Exploitation of Natural Killer (NK) cells for the treatment of acute leukemia.

Rupert Handgretinger¹, Peter Lang¹ and Maya C. André ¹,².

¹ University Children´s Hospital, Dep. of Pediatric Hematology and Oncology, University of Tuebingen, Germany
² University Children´s Hospital, Dep. of Pediatric Intensive Care, Basel, Switzerland

* To whom correspondence should be addressed

Running Title: NK cell therapy for leukemia

Corresponding author: Rupert Handgretinger, MD, PhD, University Children´s Hospital, Department of Pediatric Hematology and Oncology, Hoppe-Seyler-Str.1, D-72076 Tuebingen, Germany, email: rupert.handgretinger@med.uni-tuebingen.de, phone +49-7071-2980894, fax +49-7071-294713.
ABSTRACT

Natural Killer (NK) cells play an important role in surveillance and elimination of malignant cells. Their spontaneous cytotoxicity was first demonstrated in vitro against leukemia cell lines and NK cells might play a crucial role in the therapy of leukemia. NK cell activity is controlled by an array of germline-encoded activating and inhibitory receptors as well as modulating co-receptors. This biologic feature can be exploited in allogeneic cell therapy, and the recognition of “missing-self” on target cells is crucial for promoting NK cell-mediated Graft-versus-Leukemia (GvL) effects. In this regard, NK cells that express an inhibitory killer immunoglobulin-like receptor (KIR) for which the respective MHC class I ligand is absent on leukemic target cells can exert alloreactivity in vitro and in vivo. Several models regarding potential donor-patient constellations have been described which have demonstrated the clinical benefit of such alloreactivity of the donor-derived NK cell system in patients with adult acute myeloid leukemia (AML) and pediatric acute B cell precursor lymphoblastic leukemia (BCP-ALL) after allogeneic stem cell transplantation. Moreover, adoptive transfer of mature allogeneic NK cells in the non-transplant or transplant setting has been shown to be safe and feasible, whereas its effectivity needs further evaluation. NK cell therapy can be further improved by optimal donor selection based on phenotypic and genotypic properties, by adoptive transfer of NK cells with ex vivo or in vivo cytokine stimulation, by the use of antibodies to induce antibody-dependent cellular cytotoxicity (ADCC) or to block inhibitory KIRs or by transduction of chimeric antigen receptors (CARs).
Introduction

Natural killer (NK) cells belong to innate lymphoid immune cells that contribute to anti-tumor responses without prior sensitization. According to the currently applied innate lymphoid cell (ILC) nomenclature, NK cells are a prototypical member of the group 1 ILCs in so far as they produce IFN-γ and developmentally require the common cytokine receptor gamma chain (γc), the transcriptional repressor inhibitor of DNA binding 2 (ID2) and T-bet \(^1\). During maturation, NK cells sequentially acquire lineage-specific markers such as CD94, Nkp46, CD56 and CD16. Based on the surface density of CD56, the primarily cytokine-producing CD56\(^{\text{bright}}\)CD16\(^{-}\) NK cell subset is distinguishable from the predominantly cytotoxic CD56\(^{\text{dim}}\)CD16\(^{+}\) subset. While CD56\(^{\text{bright}}\)CD16\(^{-}\) NK cells constitute a minor fraction of NK cells in the peripheral blood (around 10%), they are enriched in the secondary lymphoid organs and presumably differentiate here to CD56\(^{\text{dim}}\)CD16\(^{+}\) NK cells. During this maturational process they up-regulate natural cytotoxicity receptors (NCRs), express perforin, proliferate and acquire cytolytic activity upon stimulation with the T cell-derived cytokine Interleukin 2 (IL-2). In contrast, the majority (approximately 90%) of the peripheral blood NK cell compartment consists of CD56\(^{\text{dim}}\)CD16\(^{+}\) NK cells which display markers of late maturity such as inhibitory killer immunoglobulin-like receptors (iKIRs) and CD57, vividly produce perforin to exert effector functions but display little proliferative capacity upon IL-2 stimulation \(^2\).

NK cells use different innate receptors to sense their environment and respond to alterations induced by infections or by malignant cell transformation. In a process termed 'licensing', NK cells use iKIRs for self-MHC class molecules to maintain a state of responsiveness and to kill target cells which have lost MHC class I \(^3\). In a balanced state, healthy tissue that expresses the “self” ligands to inhibitory NK cell receptors, i.e. certain MHC class I molecules, will be spared from being killed. In contrast, the recognition of missing or down-regulated “self”-MHC class I molecules on tumor cells by licensed NK cells shifts the NK cell receptor balance towards activation. Altogether, the modulation of NK cell activity is controlled by an array of germline-encoded activating and inhibitory receptors as well as modulating coreceptors which are summarized in Figure 1 and reviewed by \(^4\).
The biological basis for NK cell alloreactivity

Since attempts to exploit autologous NK cells either by *in vivo* stimulation with IL-2 or the infusion of *ex vivo* stimulated autologous NK cells showed limited clinical efficacy, the focus of research was directed towards the use of NK cells from healthy related or unrelated donors in the context of allogeneic cell therapy.

In this context, donor NK cells that express an inhibitory KIR for which the respective ligand is absent on the recipient's leukemic cells and that concomitantly lack the expression of CD94/NKG2A - as HLA-E molecules are present on all cells and will as a result inhibit NK cell functionality - are defined as being alloreactive. KIRs are genetically determined and highly polymorphic receptors that recognize allotypic variants of MHC class I alleles (KIR Ligands, KIRL). Molecular cloning has revealed that distinct groups of family members comprise two or three extracellular immunoglobulin-like domains and hence are designated KIR2D and KIR3D, respectively. KIRs with inhibitory activity are long-tailed in their intracytoplasmic region as indicated by the letter “L” (i.e. 2DL1) whereas their activating KIR counterparts are short-tailed as indicated by the letter “S” (i.e. 2DS1). KIR2D family members recognize HLA-C alleles with Lys (C2 epitope) or Asn (C1 epitope) residues, whereas KIR3D family members recognize HLA-B alleles with a Bw4 supertypic specificity. Based on the analysis of the 17 KIR genes and pseudogenes, two donor haplotypes are distinguished: KIR A haplotype donors express a canonical gene content including 6 inhibitory KIRs (2DL1, 3, 4, 3DL1, 2 and 3) and the activating KIR2DS4, whereas KIR B haplotype donors have a variable gene content and express in addition to the above mentioned inhibitory KIRs one or more of the B-specific genes: KIR2DS1, 2, 3, 5, KIR2DL2 and/or KIR2DL5. The considerable differences that exist in KIR gene content and copy number together with the extensive allelic polymorphisms account for the high variability that exist in the genomic KIR region of two different individuals. As HLA and KIR segregate independently on different chromosomes and as the expression of KIR genes follows a random stochastic distribution,
the repertoire of NK cells with diverging combinations of KIRs is large and only a minority of HLA-matched donors will also be KIR-KIRL matched.

**Models to define NK cell alloreactivity in the context of allogeneic cell therapy**

The beneficial effect of a KIR-KIRL mismatched donor-recipient constellation in leukemia has been initially described by the Perugia group. In pioneering studies they provided evidence in adult AML but not in adult BCP-ALL that transplantation of grafts from KIR-KIRL mismatched donors enhanced survival rates. This GvL effect was attributed to NK cell alloreactivity and predicted by analysis of the HLA types of donor and recipient. Since then, a number of clinical studies confirmed these observations, while other studies were not able to demonstrate any benefit from selecting a KIR-KIRL mismatched donor when transplanting AML patients. To explain these potential differences and to dichotomize the relative risk of relapse as a result of a given KIR-KIRL constellation, various models have been proposed: the "Ligand-ligand" model suggested by the Perugia group predicts the relative risk of relapse considering mismatches between donor and recipient’s inhibitory KIR ligands (i.e. HLA disparities in GvHD direction). Others failed to demonstrate improved survival when applying this model and performing haploidentical transplantations with less vigorous modes of T cell-depletion. The "receptor-ligand" model suggested by the Memphis group considers the presence of inhibitory KIRs on donor NK cells together with the absence of corresponding KIR ligands in the recipient's HLA repertoire. This model has so far been the most accurate when performing correlative analyses between the donor KIR phenotype and the risk of relapse in pediatric BCP-ALL patients, in T cell-depleted matched sibling transplantations and in unrelated donor transplantations of adult AML patients. The less commonly applied “Gene-gene model” accounts for mismatches on donor and recipient’s KIR genes and has accurately predicted survival rates in KIR haplotype B donors after non-myeloablative HSCT. The Tübingen group observed that patients homozygous for a HLA-C1 alleles have a poorer outcome than other patients. As a result
of these conceptual differences the term “KIR-KIRL mismatch” is used inconsistently and this inconsistency may in part explain why some of the models are more or less able to predict outcome. In Figure 2, possible constellations of alloreactivity are shown.

Recently emerging evidence indicates that not only the presence of iKIRs but also the presence of the activating KIR2DS1 receptor regulates NK cell cytotoxicity against lymphoblastoid cell lines, adult acute AML and BCP-ALL and pediatric acute BCP-ALL and AML. The inclusion of KIR haplotype variability has added an additional layer of complexity to the question which donor will be the “optimal” donor. Elegant studies in adult AML patients demonstrated that the selection of an HLA-matched unrelated donor who possesses activating (aKIRs) next to iKIRs will confer a significantly higher event-free survival. Depending on their exact position on the KIR locus, distinct centromeric (Cen) and telomeric (Tel) gene-content motifs are described in KIR haplotype A and B donors. In a large cohort study centromeric and telomeric B motifs both contributed to relapse protection but Cen-B homozygosity had the strongest independent protective effect. This was particularly true for recipients with one or two C1-bearing HLA-C allotypes. In an attempt to calculate the relative risk of relapse after transplantation, the so-called KIR B content score can be calculated (http://www.ebi.ac.uk/ipd/kir/donor_b_content.html) by scoring the number of Cen-B and/or Tel-B motifs in each genotype, thus allowing the grouping of donors into “neutral, better and best”. In a large adult cohort study matched unrelated donors with a KIR B content score of 2 or more conferred a distinctly higher event-free survival in patients with AML, but not ALL. A similar result was obtained in children with BCP-ALL after haploidentical T cell-depleted transplantation and not only the presence of KIR haplotype B but more so the selection of donors with a high KIR B content score >2 conferred better protection against relapse. In conclusion, quite a number of association studies suggest that a donor with an inhibitory KIR-KIRL mismatch towards the recipient, who possesses aKIRs next to iKIRs and who ideally expresses not one but multiple aKIRs will be a potentially “optimal” donor that may promote clinically relevant NK cell alloreactivity (Figure 3). However, given the complexity of the transplant procedure and the strong linkage
disequilibrium of selected aKIR genes to other KIR genes, further clinical studies incorporating functional NK cell analysis are needed to conclusively answer the question whether aKIRs directly contribute to NK cell alloreactivity.

**Therapeutic use of allogeneic NK cells**

There are two approaches to exploit the anti-leukemic effect of alloreactive NK cells. One is the adoptive transfer of NK cells from an alloreactive donor with moderate lympho-depleting chemotherapy to induce homeostatic lymphocyte proliferation with transient expansion of the transferred allogeneic NK cells without the establishment of a permanent donor hematopoiesis. The other is the transplantation of hematopoietic stem cells after myeloablative therapy and the permanent establishment of donor hematopoiesis and permanent engraftment of donor NK cells, thus establishing the donor KIR phenotype in the patient. In Figure 4, the two approaches are depicted.

**Adoptive NK cell therapy**

The adoptive transfer of allogeneic NK cells in a non-transplant or transplant setting has been and is currently investigated in clinical trials. In a pioneering clinical study, Miller and coworkers treated extensively lympho-depleted adult AML patients in the non-transplant setting with *ex vivo* expanded haploidentical NK cells and administered IL-2 *in vivo* to promote NK cell functionality 44. Remarkably, NK cells persisted and expanded in that subgroup of patients that had been treated with a high-intensity conditioning regimen and that exhibited high intrinsic IL-15 concentrations. In a pilot study in 10 favourable prognosis pediatric AML patients, Rubnitz and coworkers were able to show that the combination of low-dose immune-suppression with the adoptive transfer of KIR-KIRL mismatched NK cells and *in vivo* IL-2 treatment after standard chemotherapy was safe and feasible with long-term survival of all 10 participants 45. Our group recently demonstrated in 8 children with poor prognosis AML, BCP-ALL and T-ALL that the infusion of *ex vivo* IL-15-stimulated CD3/CD19-
depleted stem cell grafts (containing high numbers of NK cells) was safe and feasible in the haploidentical setting \(^{46}\).

As NK cells in the non-transplant setting are ultimately rejected, other phase I or II trials have used \textit{ex vivo} expanded donor NK cells in patients after more intensive conditioning. Expansion can either be performed using \textit{ex vivo} cytokine stimulation or activation via the mbIL15-41BBL-expressing K562 transfectant \(^{47}\). As sources both peripheral blood mononuclear cells and umbilical cord blood (UCB) cells have been used. Although the adoptive transfer of \textit{ex vivo} expanded NK cells is to date considered being safe, the clinical benefit has still to be shown. Given that NK cells early after transplantation are dysfunctional without the supportive application of IL-2 \(^{48}\), this cytokine was as a result used in combination with adoptive NK cell transfer \(^{44,45,49}\). However, to achieve \textit{in vivo} NK cell expansion with IL-2 as the only cytokine, high doses of IL-2 are necessary that may induce significant toxicity. As low dose IL-2 therapy significantly expands regulatory T cell numbers and as such limits NK cell functionality an alternative treatment strategy with IL-15 has been conceived. Considering that NK cells crucially depend on IL-15 to achieve optimal differentiation, survival and effector function \(^{50}\) the approach to administer IL-15 \textit{in vivo} is particularly interesting for patients in whom NK cell maturation appears to be blocked in an immature state early after transplantation. However, the first-in-human IL-15 study (in malignant melanoma and metastatic renal cell cancer patients) demonstrated significant IL-15 toxicities as a result of elevated pro-inflammatory cytokine levels \(^{51}\) and the optimal doses have yet to be defined. Another cytokine-based strategy improves anti-tumor properties of NK cells using a brief \textit{in vitro} priming with IL-12, IL-15 and IL-18 \(^{52}\). These so-called cytokine-activated memory-like (CIML) NK cells are interesting in so far as they exhibit long-lasting increased anti-tumor properties. In this regard, up-regulated IL-2 receptor (CD25) expression renders CIML-NK cells particularly sensitive to picomolar concentrations of IL-2 and through this promotes enhanced IFN-\(\gamma\) production upon re-stimulation with cytokines or tumor cells \(^{53}\). Interestingly, murine and preclinical \textit{in vitro} studies suggest that NK cells are actively suppressed by regulatory T cells \(^{54}\) and it has been assumed that this suppression also
occurs early post transplantation. Hence, one recent study of the Miller group used adoptive NK cell transfer and \textit{in vivo} IL-2 administration but concomitantly depleted regulatory T cells with a IL-2 diphtheria-toxin fusion protein \textsuperscript{55}.

\textbf{Allogeneic stem cell transplantation and NK cell reconstitution}

To predict whether a theoretically existing KIR-KIRL mismatch will actually translate into NK cell alloreactivity and thus increase GvL effects, it might be useful to integrate the complexity and the kinetics of NK cell reconstitution into the picture. Donor NK cells which reconstitute, develop and mature in a HLA-disparate recipient will be shaped in the predominantly donor-type-like hematopoietic niche \textsuperscript{56} and are thus donor-tolerant and recipient-alloreactive at least in the first months after transplantation \textsuperscript{26,33,40,57}. Keeping the complex process of “licencing” or “education” of NK cells in mind, it is obvious that the density of donor-derived HLA class I molecules is crucial to the question whether emerging NK cells will be alloreactive or not. In line with this it has been postulated that the infusion of “megadoses” of stem cells will enable better acquisition of NK cell maturity than the infusion of usually applied doses \textsuperscript{9}. However, many aspects in the processes that are involved in the maturation and education of NK cell populations after HSCT are at present unclear. It has been shown that NK cells are among the first lymphocytes to reconstitute to desirable cell numbers (of more than 0.1x 10\textsuperscript{9}/L CD56\textsuperscript{*} cells) within the first month after transplantation both in adults \textsuperscript{58,59} and in children \textsuperscript{33,40,60} and that the reconstitution kinetics may inversely correlate to relapse probability \textsuperscript{59,61}. However, the phenotype and cytolytic activity of reconstituting NK cells significantly depends on the pre-conditioning regime, the source of the graft and the mode of transplantation. NK cell reconstitution was shown to be impaired in patients that had been exposed to reduced-intensity (non-myeloablative) conditioning regimens \textsuperscript{62} but was enhanced in unrelated cord blood as compared to bone marrow recipients \textsuperscript{63,64} and in patients that had received selectively CD3\textsuperscript{*} or TcR\textsubscript{αβ} T cell-depleted grafts which genuinely contain high numbers of donor-derived NK cells with a mature phenotypical and functional profile \textsuperscript{33,65}. However,
despite the presence of acceptable numbers of NK cells, a large number of patients will exhibit a skewing of the NK cell repertoire in so far as a phenotypically immature subset with little capacity for IFN-γ production but some cytotoxic function will prevail \(^{66}\). Interestingly enough, individuals with a large pool of NKG2A⁺KIR⁻ NK cells, few cytotoxic CD3⁺CD56\(^{dim}\) NK cells and little expression of inhibitory KIRs and NKp30 are prone to leukemic relapse \(^{67,68}\). This would support the observation that KIR-negative unlicensed NK cells early after transplantation are hyporesponsive (Figure 2d). In line with the definition that NK cell alloreactivity implies the presence of iKIRs with varying HLA-class I specificities and the concomitant lack of CD94/NKG2A, analysis of the size and the characteristics of the iKIR NK cell subset is key to the question in whom clinically relevant GvL effects will occur \(^9,69\). Although alloreactive NK cells have been shown to persist and expand in recipients for up to years \(^{40}\), the size of alloreactive NK cell subsets varies somewhat unpredictably both in donors and patients. Phenotypically, the pool of alloreactive NK cells may in the first place comprise varying proportions KIR2DL1\(^+\), KIR2DL2/3\(^+\), KIR3DL1\(^+\) and/or KIR2DS1\(^+\) subsets. In this regard, KIR2DL1\(^+\) NK cells will be inhibited by HLA-C2 recipient’s cells whereas they will lyse leukemia in C1/C1 recipients. KIR2DL2/3\(^+\) NK cells will recognize HLA-C1 but peculiarly enough also with low affinity HLA-C2 \(^{40}\), thus leukemia of homozygous C2 recipients will only partially be lysed. KIR3DL1\(^+\) NK cells have HLA-A and B determined Bw4 supertypic specificity and will thus lyse Bw4⁺ leukemia. And lastly, KIR2DS1\(^+\) NK cells will kill leukemia in homozygous HLA-C2 recipients, however, only if the donor is HLA-C1 homozygous. Here, the activating signal of KIR2DS1 will overcome the inhibition of KIR2DL2/3 whereas in HLA-C2 donors KIR2DS1 will dampen NK cell functionality to prevent auto-reactivity \(^{70}\). A summary of the KIR-KIRL combinations that effectively contribute to NK cell alloreactivity is shown in Table 1.

In our observation, a substantial number of potentially alloreactive donor-derived NK cells expressing only a single iKIR were detectable after HLA-mismatched HSCT. Interestingly, KIR2DL2/3 was predominantly expressed irrespective of the patients’ HLA type. This may explain why patients homozygous for the C1 group (C1/C1) and therefore expressing more
inhibitory ligands for KIR2DL2/3 had a poorer survival than patients with a C1/C2 or C2/C2 HLA type 33.

Next to these well-defined phenotypical properties of human NK cells with inherent potent GvL functionality, additional adaptive NK cell responses have been postulated when a link between post-transplant cytomegalovirus (CMV)-infection and relapse protection was documented in AML patients 71,72. It was shown that CMV infection stably imprints the NK cell repertoire 73, thus promoting the clonal expansion of an NKG2C+aKIR+ NK cell compartment 57,74,75. This potentially alloreactive NK cell subset is CD56 dim, simultaneously expresses CD57+ and secretes IFN-γ in response to K562 stimulation 76. At the molecular level the methylation-induced silencing of FcεRγ, SYK, and EAT-2 DNA promoter regions triggers this adaptive CMV-induced conversion of NK cells 76. Functionally, CD56 dim FcεRγ- NK cells are prone to secrete cytokines and to exert antibody-dependent cellular cytotoxicity which is not restricted to CMV-infected target cells 76-78. It has been speculated that either MHC-class I like molecules such as HLA-E whose expression is retained on leukemic cells 67 trigger NK cell activity directly via NKG2C 78 or that virally-encoded or so far unknown non-HLA ligands trigger other activating NK cell receptors 71, such as for example aKIRs 74. Although the precise mechanisms of adaptive NK cell conversion remain elusive, a simplified view could be that in the context of viral infection a pro-inflammatory stimulus disrupts the tumor-induced immunosuppression and promotes NK cell alloreactivity towards leukemia.

Recognition of acute leukemia by alloreactive NK cells

Despite many publications that provide evidence for the relevance of NK cell alloreactivity in the elimination of adult AML but not BPC-ALL, less knowledge exists as to the significance of NK cell alloreactivity in the context of pediatric leukemia. In line with the data obtained in adult patients, pediatric AML appears to be a target of alloreactive NK cells 26,45,79,80. In contrast to adult BCB-ALL, pediatric BCP-ALL seems to be susceptible to NK cell-mediated target cell lysis 26,27,89,81. The difference in the susceptibility of adult and childhood BCP-ALL
to NK cell-mediated lysis has in part been ascribed to differing expression of cell adhesion molecules of the β1 (CD29, CD49d) and β2 integrin (leukocyte functional antigen-1) family and the Ig superfamily (ICAM-1, leukocyte functional antigen-3) \(^{22,82}\), that essentially results in a reduced NK cell-target conjugate formation and activation in the case of adult BCP-ALL. However, with respect to KIR-KIRL incompatibility it is probably more important that the surface density of MHC class I ligands is higher in pediatric BCP-ALL as compared to AML \(^{79}\) although it has been demonstrated that the \textit{in vitro} cytolytic activity of NK cells against BCP-ALL in part correlates to the extent of MHC expression \(^{81,83}\). To allow the prediction whether a given leukemia will indeed be the target of alloreactive NK cells, the pre-clinical testing should probably incorporate phenotypical and functional analyses on the clonal NK cell population level \(^{26}\) and functional binding and re-directed cytotoxicity assays in the presence of blocking antibodies to well-defined KIR epitopes \(^{40,79}\), ideally using patient-specific leukemia as target cells. In this regard, the recent generation of antibodies that can discriminate KIR2DS1 from KIR2DL1, KIR3DS1 from KIR3DL1, and KIR2DS2 from KIR2DL3 but not KIR2DL2 has allowed a more precise analysis of potentially alloreactive NK cell subsets that a donor might express towards a given leukemia \(^{84}\).

**Alloreactive NK cells promote GvL effects in the absence of GvHD**

Why do alloreactive NK cells mediate GvL effects but obviously do not promote the induction or even prevent GvHD \(^{12,85}\) in the context of \textit{ex vivo} T cell-depleted HSCT? Experimental evidence obtained in mice demonstrates that alloreactive NK cells may lyse donor-derived antigen-presenting and T cells \(^{12,22}\), may produce TGF-β \(^{85}\) and by this and other means achieve control over the antigen-driven proliferation of CD4\(^+\) \(^{86}\) and CD8\(^+\) T cells \(^{87}\) in the bone marrow compartment. Interestingly, the targeted deletion of recipient DCs or T cells critically depends on a process called trogocytosis which enables the down-stream acquisition of CCR7, a chemokine ligand which is required for the directed lymph node migration of “licensed” NK cells in response to CCL19 und CCL21 \(^{88}\). This event is negatively
regulated by iKIRs and NKG2A but promoted by KIR2DS1. As non-hematopoetic host tissues such as the skin, which is commonly affected from T cell-mediated GvHD, lack the expression of activating NK cell receptor ligands, the skin appears to be comparatively resistant to NK cell-mediated attack. It is currently assumed that in some instances NK cells might contribute to pathology once GvHD is established. In this regard, the mode of transplantation (i.e. extent of T cell depletion) and the mode of NK cell stimulation (i.e. adoptive transfer with IL-15/4-1BBL activated and expanded NK cells) may affect GvHD occurrence.

Overcoming KIR-KIRL-mediated inhibition and increasing the anti-leukemic effects of NK cells

One approach to counteract NK cell suppression induced by the KIR-KIRL interactions is the use of IPH2101, the first-in-class, non-depleting IgG4 monoclonal antibody directed against KIR2DL1 and KIR2DL2/3. Indeed, IPH2101 therapy has been effectively able to restore NK cell alloreactivity in adult AML patients, and preliminary in vitro experiments also suggest effectiveness towards pediatric BCP-ALL specimens. An additional approach to activate alloreactive but also non-alloreactive NK cells is the use of monoclonal antibodies directed against antigens expressed on leukemic blasts. It has been shown that the activation of NK cells via the Fc-receptor CD16, which induces antibody-dependent cellular cytotoxicity (ADCC), provides a strong activatory signal and overrides the inhibitory effects of the KIR-KIRL interaction. Since the CD19 antigen is widely expressed on BCP-ALL, an Fc-optimized anti-CD19 antibody was constructed and used post-transplant in selected high-risk patients with pediatric BCP-ALL. Other approaches to activate NK cells against BCP-ALL or AML either in the autologous or allogeneic setting independent of their alloreactive status might be the future clinical use of CD16xCD19 or CD16xCD33 bispecific killer engager (BiKE) or the trispecific killer engager (TRiKE) CD16xCD19xCD22. NK cells and also established NK cell lines such as NK-92 can also be redirected towards target antigens.
expressed on leukemic blasts by chimeric antigen receptors (CARs)\textsuperscript{98}. It has been shown that CAR-NK cells can overcome inhibitory signals and can induce specific killing of leukemic cells\textsuperscript{99}. While the use of CAR-T cells has been restricted so far to autologous T cells and while CAR-T cells might persist very long resulting in on-target-off tumor effects, CAR-NK cells could be adoptively and transiently transferred from allogeneic donors or even off-the-shelf\textsuperscript{100}. In Figure 5, the various approaches to overcome the KIR-KIRL-mediated inhibition and to increase the cytotoxicity towards leukemic cells in the autologous or allogeneic setting are shown.

**PERSPECTIVE**

Substantial evidence exists that NK cell alloreactivity can be exploited in adult and pediatric patients with AML but probably also in children with BCP-ALL. However, the selection of a donor with a KIR-KIRL mismatch towards a given patient does not \textit{per se} preclude the subsequent emergence of alloreactive NK cells with high GvL potential. In this regard, additionally expressed aKIRs, the mode of pre-conditioning, varying kinetics of NK cell reconstitution, prevailing cytokine levels and presumably still undefined factors all contribute to NK cell alloreactivity. Therapeutic heightening of NK cell-mediated GvL effects can probably only be achieved with a multi-tiered approach that combines the optimal recognition of leukemia by KIR-KIRL mismatched NK cells, supportive cytokine or monoclonal antibody administration and abrogation of NK cell suppressive effects such as inhibitory KIR- or regulatory T cell-signalling or TGF-β promoted cytokine-signalling. A number of questions remain as to what the absolute number of NK cells is that is needed to achieve a clinical GvL response, how the different and sequentially appearing activating signals are integrated in the NK cell response to leukemia, what the direct functional evidence is for a role of multiple aKIRs in target cell recognition, and whether NK cells may adapt to leukemia in the sense of acquiring memory-like characteristics.
Acknowledgement

This work was supported by grants from the Deutsche Forschungsgemeinschaft (KFO 183 and SFB 685) to MCA, PL and RH, the “Else Kröner-Fresenius-Stiftung” (2012_A296) to MCA and RH, the Reinhold-Beitlich-Stiftung to PL and by grants from the “Stiftung für krebskranke Kinder Tübingen e.V.”, the Jose-Carreras Leukemia Foundation and the “Stefan-Morsch-Stiftung” to RH. We thank Peter-Michael Weber for the graphic art.

Authorship: MCA and RH wrote the manuscript, PL contributed data and critically reviewed and edited the manuscript.

Conflict of interest disclosure: The authors declare no conflict of interest.
Reference List


23. Bishara A, De SD, Witt CC et al. The beneficial role of inhibitory KIR genes of HLA class I NK epitopes in haploidically mismatched stem cell allografts may be masked by residual donor-alloreactive T cells causing GVHD. *Tissue Antigens.* 2004;63(3):204-211.


75. Cichocki F, Cooley S, Davis Z et al. CD56CD57NKG2C NK cell expansion is associated with reduced leukemia relapse after reduced intensity HCT. *Leukemia.* Prepublished on September 29, 2015, as DOI 10.1038/leu-2015-260.


Legends to Figures:

Figure 1: Overview over important activatory, inhibitory and co-stimulatory NK cell receptors. NK cells integrate various signals in response to leukemic cells. The precise function of some of the receptors is not well known yet and some activatory receptors might rather be co-stimulatory and vice versa. Please note that recent evidence suggests that NKG2D and NKp46 are co-stimulatory receptors as they are not able to induce NK cell activity on their own\(^4\). In addition, KIR2DL4 is to date considered to convey rather activatory than inhibitory signals\(^4\). Many other receptors including cytokine, chemotactic and adhesion molecule receptors as well as other co-stimulatory receptors are not shown. Ligands are shown in parentheses. CD16: FcRIII receptor, ICAM-1: Intercellular Adhesion Molecule-1, NTB: Natural Killer-T-and B-cell antigen, AICL: Activation-induced C-type lectin, MICA/B: MHC class I-related chain A/B, ULBP: UL-16-binding protein, BAT-3: HLA-B associated transcript-3, PCNA: proliferating cell nuclear antigen, LFA-1: leukocyte functional antigen-1.
**Figure 2: Possible constellations of NK cell alloreactivity are depicted.** In (a), licensed donor NK cells (i.e. NK cells that have inhibitory KIRs (iKIR) for self-HLA class I) are inhibited via engagement of the iKIR by the recipients ligands (HLA class I), which exerts a strong inhibitory signal and thus these donor NK cells cannot lyse recipients' leukemic blasts (NK cell non-alloreactivity). In (b), the iKIRs of licensed NK cells are not engaged by the KIR ligands (KIRL) and the donor NK cells are activated and lyse recipients' leukemic blasts (NK cell alloreactivity). In (c), the recipients' blasts lack HLA class I expression and therefore cannot inhibit donor NK cells, resulting in activation of donor NK cells. In (d), NK cells do not express KIRs and have therefore not been licensed. KIR-negative NK cells are one of the earliest lymphocyte populations which reconstitute after T cell-depleted hematopoietic stem cell transplantation. These NK cells are hyporesponsive but might become responsive upon cytokine stimulation.

**Figure 3: Defining the 'optimal' donor.** Based on the KIR haplotype A with a fixed gene content of 6 inhibitory receptors (iKIRs) and one activatory receptor (aKIR) and haplotype B with a highly variable gene content and up to 5 aKIRs, donors can be assigned to either KIR genotype A/A (i.e homozygous for A haplotypes) or B/x (having one or two B haplotypes). The centromeric and telomeric regions can reassemble to form recombinant haplotypes. Genetic association studies suggest that an 'optimal' alloreactive donor will be one with an inhibitory KIR-KIRL mismatch and who possesses next to iKIRs multiple aKIRs (haplotype B). The KIR B content score mainly reflects the number of aKIRs and the highest score is associated with the highest number of aKIRs. NK cells from such donors with a high KIR B content score should in principle exert a strong alloreactive anti-leukemic NK cell activity and it has been shown that transplantation with donors homozygous for centromeric KIR B haplotypes is associated with the lowest level of relapse and highest overall survival. The KIR B content score can easily be determined by KIR genotyping. Note that the majority of haplotype B donors do not express all genes shown in the figure.
**Figure 4:** The two approaches for exploiting alloreactive NK cells are shown. NK cells from a KIR-KIRL-mismatched alloreactive donor or from umbilical cord blood (UCB) can be transferred directly or after ex vivo expansion following mild chemotherapy with transient NK cell proliferation. This will only induce a short-living anti-leukemic effect. On the other hand, a patient can be permanently engrafted with hematopoietic stem cells from an alloreactive donor or UCB after myeloablative therapy, thus establishing a permanent donor KIR phenotype and anti-leukemic effect.

**Figure 5:** Strategies to overcome the KIR-KIRL-mediated inhibition of NK cells and to increase their anti-leukemic response. In (A), the KIR-KIRL interaction is blocked by a monoclonal antibody, which abolishes the inhibitory signal. In (B), the KIR-KIRL-mediated inhibition is overridden by the activation of the Fc-receptor (CD16) with an antibody directed against an antigen expressed on leukemic blasts. In (C) and (D), a bispecific killer-engager (BiKE) and trispecific killer engager (TRiKE) activate NK cells via the Fc-receptor against antigens expressed on leukemia cells. In (E), chimeric antigen receptor (CAR)-NK cells directed against the CD19 antigen are depicted.
Table 1. KIR-KIRL combinations that effectively contribute to NK cell alloreactivity.

<table>
<thead>
<tr>
<th>KIR-KIRL interaction</th>
<th>Functional consequences</th>
<th>NK cell alloreactivity towards</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIR2DL1-HLA-C2</td>
<td>Inhibition in HLA-C2⁺ patients</td>
<td>Lysis in HLA-C1⁺ patients</td>
</tr>
<tr>
<td>KIR2DL2/3-HLA-C1</td>
<td>Inhibition in HLA-C1⁺ patients</td>
<td>Partial lysis in HLA-C2⁺ patients</td>
</tr>
<tr>
<td>KIR3DL1-HLA-Bw4</td>
<td>Inhibition in patients with Bw4⁺ supertypic specificity</td>
<td>Lysis in Bw4⁻ patients</td>
</tr>
<tr>
<td>KIR2DS1-HLA-C2</td>
<td>Activation in HLA-C2⁺ patients</td>
<td>Lysis in HLA-C2⁺ patients (only when donor is HLA-C1/C1)</td>
</tr>
</tbody>
</table>
Figure 1

Activatory Receptors

- KIR 2DS1/2 (HLA-C, group 2/1)
- KIR 2DS3/5, KIR3DS1 (unknown)
- KIR 2DS4 (some HLA-A-, C alleles)
- KIR2DL4 (HLA-G)
- CD94/NKG2C (HLA-E)
- LFA-1 (ICAM-1)
- NKp30 (BAT-3)
- NKp44 (PCNA)
- NTB-A (NTB-A)
- CD2 (CD54)
- 2B4 (CD48)
- NKP80 (AICL1)

Inhibitory Receptors

- CD16 (FcR)
- KIR 2DL1 (HLA-C, group 2)
- KIR 2DL2/3 (HLA-C, group 1)
- KIR 2DL5 (unknown)
- KIR3DL1 (HLA-Bw4)
- KIR 3DL2 (HLA-A3, A11)
- CD94/NKG2A (HLA-E)
- NKG2D (MICA/B, ULBPs)
- NKp46 (viral haemagglutinins)
- CD226 (CD155, CD112)

Co-stimulatory receptors
Figure 2

(a) licensed donor NK cells

(b) no lysis

Activating Receptors
Activating ligands

(c) lysis

(d) unlicensed donor NK cells

HLA

lysis?
hyporesponsive?
### Figure 3

<table>
<thead>
<tr>
<th>Haplotype A</th>
<th>Haplotype B</th>
<th>KIR Genotype</th>
<th>Haplotype Centromeric</th>
<th>Haplotype Telomeric</th>
<th>KIR B Content Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>3DL2</td>
<td>3DL2</td>
<td>B/B</td>
<td>B/B</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>2DS4</td>
<td></td>
<td>B/B</td>
<td>A/B</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>2DS1</td>
<td></td>
<td>A/B</td>
<td>B/B</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>2DS5</td>
<td></td>
<td>A/A</td>
<td>A/A</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2DL5A</td>
<td></td>
<td>A/A</td>
<td>A/B</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

- **Inhibitory receptor**: Red
- **Activatory receptor**: Cyan
- **Framework gene**: Black

**NK** cell interactions:
- **KIR** and **HLA**
  - **Lysis**
  - **Leukemia**
Figure 4

KIR-KIRL-mismatched Donor

- Haploidentical or UCB donor
  - Non-mobilized peripheral blood cells
  - Ex vivo expansion of NK cells
- Haploidentical donor
  - Transient engraftment of alloreactive NK cells
  - Adoptive transfer of enriched NK cells or CD3- or TcRaβ-depleted cells
- Mobilized peripheral blood stem cells or bone marrow
  - Matched unrelated or UCB donor

For personal use only. on October 24, 2017. by guest
Exploitation of Natural Killer (NK) cells for the treatment of acute leukemia

Rupert Handgretinger, Peter Lang and Maya C. André