NSG mice (21), we thus assume that the suboptimal cytokine milieu and weak TCR–MHC interactions due to insufficient human MHC class I expression on a limited number of human APCs in lymphoid compartments might account for the reduced life span of CD8+ T cells in our long-term engrafted NSG mice (38). Because our T cell analyses in humanized mice were performed at later time points than previously described results (16, 20), it is difficult to compare our findings with these data.

In contrast to CD8+ T cells, mature naive B cells were found in large numbers in humanized NSG mice and were able to secrete IgM upon immunization, confirming previous findings (20). Thus, despite the fact that humanized NSG mice lack activated CD20+CD25+CD69+ or memory (CD20+CD27+) B cells, the suggested partial differentiation blockade (20) of B cell-lineage cells appears to be limited to later stages of B cell differentiation.

Despite low numbers of CD8+ T lymphocytes, functional analyses of NSG-derived CD3+ T cells demonstrated increased IFN-γ synthesis upon unspecific stimulation, indicating intact basic signal transduction mechanisms. Moreover, T cells proliferated vigorously and exhibited effector functions when stimulated with allogeneic human DCS, whereas no alloimmunity was observed following co-culture with MHC-matched murine (NSG) or allogeneic (F1) DCs, as reported earlier (19). Hence, our results support emerging data in humanized mice, which demonstrate positive selection of human HSC-derived T cells by murine thymic epithelial cells, followed by CD133+ HSCs. Gene expression profiling studies have attributed differing roles to CD34+ and CD133+ HSCs (34) in the process of cell cycle, maintenance of chromatin architecture, and DNA metabolism, and in line with this, our results are compatible with a rather undifferentiated nature of CD133+ HSCs.
FIGURE 5. NK cells generated in humanized NSG mice show a highly activated phenotype and lack inhibitory KIR expression. In parallel to HSCT in pediatric patients, NSG mice were transplanted with CD34+ PBSCs, followed by weekly i.v. injections of Fc–IL-7, as described in Materials and Methods. At week 25–30, the NCR and the inhibitory KIR repertoire in donor, pediatric patient, and NSG mice was compared. A, Whereas the donor predominantly shows resting NK cells with a CD56low phenotype, the human recipient expresses both CD56low and CD56high NK cells. Note that virtually all murine NK cells are CD56high. Comparing the NCR expression in NK cells derived from donor, patient, and pooled BM and spleen cells of NSG mice at week 27 post-HSCT, the murine NK cells show a highly activated phenotype with the majority being Nkp44+ CD56+. Results are representative of three donor–patient–mouse pairs. B, Analysis of inhibitory KIR expression in NK cells shows that the expression of KIR 2DL1 (CD158a), KIR 2DL2/DL3 (CD158b), and KIR 3DL1 (CD158e) is virtually absent. The quadrant statistics indicate the percental distribution of the respective cell populations. Data are representative of three donor–patient–mouse pairs. C, Mean and SD of NCR, inhibitory KIR, NKGA, and CD94 receptor expression as determined in 15 mice transplanted with 8 different HSC donors and analyzed at week 15–30. D, Although KIR expression is lacking on the cell surface of NSG-derived CD56+ cells, the intracellular mRNA for various KIRs is present. Data show a representative NSG mouse at 21 wk post-HSCT. The band at 400 bp represents the internal control band (β-actin).
In summary, our data suggest that Fe–IL-7 administration decidedly promotes B cell precursor and both CD4+ T cell development and differentiation toward a memory phenotype; however, Ag-specific T cell immune responses occur at a reduced efficiency when compared with human T lymphocytes.

In addition to T cells, we examined the phenotype and function of NSG-derived NK cells. To our knowledge, this is the first study investigating human NK cell immunity in humanized mice by comparing the nature of NK cells from transplanted patients with reconstituted NSG recipients. Flow cytometric analyses of patient-derived NK cells demonstrated the presence of NK cells with low levels of activation and high inhibitory KIR 2DL2/3 expression as frequently observed during the early posttransplantation period in patients (R.H., unpublished observation). In contrast, NSG-derived NK cells demonstrated signs of constitutive activation with high levels of NKp44 expression together with subnormal levels of inhibitory KIRs and selected inhibitory lectin-like receptors, such as CD94 and NKG2A. Moreover, the important cytotoxic NK cell subset of CD56dimCD16+ NK cells that was abundantly expressed in transplanted patients was virtually absent in NSG mice. Because adoptive transfer of donor NK cells into irradiated NSG mice supplemented with IL-7 did neither result in engraftment nor expansion of NK cells up to 20 wk postinfusion (our unpublished data) and NSG-derived NK cells did not display a donor-specific phenotype, we conclude that the observed immature, functionally inert CD56+CD16+ KIR− NK subpopulation did not result from cotransfer of graft-immanent NK cells, but was indeed HSC derived. Moreover, our results are supported by Huntington et al. (36), who recently reported that NK cells generated in humanized Rag2−/− IL2−γ−/− mice with additional administration of IL-15 linked to its IL-15Rα chain developmentally progressed from a CD56highCD16+ KIR− phenotype to a more mature CD56dimCD16+KIR+ phenotype. Thus, the phenotypical aberrations observed in our NSG mice may in fact result from a maturation arrest of NK cells due to lack of human IL-15.

Functional analyses of NSG-derived NK cells demonstrated that NK cells isolated from Fe–IL-7–supplemented NSG mice are functionally inert and do neither display signs of intracellular IFN-γ cytokine synthesis upon specific cell stimulation nor show in vitro cytotoxic activity against the K562 erythroleukemia cell line. Thus, our observations that NSG-derived NK cells lack essential inhibitory receptors, but are at the same time unable to exert NK cell-specific cytotoxicity, are consistent with reports that NK cells from MHC class I-deficient animals or humans may be tolerant as a result of induced hyporesponsiveness (40, 41). As described by the theory of licensing, KIRs on NK cells are able to exhibit differential effects to unspecific NK cell stimulation. For these in vitro experiments, splenocytes of humanized NSG mice were cultivated with PMA/Iono. or PMA/Iono. + IL-2, followed by intracellular IFN-γ staining, as described in Materials and Methods. C. Shows the flow cytometric data of one mouse representative of three independent experiments with a total of four mice. Included is the internal IFN-γ control staining of PBMCs of a healthy donor. The quadrant statistics indicate the percental distribution of the respective cell populations. PMA/Iono., PMA/ionomycin.
of donor-derived CD34+ PBSCs with the generation of a multi-lineage human immune system in NSG mice is feasible, but has several limitations that need to be optimized. We, therefore, propose that introduction of MHC molecules possibly expressed on a murine MHC-deficient background in addition to the continuous supply of growth, homeostasis, and differentiation-promoting cytokines may refine the generation of long-term humanized mice not only to evaluate new forms of patient-tailored immunotherapy in HSC tit, but also to improve, for example, HIV- and Ab-based cancer therapy studies in these mice.

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Disclosures

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