

## Advances in clinical NK cell studies: Donor selection, manufacturing and quality control

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### ABSTRACT

Natural killer (NK) cells are increasingly used in clinical studies in order to treat patients with various malignancies. The following review summarizes platform lectures and 2013–2015 consortium meetings on manufacturing and clinical use of NK cells in Europe and United States. A broad overview of recent pre-clinical and clinical results in NK cell therapies is provided based on unstimulated, cytokine-activated, as well as genetically engineered NK cells using chimeric antigen receptors (CAR). Differences in donor selection, manufacturing and quality control of NK cells for cancer immunotherapies are described and basic recommendations are outlined for harmonization in future NK cell studies.

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### Introduction

Significant progress has been made in NK-cell based therapies in haploidentical stem cell transplantation (haploSCT)<sup>1</sup> or in the non-transplant setting<sup>2</sup> since NK cells contribute to the graft versus leukemia/tumor (GvL/GvT) effect with general no or at least less graft vs. host disease (GvHD) compared to allogeneic T cells.<sup>3,4</sup> Based on platform lectures on the clinical workshop at the international NK2013 meeting in Heidelberg, Germany, which was the 14th meeting of the Society for Natural Immunity, the formation of an international NK cell working group was announced to address harmonization on (i) donor selection, (ii) manufacturing and (iii) quality control (QC) for the clinical use of NK cells in Europe and United States. Based on regular consortium meetings in 2013–2015 including joint sessions of the European Bone Marrow Transplantation (EBMT) and the International Society of Cellular Therapy (ISCT), the group provided an overview of recent pre-clinical and clinical results in NK-cell based donor lymphocyte infusions (NK-DLI) including unstimulated, cytokine-activated, as well as genetically engineered NK cells using CARs. In the latter case, both gene-transduced primary human NK cells and the NK92 cell line were efficiently redirected against malignancies as reviewed by group members<sup>5,6</sup> recently.

### Reports on clinical NK cell therapies

Eleven speakers presented data on the NK2013 meeting in two clinical sessions chaired by Andrea Velardi, Perugia/Italy and Rupert Handgretinger, Tuebingen/Germany.

Ulrike Koehl summarized a phase I/II study (Switzerland/Germany) in 25 patients with high-risk malignancies treated with 0.5–4.5 × 10<sup>7</sup>/kg freshly purified or IL-2-activated (1000 U/mL) NK-DLI on days +3, +40, +100 post haploSCT.<sup>1,7,8</sup> No signs of GvHD occurred, if residual T cells were <25 × 10<sup>3</sup>/kg. Elevated sMICA levels in patient's plasma correlated with impaired NK-cell cytotoxicity, but high dose of IL-2 activated NK-DLI could restore NKG2D-mediated-cytotoxicity by scavenging of sMICA.<sup>9,10</sup> Activated NK-DLI was also more resistant against mycophenolate mofetil, which was used as immunosuppressive therapy in haploSCT with CD3/CD19 depleted grafts, compared to unstimulated NK-DLI.<sup>11</sup> Finally, a strongly increased selective anticancer activity using CAR-modified NK92 cells redirected against ErB2/HER2<sup>pos</sup> breast cancer and GD2<sup>pos</sup> Neuroblastoma was presented.<sup>12,13</sup>

Christian Kalberer reported on different NK-DLI studies at the Stem Cell Transplantation unit, Kantonsspital in Basel<sup>1</sup> and on a large-scale NK cell expansion protocol yielding sufficient numbers for multiple infusions to patients with haematological malignancies.<sup>14</sup> Purity of CD56<sup>+</sup>CD3<sup>-</sup> NK cells was >94% with less than 0.01% T cell contamination.<sup>15</sup> Currently in trials with

activated NK-DLI post haploSCT in AML and Multiple Myeloma patients several applications with  $8.5 \times 10^9$ – $44 \times 10^9$  NK-DLI were given. NK cells were expanded in air-permeable bags with serum-free culture medium supplemented with GMP-grade IL-2/IL-15, human AB<sup>+</sup> serum, anti-CD3 antibody and irradiated autologous cells achieving expansion rates of up to 76-fold. Aliquots with  $1.0 \times 10^8$  cells/kg were cryopreserved.

Evren Alici showed data on *ex-vivo*-expanded NK cells from both healthy donors and patients.<sup>16,17</sup> Comparing bags, an automated bioreactor and flask-based culture systems, significant expansion of NK cells was obtained with all systems under feeder-free GMP conditions. However, the bioreactor yielded in a product highly enriched in NK cells (mean:  $9.8 \times 10^9$ ) and improved cytotoxicity. Alici also presented data on NK cells transduced with a membrane bound form of IL-6 fused with NKp30 NK cell activation receptor as a control reaching transduction rates of 18% and 29%, respectively. Lytic activity of the CAR-expressing cells against malignant Namalwa B cells was 80% compared to 55% for untransduced cells at 10:1 ratio. Finally, Alici showed his genetic and functional screening method about the complex network of interactions between the patient's autologous NK cells and the respective tumor cell.

Jan Spanholtz gave a talk on NK cell immunotherapy against AML using CD34<sup>+</sup>-derived NK cells.<sup>18</sup> In order to obtain large numbers of functional NK cells, he used an efficient cytokine-based culture system for *ex vivo* expansion and differentiation of NK cells from umbilical cord blood (UCB).<sup>19,20</sup> The expansion in a bioreactor yielded more than 2,000-fold expansion, generating doses of more  $1 \times 10^{10}$  NK cells and a purity of >90%.<sup>21</sup> These NK cell products are currently used for immunotherapy in elderly AML patients not eligible for transplantation.<sup>22</sup>

Dean Lee reported on clinical translation of *ex vivo* NK cell expansion with membrane-bound IL-21. He developed a system for *ex vivo* expansion of NK cells that supports greater than 30,000-fold expansion in 3 weeks, enabling a single donor phlebotomy to yield cell doses of 50–100 times greater than that achievable by apheresis and CD3-depletion.<sup>23,24</sup> The method generates NK cells with reduced senescence, high cytotoxicity, serial killing ability, and endogenous cytokine production for improved survival, proliferation, and function.<sup>25</sup> This has been translated to GMP-compliant procedures and clinical trials are under way to apply this approach to autologous, allogeneic and UCB NK-DLI in transplant and non-transplant settings.<sup>26</sup>

Jeffrey Miller presented data on haploidentical NK-DLI with exogenous IL-2 to treat patients with AML, NHL and ovarian cancer.<sup>2,27,28</sup> *In vivo* persistence of NK cells 7 d after infusion, and successful *in vivo* expansion at day 14 ( $100 \text{ NK-DLI}/\mu\text{L}$ ) correlated with leukemia clearance. Expansion of host Tregs was associated with lack of NK cells. He also demonstrated data on the use of bispecific killer engagers (BIKEs), which are able to impart antigen-specific selectivity. A BIKE created from single chain Fv (scFv) specific for CD16 on NK cells fused by a linker to a scFv against CD33 on AML targets can create an immune synapse and trigger CD16 on NK cells to kill primary AML.<sup>29</sup> Finally, Miller suggested that NK-DLI will be most

effective if given with optimal cytokines (IL-15) to induce *in vivo* expansion and agents to enhance target specificity.<sup>30</sup>

Mark Lowdell reported on two-stage priming of allogeneic NK cells for patients with AML.<sup>31</sup> Resting NK cells require a “priming” and “triggering” process. While NK-sensitive tumors provide both signals, NK-resistant tumors evade lysis by failure to prime. M. Lowdell showed data on a tumor cell line (CTV-1) that primes resting NK cells but fails to trigger lysis.<sup>32,33</sup> These tumor-activated NK cells (TaNK) then retain the ability to lyse NK-resistant leukemias.<sup>34</sup> He created a GMP-compliant manufacturing process for TaNK cells as cellular medicines and designed a clinical trial to determine the toxicity of infusions of TaNK cells from related haploidentical donors in a cohort of eight patients with AML at different disease stages.<sup>31</sup>

Lutz Uharek showed data on early adoptive transfer of allogeneic NK-DLI among a prospective phase I/II trial. Twenty-five patients with AML, ALL, CML, Hodgkin's disease and MDS received a mean of  $9.8 \times 10^6$  CD56<sup>+</sup>CD3<sup>-</sup> NK-DLI/kg at day +2 post haploSCT. NK-DLI showed promising survival rates in patients lacking other treatment options.<sup>35,36</sup> Best results were achieved in patients with AML in remission, but responses were also seen in patients with refractory disease.

Hans Klingemann reported on the highly cytotoxic NK-92 cell line as an alternative option for cancer treatment. Phase I trials showed that irradiated NK-92 cell infusions were well tolerated up to a tested dose range of  $1 \times 10^{10}/\text{m}^2$ .<sup>5,37,38</sup> Some clinically responses were seen in patients with advanced lung cancer, melanoma and lymphoma. NK-92 cell expansion was performed in VueLife bags or in G-Rex bioreactors for an ongoing trial.<sup>39,40</sup> Cells were shipped in fresh IL-2 at room temperature.<sup>41,42</sup> Finally, Klingemann presented data on the generation of several NK-92 CAR variants<sup>5,6,13</sup> and postulated combination with checkpoint inhibitors to further improve efficacy.<sup>43</sup>

Antonio Curti showed data on 14 elderly patients with high-risk AML receiving purified CD56<sup>+</sup>CD3<sup>-</sup> NK-DLI from haploidentical KIR-ligand mismatched donors.<sup>44</sup> The median number of infused NK cells was  $2.74 \times 10^6/\text{Kg}$ .<sup>45</sup> No NK cell-related toxicity, including GVHD, was observed. Two patients in molecular relapse achieved molecular CR lasting 9 mo for both patients. 7/12 patients in morphological CR, are disease-free (median 28 mo; range 9–63). After NK-DLI, donor NK cells were found in the peripheral blood of all evaluable patients (peak value on day 10). They were also detected in the bone marrow in some cases (peak value on day 5). In addition, a rise in IL-15 serum level was followed by increase in donor chimerism.

Wing Leung presented his approach to optimize NK-DLI for childhood malignancies.<sup>46</sup> The first step is donor selection including high resolution KIR typing. Clinical results were summarized to underscore the importance of KIR and HLA in allogeneic NK-DLI. Next, novel techniques were presented, including newer cytokines (IL-12, IL-15, IL-18, IL-21), artificial presenting cells, antibodies, immunocytokines, and CAR to optimize NK cell numbers, purity and potency. For clinical application, careful selection of patient populations and timing of NK-DLI were outlined. Data were reported on the use of NK-DLI for the induction of remission in patients with

refractory leukemia, for consolidation after SCT in patients with poor-prognosis diseases, and for the replacement of SCT in hematologic malignancies with high-risk features in clinical remission.<sup>47,48</sup>

## Donor Selection

Typing of KIR, HLA, and FcγR are important for donor selection because their polymorphisms affect NK cell function and thereby the clinical outcomes of NK cell therapy. The KIR gene family is as highly polymorphic as the HLA family; therefore, tissue typing for these two families of genes have evolved substantially over the years (Fig. 1) and even high-resolution analysis of KIR repertoire is investigated on NK cell subsets.<sup>49</sup> There are three levels of KIR typing.

The first level of KIR typing is genotyping to assess the gene content.<sup>47</sup> For example, approximately 95% of individuals have inhibitory KIR2DL1, 50% have KIR2DL2, 85% have KIR2DL3, and 95% have KIR3DL1. For activating receptors, KIR2DS1 is present in approximately 35% of individuals, KIR2DS2 in 50%, and KIR3DS1 in 35%.

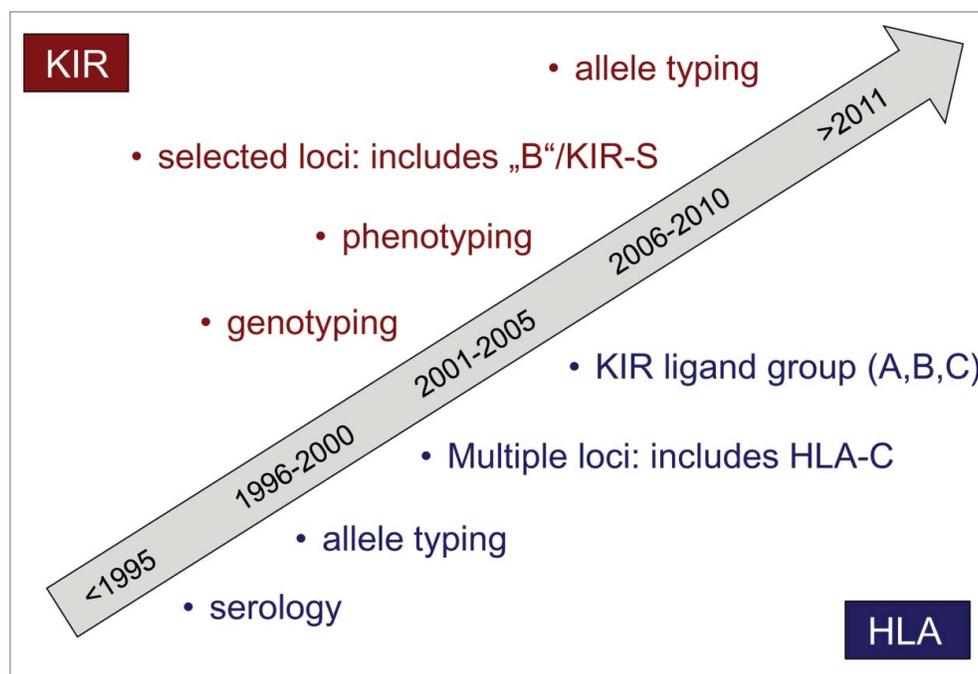
Based on the genotyping results, individuals may be classified on the basis of activating KIR content (KIR “A” vs. “B”), or on the basis of inhibitory KIR. KIR2DL3 is a hallmark gene in the centromeric (Cen) motif of “A” haplotypes, and it segregates as allele with KIR2DL2 which is a hallmark gene of “B”-haplotypes. Similarly, the telomeric motifs of “A” haplotypes typically contain KIR3DL1, which segregates as allele with KIR3DS1 in Tel-B motifs. Therefore, a simple 4-gene typing may be used clinically to classify the donors as Cen-A/A(KIR2DL2<sup>-</sup>KIR2DL3<sup>+</sup>), Cen-A/B(KIR2DL2<sup>+</sup>KIR2DL3<sup>+</sup>), Cen-B/B(KIR2DL2<sup>+</sup>KIR2DL3<sup>-</sup>), Tel-A/A(KIR3DS1<sup>-</sup>KIR3DL1<sup>+</sup>), Tel-A/B(KIR3DS1<sup>+</sup>KIR3DL1<sup>+</sup>),

or Tel-B/B(KIR3DS1<sup>+</sup>KIR3DL1<sup>-</sup>). The B-score is the sum of the number of Cen-B and Tel-B gene-content motifs (range 0–4).<sup>46,50</sup> If two or more similar donors are available, those with Cen-B and Tel-B genotypes with highest B-score are favored.<sup>51–54</sup>

Preferable donors on the basis of inhibitory KIR are those which possess a KIR for which the cognate ligand is absent in the recipient (i.e., receptor-ligand mismatch according to the Memphis model)<sup>48</sup> in particular when the donors also possess the corresponding ligand themselves (i.e., is “licensed”, per the ligand mismatch according to the Perugia model).<sup>3</sup>

The second level of KIR typing is phenotyping by flow cytometry to measure the number of KIR mismatched NK cells and by quantitative PCR to measure the frequency of gene expression.<sup>55</sup> There is considerable heterogeneity in gene expression among normal donors, with more than 10-fold difference commonly observed. Because the size of receptor-ligand mismatched NK cells in the donors corresponds to the alloreactivity of their NK cells against target cells without the cognate ligands, the donors with the largest number by phenotyping are preferable.

The third level of KIR typing is allelotyping. Different alleles of a KIR gene have different functional properties. For example, KIR3DL1\*004 is not expressed on NK cell surface and cannot educate NK cells. In comparison to KIR2DL1 alleles that have cysteine at position 245 in the transmembrane domain (KIR2DL1-C245), KIR2DL1 alleles that have arginine at the same position (KIR2DL1-R245) have stronger licensing capability, longer durability of surface expression after ligand interaction, and more recruitment of Src-homology-2 domain-containing protein tyrosine phosphatase 2 and β-arrestin 2.<sup>56</sup> Patients who received a donor haematopoietic graft containing KIR2DL1-R245 had better survival and



**Figure 1.** Evolution of tissue typing. Typing of KIR and HLA are important for donor selection because their polymorphisms affect NK cell function. HLA typing is done at a resolution that allows discernment of the KIR ligand groups in HLA-A, B, and C. KIR typing involves three levels: genotyping for gene content and B-scoring, phenotyping for gene expression, and allelotyping for allele polymorphisms.

lower cumulative incidence of disease progression than those patients who received a donor graft that contained only KIR2DL1-C245.<sup>51</sup> Patients who received a KIR2DL1-R245-positive graft with HLA-C receptor-ligand mismatch had the best survival. Therefore, donors with stronger KIR alleles should be chosen if available.

Finally, typing of FcγR polymorphisms is essential, in particular when NK cell therapy is used in combination with antibodies in which ADCC is a key mechanism of action. FcγRIIIa of 158V alleles binds IgG1 better than does those encoded by 158F alleles, resulting in enhanced activation of NK cells and better ADCC.<sup>57</sup>

### Clinical-grade purification and ex vivo expansion of NK cells

Preclinical NK-cell studies demonstrated that it is possible to produce a sufficient number of NK-DLI with effective tumor cytotoxicity. This has led to the translation of several preclinical NK-cell studies into GMP quality clinical-scale manufacturing of NK-DLI that are used in clinical trials. Both autologous and allogeneic NK-cell products are manufactured and have been used against numerous types of malignancies such as melanoma, leukemia and various types of carcinomas (Table 1).<sup>14,16,17,21,39,58-70</sup>

Even though manufacturing of NK-DLI is quite variable, the infusion of NK-DLI is well tolerated by patients. While febrile reactions are the most commonly observed complications, in general NK-DLI have a promising safety profile and most importantly they have an encouraging therapeutic effect that has been observed in some studies. More specifically, NK-DLI administered to patients with myeloid neoplasms appear to be the most effective therapy among many very early (Phase I/II) clinical trials.<sup>1,2,71,72</sup> While most studies so far have used fresh, immunomagnetically isolated NK cells, some early clinical studies were performed with in-vitro activated and expanded NK-DLI (Table 2).<sup>8,27,37,58,61,63,73-80</sup>

Even though promising results regarding safety, viability and antitumor responses have been reported, differences in NK-DLI manufacturing used in several clinical trials makes it challenging to determine the preferred source of NK-DLI and expanding conditions. While most of these studies using activated and expanded NK cells did not lead to development of GvHD, Shah et al reported on severe GvHD in 5/9 patients after treatment with donor-derived IL-15/4-1BBL-activated NK cells following HLA-matched, T-cell-depleted SCT. This gives rise for several questions such as to the safety in using manipulated feeder cells.<sup>80,81</sup> The key to future success is to optimize NK cell processing methods in order to achieve sufficient numbers of NK-DLI with the most efficient tumor cytotoxicity and clinical responses.

The majority of NK-DLI were generated through utilization of peripheral blood mononuclear cells (PBMC) either by apheresis or ficoll separation under GMP conditions. Although PBMC consists of 5–20 % NK cells, it is not possible to achieve always sufficient numbers of potent NK cells for multiple applications. Thus, various techniques to expand NK cells *ex vivo* have been developed (Table 2). For example, Alici has designed a system where they can

expand NK-DLI in a clinically compatible manner without feeders reaching highly cytotoxic NK cells.<sup>16</sup> They completed a clinical trial using donor-derived *ex vivo*-expanded NK cells in terminal cancer patients that had CLL, kidney cancer, colorectal cancer, and hepatocellular carcinoma with promising results.<sup>76</sup> Having optimized the procedure for NK cell expansion in a closed-automated bioreactor a first-in-man phase I/II clinical trial was initiated (EudraCT: 2010-022330-83).<sup>17,82</sup>

Another approach in expanding NK cells *ex vivo* makes use of feeder cells that provide essential stimulatory signals for NK cells proliferation. Monocytes, irradiated PBMC, feeder cell lines and engineered feeder cell lines are the most commonly used sources for stimulation of NK-cell expansion through humoral signals and cell-to-cell contact. On the other hand, UCB is also an essential source for achieving clinically relevant doses of NK cells when autologous cells are not optimal or readily available. Recently, expansion of active, tumor cytotoxic and pure NK cells under GMP conditions was demonstrated through use of UCB as a source.<sup>21</sup>

Moreover, the cell line NK92 which is a human NK cell line and is cytotoxic to a wide range of malignant cells,<sup>21</sup> has also been used as a source of NK cells for GMP-grade cellular therapy products.<sup>39</sup> After irradiation (to prevent proliferation), these cells can be used effectively in immunotherapeutic approaches without compromising their cytotoxic function. For example, clinical-grade NK92 cells have been manufactured and were recently safely used as antitumor therapy for end-stage patients with different tumors.<sup>37</sup> One of the advantages of using such master cell bank is an appealing opportunity in the manufacture of cellular therapy products since it is possible to establish a comprehensive standardization and characterization of the cell source.

Many of the NK expansion protocols are based on initial enrichment of NK cells either through cell selection or sorting in order to achieve pure cell therapy product and avoid unwanted side effects especially stemming from T cells. One of the methods of enriching initial NK cell numbers and final NK cell purity is the clinical grade immunomagnetic depletion of T cells and/or B cells and myeloid cells as shown by Miller et al.<sup>2</sup> Additionally, direct immunomagnetic selection of NK cells for production of NK cell therapy products, is another way to enrich initial content of NK cells. Nevertheless, in order to get activated, NK cells might require physical and cytokine-dependent communication with other cells such as monocytes;<sup>83</sup> thus, it is essential to fine-tune the enrichment of NK cells or make use of feeder cells or optimize the cytokine cocktail used in *ex vivo* NK cell expansion protocols.

Most GMP-grade NK cell therapy protocols include IL-2 as a main cytokine to stimulate NK cell activation and proliferation. Recent advances in production of GMP quality cytokines enabled further optimization of cytokine supplementation during NK cell expansion. For example, use of IL-15 in combination with IL-2 improved product viability and NK cell proliferation<sup>14</sup> that highlights necessity of other cytokines to achieve NK-cell product potency especially when it comes to the NK cell expansion protocols that are not using feeder support. In addition, it could be shown,

**Table 1.** Expansion protocol for manufacturing of NK cell products.

Source	Summary of protocol	Product specifications	Reference
Cell line	Cell line (NK-92) from a working cell bank are incubated in culture bags with IL-2 (t = 15–17 d).	$1.5 \times 10^9$ cells/L, >80% viability; >200-fold NK-cell expansion.	Tam et al. [39] Arai et al. [63]
UCB	CD34 <sup>+</sup> cells are enriched from cryopreserved volume-reduced cord blood using immunomagnetic bead selection; expanded <i>ex vivo</i> in culture bag using CD34 <sup>+</sup> expansion medium (t = 14 d); differentiated into CD56 <sup>+</sup> CD3 <sup>-</sup> cells using a bioreactor.	n = 4 final products; $1.6 \times 10^9$ – $3.7 \times 10^9$ CD56 <sup>+</sup> CD3 <sup>-</sup> cells, 90%–95% pure, >93% viability, normal karyotype, undetectable CD3 <sup>+</sup> or CD19 <sup>+</sup> cells.	Spanholtz et al. [21]
UCB	CB cells are incubated with IL-2, IL-15, tacrolimus and dalteparin sodium without feeder cells for 3 weeks.	72.8±9.6 % CD56 <sup>+</sup> CD3 cells and 1706±389 fold NK cell expansion.	Tanaka et al. [69]
PBMC (autol.)	Apheresis product is CD3 <sup>+</sup> depleted using immunomagnetic bead selection and incubated with irradiated autologous PBMCs as feeder cells with IL-2 and anti-CD3 (OKT3) in flasks (t = 21 d average).	n = 8 final products; $1.88 \times 10^{10}$ – $7.60 \times 10^{10}$ CD56 <sup>+</sup> CD3 <sup>-</sup> cells, >93% purity.	Parkhurst et al. [58]
PBMC (autol.)	Apheresis product is CD3 <sup>+</sup> depleted followed by CD56 <sup>+</sup> enrichment with immunomagnetic bead separation, combined with irradiated feeder cell line (EBV-TM-LCL), and incubated in culture bag with IL-2 (t = 28 d).	>96% CD56 <sup>+</sup> CD3 <sup>-</sup> cells, >71% viability (7-AAD negative), 0% CD3 <sup>+</sup> cells.	Berg et al. [60] Lundqvist et al. [61]
PBMC	Apheresis product is CD3 <sup>+</sup> , CD19 <sup>+</sup> , CD4 <sup>+</sup> , and CD33 <sup>+</sup> depleted using immunomagnetic bead separation; incubated with irradiated autologous PBMCs as feeder cells, IL-2, and IL-15 in a culture bag (t = 7–21 d); and then washed.	91% CD56 <sup>+</sup> cells, 75% CD16 <sup>+</sup> cells, 0.3% CD3 <sup>+</sup> cells, 37% CD25 <sup>+</sup> cells; 100-fold NK-cell expansion 16 d.	Luhm et al. [62]
PBMC	Apheresis product combined with irradiated feeder cell line (K562-mb15–41BBL) and IL-2 in culture bag, incubated (t = 7 d), CD3 <sup>+</sup> depleted using immunomagnetic bead separation, and then washed.	n = 12 final products; >72.9% CD56 <sup>+</sup> CD3 <sup>-</sup> cells, <13.5% CD3 <sup>+</sup> cells.	Fujisaki et al. [59]
PBMC	Apheresis product is added to culture flasks with anti-CD3 (OKT3) and IL-2, incubated (t = 20 d), washed, and incubated with IL-2.	Average of 65% CD56 <sup>+</sup> CD3 <sup>-</sup> cells.	Alici et al. [16]
PBMC	Apheresis product is CD3 <sup>+</sup> depleted followed by CD56 <sup>+</sup> enrichment with immunomagnetic bead separation; incubated (t = 19 d) in culture bags with IL-2, anti-CD3 (OKT3), with or without IL-15, and autologous irradiated PBMCs as feeder cells; and then washed.	62.7-fold NK-cell expansion.	Siegler et al. [14]
PBMC	Apheresis product is cultured with IL-2 and OKT3 in flasks, culture bags, and bioreactors (20 d).	530- to 1100-fold NK-cell expansion, 31%–51% NK cells.	Sutlu et al. [17]
PBMC	PBMC from ficolled whole blood is CD56 <sup>+</sup> enriched using immunomagnetic bead selection and incubated with IL-2, with or without IL-15 (t = 14 d).	67% CD56 <sup>+</sup> CD3 cells.	Klingemann et al. [64]
PBMC	Apheresis product is CD3 <sup>+</sup> depleted followed by CD56 <sup>+</sup> enrichment with immunomagnetic bead separation. No incubation was performed.	$1.1 \times 10^8$ – $8.8 \times 10^8$ CD56 <sup>+</sup> CD3 <sup>-</sup> cells.	Iyengar et al. [65]
PBMC	Apheresis product is CD3 <sup>+</sup> depleted and incubated with IL-2 and OKT3 for 14 d in culture bags.	CD3 <sup>-</sup> CD56 <sup>+</sup> (98.10±0.88%) or CD56 <sup>+</sup> CD16 <sup>+</sup> (97.43±1.66%) and 691.4±170.2-fold NK-cell expansion.	Lim et al. [66]
PBMC	Apheresis product is CD3 <sup>+</sup> depleted followed by a CD56 cell-enrichment step incubated in culture bags in presence of 1000 U/mL IL-2 for 12 d.	Up to 30-fold NK cell expansion, median 5-fold; purity: median 93% CD3 <sup>-</sup> CD56 <sup>+</sup> and 0.04% CD3 <sup>+</sup> cells.	Koehl et al. [67]
PBMC	Apheresis product is cultured with K562-mb15–41BBL cells as feeders in a gas-permeable static cell culture flask for 8–10 d.	54–70% CD56 <sup>+</sup> CD3 <sup>-</sup> cells and 38–338-fold NK cell expansion.	Lapteva et al. [68]
PPMC	Apheresis product, TCR- $\alpha\beta$ /CD19 depleted, CD56 enriched, expanded using IL-2 and irradiated clinical-grade EBV-transformed feeder cells, for 14 d, fully automated (CliniMACS Prodigy).	Mean 850-fold expansion with $1.3 \times 10^9$ cells, 99% CD56 <sup>+</sup> CD3 <sup>-</sup> cells.	Granzin et al. [70]

UCB = Umbilical cord blood; PBMC = peripheral blood mononuclear cells.

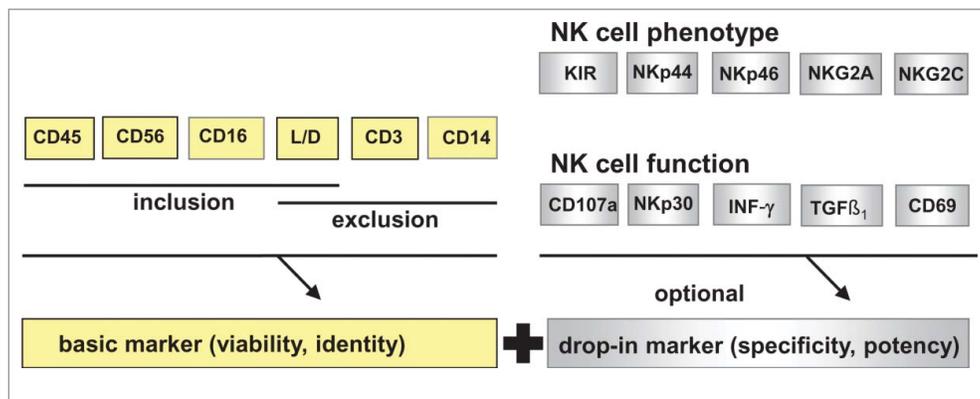
that cytokine (IL-12/IL-15)-activated NK cells transferred into naïve hosts can be detected up to 3 weeks later when they are phenotypically similar to naive cells.<sup>84</sup> Moreover, IL-12/15/18-preactivated NK cells led to sustained effector function against cancer,<sup>85</sup> which contributed to a recently opened trial of Todd Fehninger (ClinicalTrials.gov: NCT01898793) at St Louis Washington University using IL-12/15/18 stimulated NK cells in patients with AML. Besides

NK cell source, feeder support and cytokine stimulation, other parameters such as expansion platform, cell culture media and serum supplementation are also very important in achieving clinically relevant cell numbers, viability and tumor cytotoxicity. There are very few GMP quality media optimally working for *ex vivo* NK cell expansion protocols. Most commonly preferred media are X-VIVO serum-free media (BioWhittaker, Verviers, Belgium), AIM V (Life

**Table 2.** *In vitro*-activated and expanded NK cells for clinical trials.

NK cell product	Patient characteristics	Outcome	Reference
<b>NK cell line NK92:</b> Irradiated NK92 cells, doses of $1 \times 10^8$ – $3 \times 10^9$ cells/m <sup>2</sup> .	11 adult patients (10 renal cell carcinoma and 1 melanoma).	Infusion complicated with some febrile reactions. Two patients with possible tumor responses.	Arai et al. [63]
Irradiated NK92 cells, doses of $1 \times 10^9$ up to $1 \times 10^{10}$ cells/m <sup>2</sup> .	13 patients with solid tumors (7 sarcomas, 2 patients with leukemia/lymphoma).	No severe side effects, response in one patient with advanced lung cancer.	Tonn et al. [37]
<b>Autologous NK cells:</b> PBMNC derived, stimulated with IL-2 and $\alpha$ -galactosylceramide, doses: $1 \times 10^7$ – $5 \times 10^7$ cells/m <sup>2</sup> administered at 2 and 3 weeks after apheresis.	Six patients with non-small cell lung cancer (all adult).	Infusion complicated with febrile reactions, transient arrhythmia, headache. No tumor responses.	Motohashi et al. [73]
PBMNC derived, stimulated with IL-2 and a Hsp70 derived peptide, doses: $1 \times 10^6$ to $7.5 \times 10^6$ .	12 patients with colon or lung carcinoma (all adult).	Infusion complicated by itching in one patient. No significant tumor responses.	Krause et al. [74]
PBMNC derived; CD3 <sup>+</sup> depleted; stimulated with irradiated autologous PBMNCs as feeder cells, IL-2, and OKT3, doses: $1.88 \times 10^{10}$ to $7.6 \times 10^{10}$ NK cells.	Eight patients with melanoma or renal cell carcinoma (all adult).	Infusions complicated by shortness of breath in one patient, otherwise no reactions. No tumor response.	Parkhurst et al. [58]
PBMNC CD3 <sup>+</sup> depleted, CD56 <sup>+</sup> enriched, stimulated with irradiated feeder cell line EBV-TM-LCL and IL-2, doses: up to $1.88 \times 10^9$ /kg.	n = 14 patients (CLL and solid tumors).	Thyroiditis and constitutional symptoms.	Lundqvist et al. [61]
<b>Allogeneic NK cells:</b> Related allogeneic NK cells (CD34 <sup>+</sup> progenitor cells from donor were expanded and differentiated into NK cells with IL-15, IL-21, and hydrocortisone) at doses of $1.8 \times 10^8$ to $6.3 \times 10^8$ cells administered 43–50 d after allogeneic SCT.	14 patients with AML, ALL, or high-grade MDS (all adult).	No infusion-related complications. Two patients with active leukemia had no benefit.	Yoon et al. [75]
Related allogeneic NK cells (PBMNC derived, stimulated with IL-2 and OKT3) at doses of $1 \times 10^6$ to $10 \times 10^6$ cells/kg, administered at 13 to 41 d after autologous SCT.	Six patients with colon carcinoma, hepatocellular carcinoma, renal cell carcinoma, or chronic lymphocytic lymphoma (all adult).	Infusions complicated by febrile reactions, nausea, coughing, hemoptysis, and melena. Patient with hepatocellular carcinoma had stable disease, otherwise no tumor response.	Barkholt et al. [76]
Haploidentical allogeneic NK cells (PBMNC derived, CD3 <sup>+</sup> depleted, CD56 <sup>+</sup> enriched, IL-2 stimulated); doses up to $30 \times 10^6$ /kg post SCT.	Two pediatric patients with high risk ALL and one patient with AML, all haploSCT in blast persistence.	All three patients reached remission for several weeks/ months, two patients died during the next relapse, one due to infection.	Koehl et al. [77]
Haploidentical allogeneic NK cells (PBMNC derived, CD3 <sup>+</sup> depleted, CD56 <sup>+</sup> enriched, IL-2 stimulated); doses: $6 \times 10^6$ to $45.1 \times 10^6$ cells/kg on days (+3), 40 and 100 post haploSCT.	Four patients with neuroblastoma, four with AML, one with ALL (all pediatric and mostly non in remission at haploSCT).	Infusion complicated with some febrile reactions and two patients with vomiting and blood pressure changes. Two patients with high-risk neuroblastoma alive at 2 y.	Brehm et al. [8]
Haploidentical allogeneic NK cells (PBMNC derived, CD3 <sup>+</sup> depleted, stimulated with IL-2 overnight) at doses of $8.33 \times 10^6$ to $3.94 \times 10^7$ cells/kg.	20 patients with breast or ovarian carcinoma (all adult).	Infusions complicated by dyspnea, hypoxia, febrile reactions, hypertension, hypotension, fatigue, edema, pneumonitis, rash, nausea, myalgia. Two patients developed passenger lymphocyte syndrome.	Geller et al. [27]
PBMNC derived (haploidentical and autologous NK cells) activated/expanded with K562, genetically modified to express 41BB-ligand and membrane-bound interleukin (IL)15; doses: up to $1 \times 10^8$ /kg.	Eight patients high risk myeloma.	Seven patients without side effects, one patients showed 7 d after cell infusion that donor NK cells comprised >90% of circulating leukocytes.	Szmania et al. [78]
Donor CD56 <sup>+</sup> NK cells were cultured for 20–23 d with interleukin-15 and hydrocortisone; doses: $0.2$ – $29 \times 10^7$ cells/kg.	16 patients with adenocarcinoma or squamous cell carcinoma.	No severe side effects, two patients with partial response and six patients with disease stabilization.	Iliopoulou et al. [79]
Donor-derived IL-15/4–1BBL-activated NK cells following HLA-matched, T-cell-depleted SCT, doses: $1$ – $10 \times 10^6$ cells/kg.	Nine pediatric patients and young adults with high risk solid tumors.	5/9 patients developed GvHD grade III/IV.	Shah et al. [80]

PBMNC = peripheral blood mononuclear cells, SCT = stem cell transplantation, haploSCT = haploidentical stem cell transplantation.



**Figure 2.** Modular quality control for NK cells. For NK cell phenotyping on accredited flow cytometer a backbone including the antibodies CD45, CD56, CD3, CD16, CD14 or CD14/DRAQ7 and live/dead staining such as 7-AAD or PI for NK cell identity should be accompanied by different variable drop in markers like NCRs or KIRs for specificity and potency. For NK cell functionality, the same backbone might be used in combination to intracellular staining or specific target labeling in case of effector: target cytotoxicity. NCR = natural cytotoxicity receptors; KIR = Killer immunoglobulin like receptors.

Technologies, Grand Island, NY), or stem cell growth medium (SCGM; CellGenix, Freiburg, Germany). Generally, medium is supplemented by human AB serum or fetal bovine serum from certified sources.

Finally, there are numerous variables that may impact quality and quantity of NK cell products. Future trials will evaluate the contribution of each factor to the product purity, potency, and safety as well as identify NK cell products that can be reproducibly manufactured with the optimal safety and antitumor responses.

### Quality control for NK cell phenotype and functionality

Multi-color flow cytometric quality control (QC) provides the platform for both quantification of NK cell phenotypes and NK cell functionality. While the ISHAGE (International Society for Hematotherapy and Graft Engineering)<sup>86</sup> protocol for CD34<sup>+</sup> cells was successfully established >15 y ago leading to a broad international use, harmonization for enumeration of NK cell products is still a major challenge. The lack of accredited QC methods makes comparison of technical and clinical results from different NK studies tedious. Ideally, a QC panel for NK cells should consist of (i) a stable validated backbone to determine cell viability, cell number and to confirm the identity/purity of the CD56<sup>+</sup>CD3<sup>-</sup> NK cells and (ii) optional, variable “drop-in” markers to further specify the cells regarding subpopulations, functionality or potency. The development of new dyes and the multitude of biomarkers available today allow the design of such a modular assay.<sup>87</sup>

Flow cytometric quantification should be performed in single platform, no-wash-preparation technique including live/dead staining in order to enumerate the absolute number of viable CD56<sup>+</sup>CD3<sup>-</sup> NK cells. Ideally, for NK cells the backbone constitutes the antibodies CD45, CD56, CD3, CD16, CD14 or CD14/DRAQ7, live/dead staining such as 7-AAD or PI and counting beads<sup>67</sup> as presented in Fig. 2. The gating strategy is based on viable lymphocyte subtyping, using low scatter, positive expression of CD56 and CD45 antigens, CD3 as a negative discrimination marker, CD14 as a dump channel and CD16 to differentiate between immune regulatory and cytotoxic NK cells. Additionally, in the same panel various “drop-in” antibodies can

be included for staining with different other markers of interest such as KIRs, NCRs, NKG2A, NKG2C, NKG2D, CD62L, CD57, CD244 and activating markers (CD69, HLA-DR, CD25). Finally, enumeration of functionality is possible as a “drop in” system as well, but this does not allow the use of the single platform no wash system. For example, CD107a can be adopted for quantification of degranulation<sup>35,36</sup> and intracellular cytokine staining (such as INF $\gamma$  or TGF- $\beta$ ) for estimation of NK cell functionality.

Advanced flow cytometric analyses in single platform no wash technique is also possible to use for a proper quantification of NK cell cytotoxicity against the MHC class I-negative cell line K562 or against the patient’s individual leukemic<sup>88</sup> or tumor cells.<sup>89</sup> Whole blood NK cells, purified or cytokine activated NK cells and malignant cells are co-cultured in different effector: target ratios. Absolute cell counts are determined using counting beads and cytotoxicity is defined as the loss of vital target cells relative to a control.

In order to harmonize QC panels for NK cells, several world-wide exchange programs have been initiated for improved harmonization and compatibility between NK-cell based immunotherapies in the future.

### Conclusion and future direction

Considerable progress has been made in clinical NK cell studies during the past decade. Improved strategies in KIR typing in order to select an auspicious NK cell donor, optimization of large scale NK cell manufacturing protocols and harmonization in QC for cell release enables a platform for future clinical studies. In addition, establishment of fully automated clinical-scale protocols gives rise for cost-effective NK cell production. Although these advances generates hope, there is also a need for caution regarding GvHD development if donor-derived IL-15/4-1BBL-activated NK cells are used. This refers to well-established application of purified activated CD56<sup>+</sup>CD3<sup>-</sup> NK-DLI, which led to high safety for the patients, even if haploidentical or third party NK cells were transfused. Highly purified NK cells may also open a new field of immunotherapy by using genetic engineered CAR expressing NK cells for redirecting against various malignancies. In contrast to CAR T cells, which have

entered clinical trials successfully, but which are still in discussion for insertion of suicide genes to improve safety, primary mature NK cells have a limited lifespan only and therefore seemed to be excellent effector cells for genetic manipulation with CARs.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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