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Ulrike Koehl[1], Ruth Esser[1], Lutz Uharek[2], Martin Stern[3], and Jakob Passweg[3]

[1] Institute of Cellular Therapeutics GMP Development Unit (OE 8885), IFB-Tx and Cellular Therapy Centre Hannover Medical School (MHH), Hannover, Germany

[2] Internal Medicine, Hematology and Oncology, Charité Berlin, Germany.

[3] Division of Hematology, University Hospital Basel, Switzerland.

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Introduction

Cell therapy may represent a promising treatment option for patients who suffer from leukemia and tumors and have a high risk for relapse after allogeneic, especially haploidentical, stem cell transplantation (SCT). While established T cell therapies, such as donor lymphocyte infusions, are associated with the risk of graft-versus-host disease (GvHD), natural killer (NK) cells may mediate graft-versus-leukemia/tumor effects without induction of GvHD. Therefore, immunotherapy with highly purified NK cells in recipients of haploidentical SCT could serve as an attractive alternative cell therapy[1,2].

Human NK cells are lymphocytes of the innate immune system involved in the early defense against infectious pathogens and against MHC class-I–negative or –low-expressing malignant targets without the requirement for prior immune sensitization of the host[3,4]. They reside mainly in the marrow, spleen, and peripheral blood, where they account for approximately 2–18% of the peripheral blood lymphocytes. NK cells are usually the first lymphoid subpopulation to recover after SCT[5]. Phenotypically they can be defined by the expression of CD56, an isoform of the neural cell adhesion molecule and the lack of the CD3 antigen on the surface. Further characterization allows the major CD56^{dim}CD16^{+} (around 90%) to be distinguished from the minor CD56^{bright}CD16^{-} NK subpopulation. The immunoregulatory CD56^{bright} NK cell subsets express the high-affinity interleukin 2 (IL-2) receptor, which enables them to proliferate in response to IL-2 and to produce high amounts of cytokines, such as IFN-γ, TNF-α, TNF-β, GM-CSF, IL-10, and IL-13. The CD56^{dim} NK cells are essentially cytotoxic cells that express low levels of the IL-2 receptor. NK cells are able to lyse targets by releasing cytotoxic granules containing perforin and granzymes and using antibody-dependent cellular cytotoxicity pathways via membrane receptor binding to the Fc portion of IgG antibody, and by the induction of apoptosis through molecules of the TNF superfamily (Fas/CD95, TRAIL).

Killing activity of NK cells is regulated by a set of surface receptors that either induce or inhibit the cytotoxic response[3,6,7]. Activation of NK cells is facilitated by the engagement of activating surface receptors through interaction with stimulatory ligands expressed by malignant cells. These immune recognition receptors include NKG2D, the natural cytotoxicity receptors (NCR) NKp30, NKp44, and NKp46, CD16, NKp80, DNAM-1, and 2B4 (CD244)[6,8]. Activation with cytokines, such as IL-2, leads to a strong up-regulation of the NCRs and NKG2D, and this correlates with increased NK cell cytotoxicity against malignant cells[9]. Inhibitory receptors comprise both killer cell immunoglobulin-like receptors (KIRs) and the heterodimeric C-type lectine receptor CD94-NKG2A/B[10]. In addition, several activating KIR and the C-type lectin receptor CD94-NKG2C/E/F are known[3]. A number of studies have demonstrated NK cell–based killing of many different mouse and human tumors and leukemias and have led to the initiation of the first clinical phase I/II trials using allogeneic NK cells for treatment of cancer.

Materials and methods

Clinical-scale NK cell enrichment

Protocols for the enrichment of NK cells from non-stimulated leukapheresis products using good manufacturing practice (GMP) procedures have already been established (table 1; fig. 1). The aim of these procedures is to obtain a highly purified NK cell product with minimal T cell contamination and conserved

NK cell cytotoxicity. NK cell enrichment usually consists of one or two rounds of $CD3^+$ cell depletion with subsequent $CD56^+$ cell enrichment[11-13].

In our procedure, after steady-state leukapheresis of unstimulated donors, the cells were washed twice for platelet reduction with CliniMACS® PBS/EDTA Buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) supplemented with 0.4% human serum albumin (Red Cross Blood Donor Service, Baden-Württemberg-Hessen, Germany). Thereafter, 5 mL of Intraglobin (Biotest, Dreieich, Germany) were added and incubated for five minutes to reduce non-specific antibody binding. Cells were labeled for 30 minutes with CliniMACS CD3 Reagent (Miltenyi Biotec), using one vial of reagent in case of total nucleated cell (TNC) numbers up to 40×10^9 or $CD3^+$ cell numbers up to 15×10^9 and two vials in case of TNC numbers up to 80×10^9 or $CD3^+$ cell numbers up to 30×10^9. After washing twice, $CD3^+$ cells were depleted with the CliniMACS Plus Instrument using the separation program "DEPLETION 2.1". If necessary, the T cell depletion step was repeated to further remove residual T cells. Thereafter, the T cell–depleted harvests were concentrated and labeled with CliniMACS CD56 Reagent (Miltenyi Biotec) for 30 minutes (one vial CD56 Reagent for TNC numbers up to 40×10^9 and $CD56^+$ cell numbers up to 10×10^9). After washing, $CD56^+CD3^-$ NK cells were enriched using the separation program "ENRICHMENT 1.1". All steps were performed in a closed system observing GMP. The study protocol was approved by the local ethics committee in Frankfurt and Basel and informed consent of the donors has been obtained.

Expansion and activation of NK cells

The purified $CD56^+CD3^-$ NK cells were suspended and seeded at a concentration of $1–2\times10^6$ cells/mL in X-VIVO™ 10 media (BioWhittaker, Verviers, Belgium) supplemented with 5% heat-inactivated human fresh frozen plasma and 1,000 U/mL rhIL-2 (Proleukin®, Novartis, Germany) under GMP-compliant conditions (fig. 1). In an early phase of the study, cells had been expanded and activated using both 175 cm^2 culture flasks (Nunc, Wiesbaden, Germany) and VueLife™ cell culture bags (CellGenix, Freiburg, Germany); for the ongoing phase I/II trial only VueLife cell culture bags were used[11]. Fresh medium was added every three days, and samples for monitoring cell content and viability were taken directly after leukapheresis, after each depletion/enrichment step, and every second day during stimulation. Phenotyping and evaluation for cytotoxicity was performed by flow cytometry. After 10 days, stimulated NK cells were administered to the patients or cryopreserved in X-VIVO 10 medium supplemented with 10% DMSO.

Phenotyping and functional characterization of the product for quality control

The absolute number of $CD56^+CD3^-$ NK cells and the number of residual T cells were determined by flow cytometry performed on a four- or a five-color flow cytometer (Epics XL or FC 500, Beckman Coulter, Krefeld, Germany) in a single-platform technique. The gating strategy was based on the ISHAGE single-platform stem cell enumeration method using low scatter, high expression of CD3 and CD45 antigens, CD16 and CD56 expression and 7-AAD staining, in a no-wash preparation with counting beads. Our previously described four-color panels[14] were extended to the following five-color panels: CD45-FITC/CD56-PE/CD3-ECD/7-AAD/CD16-PC7 and

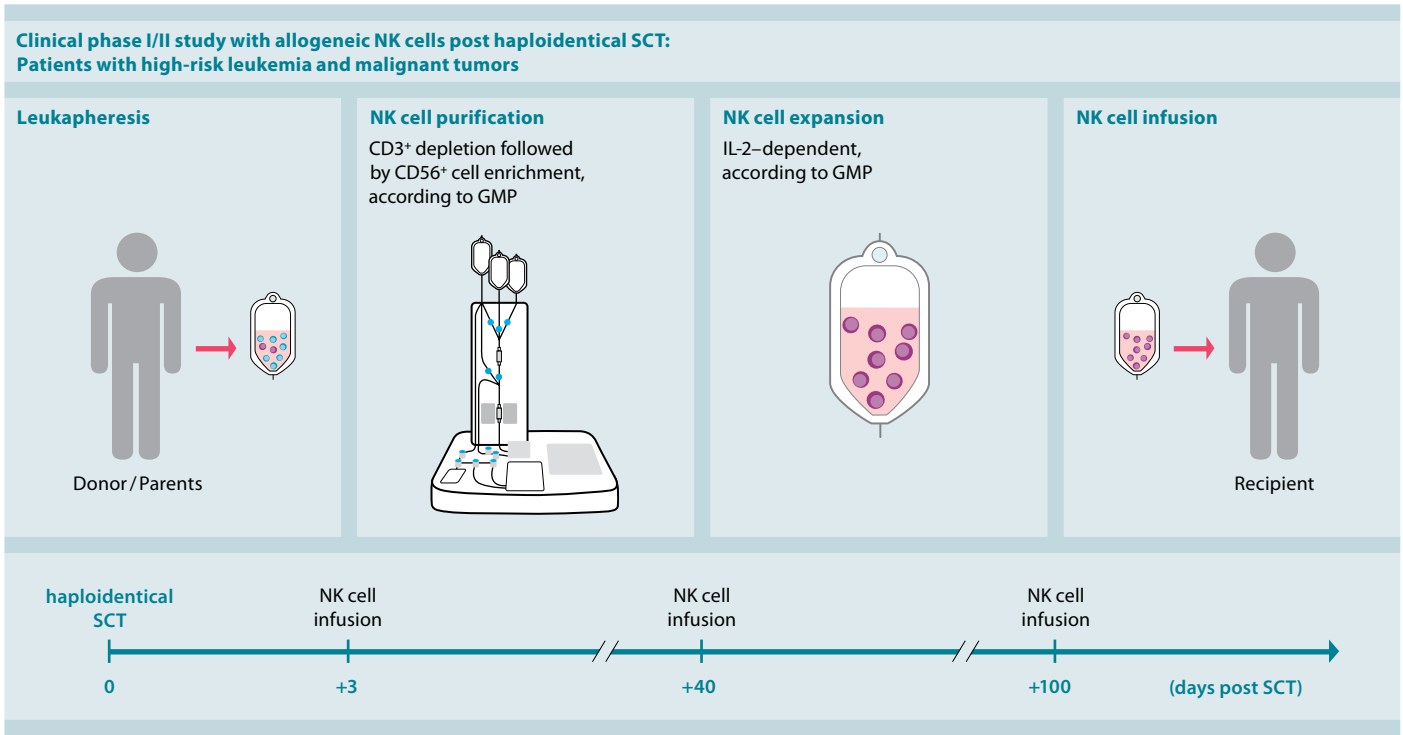

Figure 1 Purification, *ex vivo* expansion, and infusion of donor NK cells. In an ongoing clinical phase I/II trial patients receive freshly isolated NK cells on day (+3), +40, +100 or IL-2–activated NK cells on day +40 and +100 post haploidentical SCT.

CD45-FITC/CD3-PE/CD14-ECD/7-AAD/CD56-PC7. Samples were prepared in triplicate and CD45-FITC/IgG1-PE/CD14-ECD/7-AAD/CD56-PC7 served as a control. In addition, cells were labeled with appropriate combinations of fluorochrome-conjugated antibodies (MAb) to monitor NK cell subsets, activating and inhibitory NK cell receptors, and activation status. MAbs used were CD16 (clone 3G8), CD45 (clone J.33), CD56 (clone N901), HLA-DR (clone Immu-357), CD69 (clone TP1.55.3), CD158a,h (KIR2DL1/S1, clone EB6B), CD158b1/b2,j (KIR2DL2/3/S2, clone GL183), CD158e1/e2 (KIRp70, KIR3DL1/S1, clone Z27.3.7), CD158i (KARp50.3, KIR2DS4, clone FES172), CD337 (NKp30, clone Z25), CD336 (NKp44, clone Z231), CD335 (NKp46, clone BAB281), and CD314 (NKG2D, clone ON72), all supplied by Beckman Coulter (Marseille, France), and CD3 (clone SK7) supplied by BD® Biosciences.

The cytotoxicity of the highly enriched NK cells before and after IL-2 stimulation was tested against the MHC class I–negative cell line K562 or against the patient's individual leukemic cells using an antibody-based flow cytometric assay as described previously[15,16]. NK cells and leukemic cells were cocultured for four hours at effector-to-target ratios between 0.5 : 1 and 10 : 1. Absolute cell counts were determined using Flow-Count™ beads. Cytotoxicity was defined as the loss of viable target cells relative to the control. In other studies NK cell functionality was measured by a CD107a degranulation assay[17,18].

Results and discussion

Technical aspects – clinical-scale NK cell product manufacture and NK cell activation

A number of studies have shown that clinical-scale NK cell product manufacture from non-stimulated leukapheresis products, using a $CD3^+$ cell depletion step followed by $CD56^+$ cell enrichment, leads to highly purified $CD56^+CD3^-$ NK cell products with a purity ranging from 89.7 to 98.6% (table 1)[11-13,19-25]. Purity of NK cell products was lower if the starting product was the negative fraction after $CD34^+$ cell selection[23]. The high NK cell purity and extensive T cell depletion is at the expense of a considerable loss of NK cells during isolation. The final recovery of $CD3^-CD56^+$ NK cells ranged between 19.4% and 58%. Overnight storage of the leukapheresis product led to a greater loss of NK cells during the NK cell enrichment process compared to processing of fresh harvests[13]. A much higher NK cell recovery was obtained by using only a $CD3^+$ cell depletion step, without further $CD56^+$ cell enrichment. However, such a product was associated with low purity and less T cell depletion[20]. Similarly, the final T cell number was much higher if a $CD56^+$ cell selection was used alone, but this kind of purification was associated with an increased NK cell recovery and purity[25]. In contrast, the two-step NK cell product manufacture described here led to efficient T cell depletion of 3.6 to 5.3 orders of magnitude (table 1), and this could be further increased by performing the $CD3^+$ cell depletion step twice[11]. A residual T cell contamination between 0.01 and 0.09% in the final product allows the infusion of NK cell products of more than 1.0×10^7 $CD56^+CD3^-$ NK cells/kg BW with less than 5.0×10^4 $CD3^+$ cells/kg BW and often less than 2.5×10^4 $CD3^+$ T cells/kg BW[1].

The objective of NK cell purification is not only to remove potentially unwanted T cells but also to enable activation and expansion of the NK cells. Indeed, enriched NK cells can be infused without any additional manipulation, or after overnight culture in high-dose IL-2. They can also be expanded in IL-2 or other cytokines, such as IL-15, alone or in combination, for two to several weeks in cell culture bags or in a bioreactor[11,12,26]. Similarly, it is possible to expand single KIR^+ NK cells[27]. *In vitro* expansion has two aims, to activate the selected $CD56^+CD3^-$ cells, and to increase the total number of NK cells. Using CD69 as an activation marker, activation of NK cells was found to occur within 1–3 days of incubation with IL-2[9,21]. When enriched $CD56^+CD3^-$ NK cells were cultured with IL-2, a significant expansion was observed although there was a lag of 3–5 days before the NK cells started to proliferate[11]. On day 5, expansion occurred and led to a two- to tenfold increase of $CD56^+CD3^-$ NK cells after 10–14 days. Although NK cells were viable immediately after purification (>90%), the vital NK cell count decreased by 30–50% during the first three to five days following IL-2 stimulation. Afterwards, cell viability recovered to >98%, and by day 10–14, a maximal NK cell expansion was obtained. No overgrowth of the remaining T cells was observed during expansion and activation

Author	Method	Donors (n)	NK cell purity (%)	Recovery (%)	Log T cell depletion
[19]Lang *et al.* (2002)	CD56 enrichment, followed by CD3 depletion	4	98.6 (m)	42.0 (m)	3.6×10^4-fold
[20]McKenna *et al.* (2007)	CD3 depletion	36	37.7 (x)	78.8	2.7
[20]McKenna *et al.* (2007)	CD3 depletion, followed by CD56 enrichment	13	89.7 (x)	19.4	4.3
[21]Koehl *et al.* (2004)	CD3 depletion, followed by CD56 enrichment	6	95.0 (m)	37.0 (m)	4.5 (m)
[12]Koehl *et al.* (2005)	CD3 depletion twice, followed by CD56 enrichment	15	94.9 (m)	33.0 (m)	5.0 (m)
[22]Iyengar *et al.* (2003)	CD3 depletion, followed by CD56 enrichment	12	91.0 (m)	48.7 (m)	5.3 (m)
[23]Uharek *et al.* (2003)	CD34 neg fraction: CD3 depletion, CD56 enrichment	7	75.0 (m)	42.0 (m)	4.0
[24]Passweg *et al.* (2004)	CD3 depletion, followed by CD56 enrichment	6	97.3 (m)	35.5 (m)	3.6
[13]Meyer-Monard *et al.* (2009)	CD3 depletion, followed by CD56 enrichment	24	94.5 (m)	58.0 (m)	4.2 (m)
[25]Rizzieri *et al.* (2010)	CD56 enrichment	51	96.5	80	

m: median; x: mean

Table 1 Clinical-grade NK cell enrichment using the CliniMACS® System.

with IL-2. With a protocol that enables the generation of NK cells on a clinical scale, using a closed system that conforms to GMP guidelines, the expanded NK cells were highly cytotoxic against different leukemic and tumor target cells[9,21]. Importantly, no non-specific activation against normal allogeneic lymphocytes occurred[19]. In addition we were able to demonstrate that IL-2 stimulation led to up-regulation of all natural cytotoxicity receptors (NCRs) and the activating receptor NKG2D, which might explain the observed increased cytotoxicity against MHC-I-negative targets[9]. There is evidence that a combination of cytokines, such as IL-2, IL-12, IL-15, and IL-21, may further increase cytotoxic activity of NK cells. In addition to NK cell enrichment from leukapheresis products as summarized in table 1, NK cells can also be generated from cord blood[28].

Clinical-scale collection, enrichment, activation, and expansion of purified NK cells are feasible. Most of the technical aspects for adoptive NK cell therapy have been developed for clinical applications. However, these laboratory procedures are time consuming and expensive, need particular skills, and must be performed according to a GMP-compliant protocol.

Clinical studies using freshly purified or IL-2–activated NK cells

Previous trials and ongoing clinical phase I/II studies have shown the feasibility of using freshly purified or IL-2–activated donor NK cells for the treatment of high-risk patients suffering from leukemia or tumors in both non-transplant settings and after haploidentical SCT as an additional immunotherapy[21,24,25,29-32]. NK cell products were infused as a single dose rate or as multiple applications with doses between 0.2×10^7 and 8.1×10^7 $CD56^+CD3^-$ NK cells/kg BW, mostly with less than 2.5×10^4 $CD3^+$ T cells/kg BW[1,9,25,32]. These first immunotherapy trials show that NK cells can be administered without immediate adverse events, that they are well tolerated by the patients and do not induce GvHD > grade II. However, some cases of GvHD have been observed after NK cell infusion. In some instances this has been associated with a less efficient T cell depletion. Whether GvHD is attributable to contamination by T cells or is due to the effects of NK cells cannot be determined on the basis of this clinical data. The fact that (at least in some cases of GvHD) the T cell content was higher than in cases without GvHD, seems to favor a T cell effect. With regard to NK cell efficiency, Rubnitz *et al.*[32] recently reported that NK cell administration to ten pediatric patients with AML in first complete remission led to a two-year event-free survival of 100%, with all patients still in complete remission. An earlier study demonstrated that patients with AML had a lower rate of leukemia relapse compared to the expected rate, a lower rate of graft rejection, and a paradoxical reduction in GvHD post-haploidentical SCT, when the NK cells possessed inhibitory KIRs for which the recipient had no ligand[33]. We could show an increased cytotoxic activity of stimulated NK cells against high-risk neuroblastoma (NB) due to IL-2–mediated up-regulation of the activating receptors NKp30, NKp44, NKp46, and NKG2D[9]. However, we have also been able to demonstrate tumor escape from immune surveillance by release of soluble MICA (ligand MHC class I–chain-related gene A) compromising NKG2D-dependent NK cell cytotoxicity in patients with NB. Elevated sMICA levels in patients' plasma correlated significantly with impaired NK cell–mediated cytotoxicity of the infused donor NK cells[34].

Future perspectives

Future studies should improve NK cell immunotherapy by increasing the understanding of the conditions leading to tumor cell kill by NK cells, by increasing the cytotoxicity of NK cells against various malignancies, and by optimizing the schedule of the NK administration based on results of ongoing phase I/II studies. Given the plausible benefit of IL-2–stimulated NK cells compared to freshly isolated, resting NK cells with regard to cytotoxicity, it may be possible to increase cytotoxicity by activation with cytokine combinations like IL-2/IL-15 or by cross-talk with dendritic cells (DCs). Additional investigation is necessary to develop strategies to overcome tumor immune escape mechanisms. Options may encompass development of MAb against sMICA, genetic engineering of NK cells by introduction of chimeric receptors for tumor retargeting, or enhancing tumor cell recognition by using small interfering RNA to silence inhibitory receptors. Open issues in clinical studies also include NK cell dose rate, time schedule, appropriate selection of donor/recipients, and also the types of tumors to be considered for treatment, because it is already known that certain types of malignant cells may be more responsive to NK cell therapy than others. Ultimately, expansion of tumor-reactive NK cells within the patient might prove to be feasible. It is possible to transfuse NK cells simultaneously with the transplants, and the first clinical trials indicate that an early NK application post SCT may be most effective in attacking minimal residual disease.

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