Ex vivo Expansion of Highly Purified NK Cells for Immunotherapy after Haploidentical Stem Cell Transplantation in Children

Abstract

Background: Allogeneic natural killer (NK) cells are known to show medium to high cytotoxic activity against HLA-nonidentical leukemia or tumor cells. For a possible benefit of posttransplantation treatment with NK cells after haploidentical stem cell transplantation (haplo-SCT) we developed a clinical scale procedure for NK cell processing observing Good Manufacturing Practice (GMP). Methods: Allogeneic donor NK cells were selected from 15 unstimulated leukaphereses using two rounds of immunomagnetic T-cell depletion, followed by an NK cell enrichment step. CD56⁺CD3⁻ NK cells were stimulated and expanded in vitro according to GMP. Quality control of NK cell purity, residual T-cells and cytotoxic activity was done by multi-coloured flow-cytometric analyses. Results: Purification led to an absolute number of 234–1237 × 10⁶ CD56⁺CD3⁻ NK cells from leukapheresis harvests with a median purity of 95% and a 4 to 6½ log depletion of T-cells. After two weeks stimulation with IL-2 a five-fold expansion of NK cells with a T-cell contamination below 0.1% was reached. Median cell viability was 95% after purification and 99% after expansion. The IL-2 stimulated NK cells showed a highly increased lytic activity against the MHC-I deficient K562 cells compared to freshly isolated NK cells and a medium cytotoxicity against patients’ leukemic cells. Conclusions: Clinical scale enrichment and activation of allogeneic donor NK cells is feasible. High dose NK cell application may be a new treatment option for pediatric patients with leukemia or solid tumors.


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Bibliography

Introduction

Posttransplant relapse remains a major obstacle in pediatric patients with refractory leukemia even after haploidentical stem cell transplantation [10]. Immunotherapy with selected donor cells or targeted therapy [23] may improve outcome after HLA-identical or mismatched allogeneic hematopoietic stem cell transplantation by enhancing the graft versus leukemia (GvL) effect. However, donor T cells can cause severe graft versus host disease (GvHD) [1]. By contrast, natural killer (NK) cells may mediate GvL without GvHD [3, 20, 24]. NK cells play an important role in innate defence against virally infected and malignant cells without prior priming or sensitization [17]. In humans, they comprise approximately 10% of peripheral blood lymphocytes. CD56+ NK cells do not express the T cell marker CD3+, distinguishing them from CD56+CD3– T cells [17]. IL-2 and IL-12 stimulation lead to activation and expansion of a CD56 population with high cytolytic activity against NK cell targets [2, 18]. The cytolytic function of NK cells is regulated by a balance of signals transmitted by killer cell inhibitory (KIR) and activating receptors. Inhibitory receptors bind to specific HLA molecules, while the various activating receptors bind to both, these HLA epitopes and other ligands on the target cells. [3]. A benefit of NK cells in reduction of leukemia relapse and graft rejection as well as in protection of patients against GvHD in certain transplant settings has been shown [20, 24]. In the haploidentical setting, NK cells seem to exert anti-leukemic effects against AML cells if a KIR ligand incompatibility exists in the graft versus host (GvH) direction resulting in a reduced risk of relapse compared to KIR compatible transplants [4, 19]. Adoptive immunotherapy using natural killer cells may be useful to prevent graft rejection by improvement of hematopoietic engraftment and/or exerting GvL effect [15, 16]. Here, we present a new protocol allowing the clinical scale NK cell isolation of allogeneic donors and expansion according to the principles of the GMP.

Material and methods

Clinical scale NK cell selection

NK cell enrichment included two rounds of CD3+ depletion with ensuing CD56 selection. Briefly, steady state leukapheresis was performed in unstimulated donors. The cells were washed twice for platelet reduction with CliniMacs 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 to reduce unspecific antibody binding and incubated for 5 minutes. Cells were labeled for 30 min with clinical grade CD3+ MicroBeads® kindly provided by Miltenyi Biotec. After washing twice, CD3+ cells were depleted with the CliniMacs device (Miltenyi) using the program “DEPLETION 2.1”. The depletion run was repeated on the negative fraction to remove residual T cells. Thereafter, the T cell depleted harvests were concentrated and labelled with clinical grade CD56 MicroBeads® (Miltenyi) for 30 min, washed and enriched of the CD56–CD3+ NK cells was performed using the program „ENRICHMENT 1.1“.

Expansion, activation and cryopreservation of NK cells

The isolated CD56–CD3+ NK cell (1 ×10⁶ cells/ml) were suspended in X-vivo 10 media (BioWhittacker, Verviers, Belgium) supplemented with 5% heat-inactivated human fresh frozen plasma (FFP) and 1000 U/ml rhIL-2 (Proleukin, Chiron, Ratingen, Germany) in a GMP surrounding (Fig. 1). Cells were expanded and activated using both 175 cm³ culture flasks (Nunc, Wiesbaden, Germany) for the first experiments and VueLife bags (Cellgenix, Freiburg, Germany) for the ongoing cell expansion. Fresh media was added every three days. Samples were drawn after leukapheresis and each depletion/selection step as well as every second day during stimulation to monitor cell content and viability. Phenotyping and evaluation for cytotoxicity was performed by flow cytometry.

Stimulated NK cells were cryopreserved in X-vivo 10 media supplemented with 20% DMSO.

Flow cytometric analysis

Flow cytometric analysis was performed both, on a four and a five colour-flow cytometer (Epics XL or FC 500, Beckman Coulter, Krefeld, Germany) in dual and single platform technique to determine both absolute and relative NK cell content. Cells were labelled with appropriate combinations of fluorescence conjugated antibodies against CD3, CD14, CD16, CD45, CD56, CD57, CD69, and HLA-DR to monitor T cell content, NK cell subsets and activation status. Controls were set up using isotypic antibodies. Additionally, 7-AAD was used together with antibodies in a modified ISHAGE protocol for stem cell measurement to deter-

Key words

NK cells · haploidentical stem cell transplantation · CD56 selection · CD3 depletion

Schlüsselwörter

NK-Zellen · haploidente Stammzelltransplantation · CD56-Selektion · CD3 Depletion
mine viability of NK cells and residual T cells. Flow-Count beads® (Coulter Immunotec, Marseille; France) served as internal standard.

**Cytotoxicity assay**

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, both with an Europium and an antibody-based flow cytometric assay as described previously [13, 25]. NK cells and leukemic cells were co-cultured for 4 h at 1:1, 5:1, 10:1, 20:1 and 40:1 effector:target ratios. Specific lyses in the Europium assay was calculated as % specific lysis = (experimental release – spontaneous release)/(maximum release – spontaneous release)×100. In the flow cytometric assay, absolute cell counts were determined using Flow-Count beads®. Cytotoxicity was defined as the loss of vital target cells relative to the control. The cytotoxicity of the NK cell line NK92 against K562 served as a positive control [5, 21].

### Results

**Purity and recovery of NK cells**

A total of 15 unstimulated leukapheresis was performed, resulting in a total of 11 immunomagnetic separation procedures (7 single, 4 pooled). The characteristics of these are given in Table 1. Starting with a median of $18.1 \times 10^9$ leukocytes and 8.2% NK cells, median CD56+CD3– NK cell content was 95% after the final selection step with a median recovery of 33%. The first and the second T cell depletion round resulted in NK cell recoveries of 69% and 61% compared to the starting CD56+CD3– cell content, respectively. Overall a 4.2 up to 6.6 log (median 5 log) depletion of T cells was observed. Median viability of the NK cells was 99% prior to and 95% after purification. The major population of contaminating cells after selection could be shown to be CD14+ monocytes with a median of 5% of all CD45+ leukocytes.

**Expansion, activation and cryopreservation of donor derived NK cells**

During the first five days after IL-2 stimulation, vital NK cell counts decreased by 30%. Afterwards, the cells recovered and by day 14, a median 5-fold expansion was seen (range: 4- to 9-fold, Fig. 2A). At that time point, viability of the NK cells has been increased for up to >99%. An early increase in the activation marker CD69 was observed on viable NK cells which
reached a plateau expression level of >90% after 24 hours, which remained high during the next 14 days (Fig. 2B; Fig. 3C). Our immunomagnetic selection strategy led to a T cell contamination below 0.1% (Fig. 3A), which remained stable throughout expansion in the presence of IL-2 after 10–14 days (Fig. 3B). Thawing of cryopreserved NK cells was associated with a median viability of 77%.

**Cytotoxicity**

For all CD56^+CD3^- NK cell enriched products, cytotoxicity of the freshly isolated cells against K562 was low at effector:target ratio of 1:1 and increased to a median activity of >10% at ratios above 10:1. After 10 days of IL-2 stimulation the cytotoxic activity of NK cells peaked and showed 75% killing of K562 cells and an observable cytotoxicity in the 1:1 ratio (Fig. 4). The lytic activity of the control NK cell line NK92 was 70% at a ratio of 10:1 (not shown). The cytotoxicity of haploidentical donor NK cells against the individual leukemic cells in samples of three pediatric patients with a content of 95%, 99% and 100% leukemic cells respectively was low in the 1:1 ratio and increased to a medium lytic activity in the 40:1 ratio.
CD56+CD3− NK cells. The purity obtained with this procedure is richment procedure which leads to a median purity of 95 with two rounds of CD3 depletion and a concomitant CD56 en-
taminating T cells. Here, we report on a GMP compliant system of GvHD, it was our aim to establish a clinical scale enrichment
cial effects on both, engraftment and GvL, without the induction
that yield mixtures of CD56+CD3− NK and CD56+CD3+ T cell popu-
To date, several studies reported NK cell preparation methods
NK cell immunotherapy may promote hematopoietic engraft-
against melanoma and renal carcinoma cells [8]. Moreover,
tro expanded NK cells are able to kill AML blasts when KIR in-
the GvH direction is present [19]. They demonstrated that in vi-
to Cytotoxic activity of activated and non-activated NK cells
Fig. 4 Cytotoxic activity of activated and non-activated NK cells
against K562 cells and haploidentical primary leukemia targets. The lytic activity of non-stimulated (open squares) and IL-2 stimulated (closed squares) NK cells of several donors (n = 11) against MHC-I class I negative K562 cells was compared to the cytotoxic activity of IL-2 stim-
ulated NK cells from haploidentical donors against the respective pa-
tients’ leukemic cells (n = 3; closed circle). Shown is the lytic activity in % dead cells of total (y-achsisis) at effector to target ratios ranging from 1:1–40:1 (x-achsisis).

Discussion
NK cells are known to play an important role in the control of leukemia and solid tumors [2, 3, 14, 19, 22]. They are increasingly hedged as a possible means for cellular immunotherapy after allogenic SCT, especially after haploidentical SCT. Ruggieri et al. have shown that the risk for patients suffering from AML relapse after haploidentical or partially mismatched unrelated allogeneic SCT is significantly reduced when a KIR epitope mismatch in the GvH direction is present [19]. They demonstrated that in vitro expanded NK cells are able to kill AML blasts when KIR incompatibility is given. A similar effect was reported in vitro against melanoma and renal carcinoma cells [8]. Moreover, NK cell immunotherapy may promote hematopoietic engraft-
ment after haploidentical SCT accompanied by enhanced GvL [15].

To date, several studies reported NK cell preparation methods that yield mixtures of CD56+CD3− NK and CD56+CD3+ T cell populations, respectively [6, 7, 11]. As these studies were done in an autologous setting or pre-clinical, the effect of the CD56+CD3+ cells in allogeneic SCT remains to be established. For the benefi-
cial effects on both, engraftment and GvL, without the induction of GvHD, it was our aim to establish a clinical scale enrichment and expansion protocol for donor derived NK cells free of cont-
taminating Tcells. Here, we report on a GMP compliant system with two rounds of CD3 depletion and a concomitant CD56 en-
richment procedure which leads to a median purity of 95% CD56+CD3− NK cells. The purity obtained with this procedure is comparable to that we reached previously after initial CD56 po-
sitive selection followed by Tcell depletion [13]. However this laboratory method was not GMP compliant. It is important to notice that all steps of our clinical scale process are easy to per-
form, although the processing is very time consuming. Therefore, this method can be applied for the treatment of haploiden-
tical transplanted patients in the clinical setting as postulated by Lyengar et al. similarly [9].

Our data indicate that within 5 days of IL-2 stimulation, NK cell proliferation occurs in the culture. A 5-fold expansion of the CD56+CD3− cell fraction was obtained within 2 weeks of cell cul-
ture, albeit lower compared to the expansion when both, NK and CD56+CD3+ T cells are present in the culture [11]. We could show that the cytotoxicity of freshly isolated NK cells was low and their induction was dependent on the activation with IL-2. NK cells reached a high lytic activity against the MHC-I deficient cell line K562 after 12 days of IL-2 stimulation. This is in accordance with observations of Farag et al. [3] and our previous experimental data [13]. Most important, in contrast to lympho-
Kine activated killer (LAK) cells, which consists of >80% T cells that might be responsible for the adverse reactions attributed to LAK infusions [21], we did not see an outgrowth of residual CD3+ T cells (<0.1 %) during IL-2 activation. We demonstrated that the major contaminating cell population after purification and after expansion are CD14+ monocytes.

Our results show that a 10–14 days expansion period with 1000 IU IL-2/ml is sufficient to achieve the desired cell dose and activity for clinical application. To date, we used this protocol for the treatment of three patients with refractory or progressive leukemia after haplo-SCT, with encouraging results as to chimerism conversion and GvHD [12]. All patients tolerated the NK immuno-
therapy well and no unexpected adverse events were recorded. In summary, we established a GMP-compliant NK cell iso-
lation and expansion protocol for clinical application in the haploidentical setting. Several clinical studies ensuing this pro-
tocol are underway [16].

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