

**Title:** Immunotherapy with alloreactive NK cells in AML: lessons for the future

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**Running Title:** NK cell-based cancer immunotherapy

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**Summary:**

The report by Curti et al. highlights the potential of NK cell-based adoptive cellular immunotherapy in oncology, currently boosted by advances in the knowledge on NK cell biology and in their ex-vivo GMP manipulation. Several issues deserve attention to fully achieve the translation of these advances to the clinic.

## Text

In this issue of Clinical Cancer Research, Curti and colleagues report their experience infusing NK cells from haploidentical KIR-HLA class I mismatched donors in elderly AML patients after first complete remission (1). Nine out of 16 cases reached disease-free survival along follow up (6-68 months), and a positive outcome was associated to greater infusion of donor alloreactive NK cell clones, detected pre- and early post-infusion (day 3). The results confirm the feasibility and safety of this adoptive cellular immunotherapy (ACI) approach, pointing out that the numbers of infused alloreactive NK cells influence on clinical efficacy (Figure 1); further studies with a larger number of cases are required to validate this observation.

The anti-leukemic effect of allogeneic hematopoietic stem cell transplantation (allo-HSCT) was early revealed by the association of Graft-vs-Host Disease (GVHD) with a reduced risk of relapse of hematological malignancies. Different approaches have been undertaken aimed to dissociate GVHD from the “graft-vs-leukemia” (GVL) effect in allo-HSCT. Studies in T cell-depleted haploidentical allo-HSCT supported the concept of harnessing GVL to control minimal residual disease focusing their attention on the role of NK cells (2).

NK cells are controlled by inhibitory receptors specific for HLA class I molecules (KIR, CD94/NKG2A and LILRB1) which prevent their activation against normal autologous cells (3). The CD94/NKG2A receptor is specific for HLA-E, conserved in all individuals, and LILRB1 interacts with a broad spectrum of HLA class I molecules. By contrast, some inhibitory killer immunoglobulin-like receptors (KIR) specifically recognize common structural features shared by different HLA class I alleles, mainly of the HLA-C and HLA-B loci. The KIR repertoire of an individual is primarily determined by inherited KIR haplotypes, which may contain different sets of genes displaying allelic polymorphisms. Along NK-cell differentiation KIR expression follows a clonal distribution pattern, leading to the development of NK cell subsets displaying different KIR combinations. NK cell maturation is favored by KIR interaction with HLA class I molecules, a process termed “licensing”. Remarkably, human cytomegalovirus (HCMV) infection promotes a persistent expansion of mature NK cells hallmarked by the expression of the CD94/NKG2C activating receptor and a predominant inhibitory KIR

specific for HLA-C (KIR2DL)(4). These “adaptive” NK cells display an enhanced cytolytic potential, cytokine production and prolonged survival. Hence, both genetic and environmental factors dictate the configuration of the individual NK cell receptor repertoire.

NK cell alloreactivity takes place upon encounter of cells lacking HLA class I molecules specifically recognized by their inhibitory KIR. In the context of allo-HSCT, KIR/HLA class I mismatches between donor (D) and recipient (R) promote NK alloreactivity. Remarkably, this effect is not encompassed by severe GVHD but favors the response against neoplastic cells. On that basis, several teams have developed allo-HSCT protocols assessing KIR and HLA class I genotypes, aimed to select for D/R mismatches promoting NK alloreactivity against minimal residual disease (MRD)(5, 6).

The general principle of ACI is infusing into the patient immune cells which have been ex vivo selected, activated, expanded and/or modified. Among a variety of approaches, pioneering studies with Interleukin 2 (IL2)-activated NK and T cells, termed lymphokine-activated cells (LAK), were developed in the 80s for cancer immunotherapy by Rosenberg et al. Currently, a substantial progress in the knowledge on NK cell biology and the development of methods allowing NK cell processing under GMP conditions have set the basis for a more accurate clinical use. In the context of allo-HSCT, Miller et al. (7) originally reported the feasibility and safety of infusing haploidentical NK cells following immunosuppressive chemotherapy in poor prognosis AML patients. Circulating donor NK cells were found up to 28 days post-infusion and some patients achieved significant clinical responses without developing toxicity or GVHD, confirmed by others. Thus, harnessing NK cell activation is currently envisaged as a valuable strategy for cancer immunotherapy, and several considerations deserve attention in this regard.

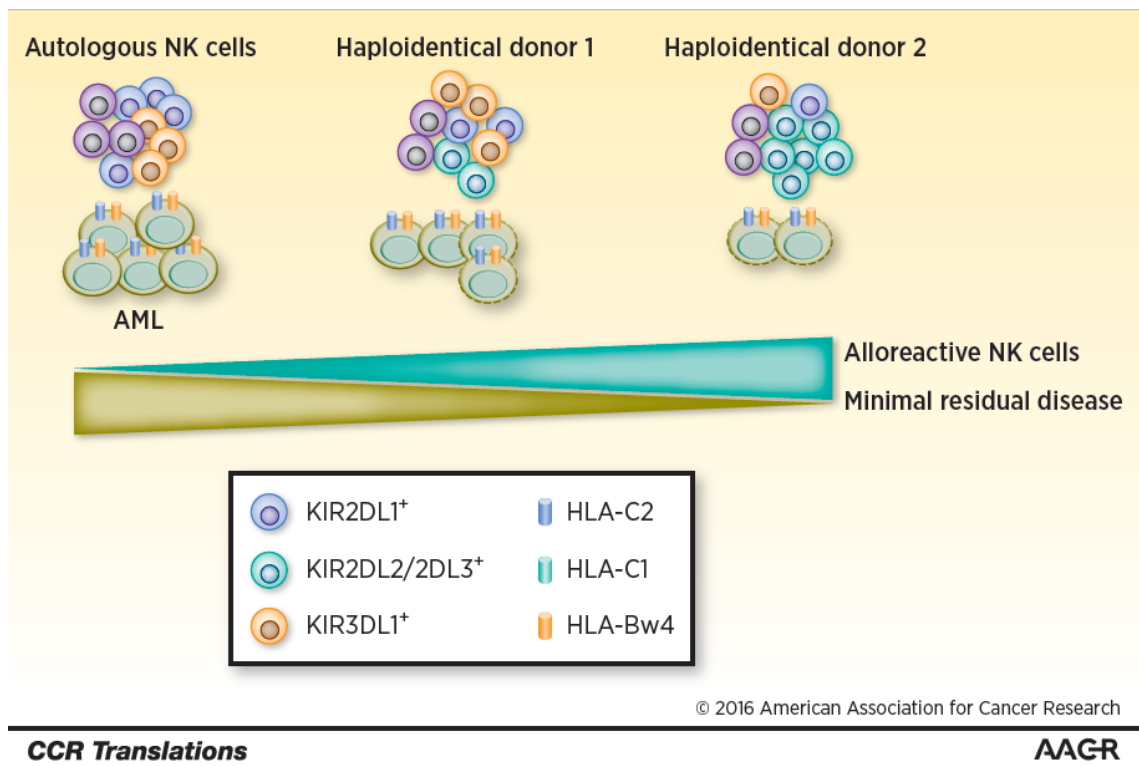
- a) As stressed by Curti et al. (1) increasing the numbers of alloreactive NK cells infused rather than simply relying on immunogenetic analysis to predict KIR-HLA ligand mismatch for donor selection, may improve clinical efficacy. Yet, a major effort to simplify and standardize technical procedures for quantifying alloreactive NK cells is warranted in order to allow a reliable comparison of different trials and development of multicenter studies.

- b) Homeostatic proliferation fostered by the production of endogenous IL-15 early after infusion favors donor NK-cell expansion and survival (7). On the other hand, regulatory T cells (Treg) may inhibit NK cell proliferation during IL-2 treatment following NK cell infusion, and Treg depletion resulted in increased donor NK-cell expansion, improved complete remission rates and disease free survival in AML patients treated with haploidentical NK cells (8). Optimizing the conditions that promote engraftment, homeostatic proliferation and survival of alloreactive NK cells is an important issue to enhance their anti-leukemic potential (9).
- c) Assessing the anti-leukemic potential of distinct NK cell-subsets is also warranted. In this regard, HCMV reactivation in HSCT recipients promotes the differentiation and expansion of adaptive NKG2C<sup>+</sup> NK cells (10) and this effect has been associated with a reduced risk of early relapse post-allo-HSCT (11). On the other hand, allografts predominantly containing naïve NKG2A<sup>+</sup> KIR<sup>-</sup> NK cells were related with lower leukemia relapse rates in HLA-matched allo-HSCT (12). Further studies are required to assess the anti-leukemic potential of adaptive NK cells, as well as to address the role played by activating KIR.
- d) The opportunity of combining NK cell-based ACI with mAbs or immunomodulatory drugs (e.g. tumor antigen-specific mAbs, immune checkpoint inhibitors, proteasome inhibitors) to enhance their response appears quite promising (13). In this regard, humanized mAb aimed to promote NK-cell activation blocking inhibitory receptors (i.e. KIR and NKG2A) have been developed to enlarge the reactive NK-cell pool overcoming the KIR-HLA mismatch requirement. Finally, the possibility of engineering chimeric antigen receptor (CAR) NK cells is currently envisaged.

In summary, a variety of approaches to infuse ex-vivo expanded NK-cell populations activated under different conditions and characterized according to the current knowledge on NK-cell biology are under development. The establishment of consensus optimized protocols and product characterization criteria are essential to formally validate these procedures in clinical practice.

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**Figure 1: Influence of the frequency of alloreactive NK cells on the control of minimal residual disease in AML.** In this schematic example, alloreactive NK cells are depicted as KIR2DL2/L3+ NK cells specific for the HLA-C1 ligand, missing in the AML patient. Anti-tumor responses of KIR2DL1+ and KIR3DL1+ NK-cells are inhibited by their respective ligands (C2 and Bw4) displayed by leukaemic blasts. NK-cell subsets lacking inhibitory KIR with alloreactive potential are colored in purple.