

## Use of Natural Killer Cells for Acute Leukemia Patients

Giovanni F. Torelli

Giovanni F. Torelli, Hematology, Department of Cellular Biotechnologies and Hematology, Sapienza University, Rome, Italy

Correspondence to: Giovanni F. Torelli, MD, PhD, Hematology, Department of Cellular Biotechnologies and Hematology, Sapienza University, Via Benevento 6, 00161 Rome, Italy.

Email: [torelli@bce.uniroma1.it](mailto:torelli@bce.uniroma1.it)

Telephone: +39-49974321 Fax: +390644241984

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### ABSTRACT

The management of acute leukemia has witnessed profound changes in recent years. The anti-leukemic potential of Natural Killer (NK) cells has raised considerable interest for the design of new therapeutic approaches based on the infusion of freshly-isolated or *ex vivo* manipulated effectors. Several points need to be addressed for the design of optimal protocols of NK infusion, including which disease can benefit most, the optimal timing during the course of the disease, the best preparative regimen and the origin of NK cells. This review aims at summarizing the biological and clinical data on the role played by NK cells in patients with acute leukemia; in addition, optimal good manufacturing practice compliant protocols for *ex vivo* expansion and activation of these effector cells are discussed.

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**Key words:** Anti-leukemic activity of NK cells; *Ex vivo* manipulation of NK cells; GMP protocols

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### DEFINITION OF NATURAL KILLER CELLS

Natural Killer (NK) cells are a subset of peripheral blood lymphocytes immunophenotypically characterized by the expression of the CD56 surface antigen and the lack of the CD3 and T cell receptor (TCR) proteins. These effectors play a central role in the defense against viral infections and tumor growth; they exert their cytotoxic capacity in a human leukocyte antigen (HLA)-unrestricted fashion, in addition to the production of various cytokines and chemokines, including tumor-necrosis-factor  $\alpha$  and interferon  $\gamma$ <sup>[1-3]</sup>. In humans, on the basis of the intensity of the CD56 antigen expression, it is possible to distinguish two main circulating NK cell subsets: CD56<sup>dim</sup> NK cells, which represent the major subset and mainly express a cytotoxic capacity, and the CD56<sup>bright</sup> subset which primarily produces cytokines<sup>[4]</sup>.

The cytolytic activity of these cells is finely regulated by the balance between inhibitory and activating signals derived from receptors expressed at the cell surface. In the late 80s, the "missing self hypothesis" was proposed by Ljunggren and Karre<sup>[5]</sup>, based on the observation that NK cells could efficiently kill targets who had lost major histocompatibility complex (MHC)-class I. Specific inhibitory receptors expressed by NK cells and recognizing allelic forms of HLA-class I were then identified and collectively termed as killer immunoglobulin-like receptors (KIRs) (KIR2DL and KIR3DL)<sup>[6,7]</sup>. Activating KIRs (KIR2DS and KIR3DS) were also discovered, similar to the corresponding inhibitory KIRs in the extracellular domain but with substantial differences in the transmembrane and cytoplasmic portion<sup>[8]</sup>. Besides activating KIRs, other receptors involved in tumor recognition include the natural cytotoxicity receptors (NCRs) (NKp30, NKp44, NKp46), NKG2D and DNAM-1<sup>[9-13]</sup>. It is the selective engagement of these receptors, in the absence of efficient inhibitory signals, that render target cells susceptible to NK-cell mediated lysis. Interestingly, the known ligands for these receptors are typically over-expressed upon cell stress, such as after tumor transformation or viral infection, when the expression of HLA-I molecules is reduced

rendering therefore the cell susceptible to NK lysis. While some of these receptors are still orphan of their ligands, MICA/B and ULBPs have been discovered to be the ligands for NKG2D<sup>[14]</sup>, whereas the Poliovirus receptor (PVR, CD155) and Nectin-2 (Nec-2, CD112) interact with DNAM-1<sup>[15,16]</sup>.

## ANTI-LEUKEMIC ACTIVITY OF NATURAL KILLER CELLS

It is from the setting of haploidentical stem cell transplantation (SCT) that the anti-leukemic capacity of NK cells became evident. In this context, alloreactive NK cells express inhibitory KIRs specific for HLA-class I alleles that are missing in the recipient, in addition to activating KIRs recognizing specific HLA ligands<sup>[17,18]</sup>; this further underlines the importance of both inhibitory and activating signals for tumor recognition.

Alloreactive NK cells have been demonstrated to positively affect the outcome of both adult with acute myeloid leukemia (AML) and children with acute lymphoid leukemia (ALL) undergoing T-cell depleted haploidentical (SCT)<sup>[19,20]</sup>. The biological reasons responsible for the different susceptibility of adult and children ALL blasts to the lytic effect played by alloreactive NK cells are so far unknown. Nonetheless, we recently reported the significant increased expression in pediatric B-lineage ALL (B-ALL) of some of the ligands for NKG2D and DNAM-1, including Nec-2<sup>[21]</sup>. In addition, these data allowed to recognize a differential expression of ligands for NK cell activating receptors in molecularly-defined subgroups of ALL patients. This is the case of *BCR-ABL*<sup>+</sup> ALL blasts, which display, together with the *MLL-AF4*<sup>-</sup> subgroup, the highest intensity of ligand expression within the B-ALL adult context. Importantly, the high expression of ligands for NK cell activating receptors correlated with the degree of susceptibility to lysis by expanded allogeneic NK cells, further supporting the role played by these receptors during the process of recognition. These results provided for the first time an explanation to the differential susceptibility of age- or molecularly-defined subgroups of acute leukemia patients to the lytic action of cytotoxic NK cells.

In the last years, the possibility of using either selected or *ex vivo* activated/expanded NK cells in the design of new immunotherapeutic strategies for the treatment of leukemic patients has gained much interest<sup>[22]</sup>. Isolated experiences are reported in the literature characterized by the infusion of NK cells derived from haploidentical donors<sup>[23-25]</sup> (see Table 1 for details) or from the hematopoietic stem cell donor when infused after a transplant<sup>[26-28]</sup> (see Table 2 for details), mainly for patient affected by AML, myelodysplastic syndrome or high risk lymphoma. Altogether these reports demonstrated that the infusion of these cells is safe and some encouraging results are described. Nonetheless several points need to be addressed for the design of optimal protocols of NK infusion, including which disease can benefit most, the optimal timing during the course of the disease, the best preparative regimen, the origin of NK cells whether autologous or allogeneic, and finally the *ex vivo* manipulation protocol.

## EX VIVO MANIPULATION OF NATURAL KILLER CELLS FOR ADOPTIVE IMMUNOTHERAPY

The need of large quantities of highly activated effector cells to produce an anti-cancer effect translates into the necessity of developing good manufacturing practice (GMP)-compliant methods for the efficient production of fully functional NK cells for clinical application. Several protocols for the *ex vivo* NK cell expansion and activation have been investigated, including the long-term culture with cytokines<sup>[29,30]</sup> and the use of different sources of allogeneic feeder cells<sup>[31-36]</sup>, very recently, a feeder-free particle-based technology which utilizes plasma membrane particles derived from transfected K562 cell line was also proposed<sup>[37]</sup>. Despite the excellent expansion fold, the majority of these protocols are not acceptable for clinical use in many countries, either because they do not utilize clinical grade materials or for the potential transmission of infectious diseases due to the use of allogeneic feeders. In addition, the proportion of contaminating CD3<sup>+</sup> T lymphocytes remains high in the majority of these approaches, with an associated risk of graft-versus-

**Table 1** Infusion of NK cells derived from haploidentical donors.

Reference	Disease	Conditioning	Ex-vivo manipulation of NK cells	Doses of NK cells	Concomitant treatment
Miller <i>et al</i> <sup>[23]</sup>	Acute myeloid leukemia, metastatic melanoma, renal carcinoma, hodgkin disease	Cyclophosphamide + steroids, fludarabine or cyclophosphamide + fludarabine	12 hour incubation with IL-2 of CD3-depleted PBMC	1×10 <sup>5</sup> - 2×10 <sup>7</sup> /Kg of BW. One dose per patient	s.c. IL-2
Rubnitz <i>et al</i> <sup>[24]</sup>	Pediatric acute myeloid leukemia	Cyclophosphamide + fludarabine	None	5×10 <sup>6</sup> - 8×10 <sup>7</sup> /Kg of BW. One dose per patient	s.c. IL-2
Curti <i>et al</i> <sup>[25]</sup>	Acute myeloid leukemia	Cyclophosphamide + fludarabine	None	5×10 <sup>6</sup> /Kg of BW. One dose per patient	s.c. IL-2

**Table 2** Infusion of NK cells derived from the hematopoietic stem cell donor.

Reference	Disease	Conditioning	Ex-vivo manipulation of NK cells	Doses of NK cells	Concomitant treatment
Yoon <i>et al</i> <sup>[26]</sup>	Acute leukemia, myelodysplastic syndrome	None	6 week culture with SCF, IL-7, Flt3L and steroids of CD34+ cells	3×10 <sup>5</sup> - 2×10 <sup>7</sup> /Kg of BW. One dose per patient at 6-7 weeks from transplant	None
Stern <i>et al</i> <sup>[27]</sup>	High risk leukemia, solid tumors	None	None	3×10 <sup>6</sup> - 4×10 <sup>7</sup> /Kg of BW. 1-3 doses per patient at 3, 40 and 100 days from transplant	None
Choi <i>et al</i> <sup>[28]</sup>	High risk hematologic malignancies	None	2 - 3 week culture with IL-15 and IL-21 of CD-3 depleted mobilized leukapheresis	2×10 <sup>7</sup> - 1×10 <sup>8</sup> /Kg of BW. 2 doses per patient at 2 and 3 weeks from transplant	None

host disease (GVHD) in the allogeneic context<sup>[38,39]</sup>; the probability of developing such a complication becomes even higher when the anti-CD3 monoclonal antibody (mAb) is added to the culture system<sup>[40,41]</sup>, running the risk of expanding and activating residual CD3<sup>+</sup> cells.

We recently developed a new GMP compliant-method for *ex-vivo* NK cell expansion<sup>[42]</sup>. Peripheral blood mononuclear cells (PBMCs) are obtained from leukapheretic procedures and processed in the GMP facility. For NK-cell enrichment, a two-step immunomagnetic procedure consisting of a CD3<sup>+</sup> T-cell depletion followed by a CD56<sup>+</sup> cell positive selection is used. Isolated NK cells are suspended in serum-free medium containing autologous plasma, interleukin (IL)-2 and IL-15 in the presence of irradiated autologous feeder cells and cultured for 14 days at 37°C.

The application of this method resulted in the efficient production of effector cells presenting a significantly increased expression of activating receptors important in tumor cell recognition, including NKG2D, DNAM1, Nkp30 and Nkp44, when compared to freshly isolated NK cells. The two-step NK cell selection procedure ensures the highest purity of the final product, as demonstrated by a minimal contamination by CD3<sup>+</sup> cells belonging to the T- and NK-T compartments; this is important in order to maximally reduce the risk of inducing a GVHD when the product is used in the allogeneic context. Furthermore, the high level of B-cell depletion reached with this system is also an important condition to prevent Epstein-Barr Virus (EBV) reactivation, which may potentially trigger lymphoproliferative diseases in immunocompromised patients.

Several other advantages are also associated with the expansion strategy utilized in this protocol; these include (1) the lack of *in vivo* cytokine infusion which can cause systemic toxicities; (2) the use of clinical grade manufactured IL-2 and IL-15, which play essential roles in NK cell development, expansion, homeostasis and activation<sup>[43,44]</sup>; (3) the use of autologous feeder cells to prevent the unknown effects associated with the use of allogeneic feeders; and d) the lack of an anti-CD3 mAb in the culture system, thus reducing the risk of GVHD in the allogeneic setting.

In our model, both cytokines and feeder cells are necessary to obtain optimal NK cell proliferation, since a lower expansion fold was observed in the presence of cytokines or feeder cells alone. Under these conditions, the degree of NK cell expansion was on average 15.7 fold. When tested in a cytotoxic assay against the K562 cell line, these expanded effectors proved to be highly active. This confirms recently published data from our group documenting the ability of expanded NK cells to recognize and kill primary acute leukemia blast cells<sup>[21,45]</sup>. Similar results were obtained when the expanded effectors were tested in a degranulation assay. Interestingly, these cells exerted a comparable cytotoxic activity when used prior to cryopreservation and after thawing; this is extremely important since *ex vivo* expanded cells must be cryopreserved before *in vivo* infusion in adoptive cell therapy protocols, in order to allow the necessary quality tests to be carried out before the batch of cells can be released for clinical use.

## CONCLUSIONS

In conclusion, new adoptive immunotherapeutic approaches based on the *in vivo* infusion of *ex vivo* expanded and activated NK cells represent promising strategies for the treatment of patients affected by acute leukemia. Several points still need to be addressed for the design of optimal protocols of NK infusion. The development of GMP methods for efficient production of fully functional NK cells is mandatory for clinical application. Future clinical trials are warranted and may solve some of the still debated points.

## CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

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- Peer reviewer:** Georgia D Kaiafa, Aristotle University, AHEPA University Hospital, 1st Propedeutic Dept of Internal Medicine, Hematology Unit, Stilonos Kyriakidi 1, PC 546 36, Thessaloniki, Greece.