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Determinants of Antileukemia Effects of Allogeneic NK Cells

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In HLA-nonidentical bone marrow transplantation, we studied the characteristics of donor NK cells, recipient leukemia cells, and the cytokine environment that predict the antileukemia effects of allogeneic NK cells. We found that the risk of relapse in pediatric patients with hematologic malignancies was best predicted by a model taking into consideration the presence of inhibitory killer cell Ig-like receptors (KIRs) on the donor’s NK cells and the absence of corresponding KIR ligand in the recipient’s HLA repertoire (a receptor-ligand model). The risk of relapse was prognosticated less precisely by the Perugia donor-recipient KIR ligand-ligand mismatch model or by a natural cytotoxicity model. In contrast to the ligand-ligand model, we found that the new receptor-ligand model was accurate when analysis was applied to patients with lymphoid malignancy. These findings corroborate our observations that the recipient’s KIR repertoire, which was derived from highly purified, HLA-disparate CD34⁺ cells, resumed a donor-specific pattern within 3 mo of transplantation, but did not correlate evidently with the donor or recipient ligand repertoire. In an in vitro assay and an in vivo mouse model, human NK cell cytotoxicity toward human leukemia cells with 11q23 chromosomal rearrangement increased with the number of receptor-ligand mismatch pairs or pretreatment with IL-12 and IL-18. These findings provide new insights into the determinants of antileukemia effects of allogeneic NK cells and therapeutic strategies.


Transplantation of allogeneic bone marrow or blood hematopoietic stem cells is an established method for the treatment of high risk hematologic malignancies (1). Less than 30% of patients have a sibling donor who is HLA identical. Transplantation using HLA-nonidentical donors, such as unrelated or mismatched family donors, is associated with increased risk of graft rejection and graft-vs-host (GvH) disease. Despite these disadvantages, transplantation using an HLA-nonidentical donor provides a unique setting in which donor NK cells may contribute to antileukemia effects (2).

Human NK cells are regulated by inhibitory receptors for HLA class I molecules (3). A family of NK receptors, the killer cell Ig-like receptors (KIRs), is specific for epitopes shared by groups of HLA class I molecules (4). To avoid autoimmunity, each NK cell usually expresses at least one inhibitory receptor that recognizes one or another self HLA molecule (4, 5). In the setting of transplantation of HLA-nonidentical cells, GvH reactions may occur when none of the KIRs in a subset of donor NK cells is engaged by the host HLA molecule, because the host’s HLA repertoire lacks the appropriate ligand (6). Similarly, graft rejection is possible on the rare occasion when the host’s NK cells survive the high dose cytoreductive therapy given before transplantation. Analogous studies of NK cell-mediated GvH reactions have demonstrated a predominant lysis of host lymphohematopoietic cells in the absence of systemic GvH reactions to other nonhemopoietic tissues (7), suggesting that clinical graft-vs-leukemia response may occur in the absence of GvH disease (8). These studies (6, 7) led to a clinical practice of selecting a ligand-ligand incompatible donor with an HLA mismatch that favors NK cell-mediated GvH reactions (2, 8). The rationale for this clinical practice, however, has been challenged by other investigators (9) because our understanding of the biological determinants of antileukemia effects of NK cells remains incomplete. In this study we conducted clinical and laboratory investigations in parallel to obtain additional fundamental insights into the antileukemia response of allogeneic NK cells.

Materials and Methods

Clinical transplantation

Routine procedures for the selection of donor, mobilization of stem cells, conditioning, and supportive care have been described previously (10). These patients (0.3–17.9 years of age) received an experimental haploidentical transplant because their estimated disease-free survival was ≤25% with chemotherapy, and they did not have an HLA-matched related or unrelated donor. The transplantations were performed in Memphis (n = 22) or Tuebingen (n = 14) for myeloid (n = 17) or lymphoid (n = 19) malignancy. All grafts were purified for CD34⁺ cells using CliniMACS systems (Miltenyi Biotec, Auburn, CA). Because all the grafts contained <3 × 10⁶ CD34⁺ cells/kg, GvH disease prophylaxis was not given to any patient after transplantation. Monitoring of the KIR repertoire and the natural cytotoxicity of NK cells was performed on blood samples obtained from patients treated in Memphis at 1, 3, 6, 9, and 12 mo after transplantation.

Statistics

Three models were used to dichotomize the risk of primary disease relapse after transplantation into high and low risk categories. A patient was categorized as being at low risk 1) by the ligand-ligand model if at least one group of donor KIR ligand was missing in the recipient’s ligand repertoire, a model proposed by Ruggeri et al. (6) of Perugia University (6, 7); 2) by the receptor-ligand model if at least one KIR gene expressed in the donor’s NK cell repertoire did not recognize any of the HLA molecules in the recipient’s ligand repertoire, a model defined a priori based on the known
HLA-C allotypes with asparagine at position 80 (HLA-C Asn80) often hamper the development of novel therapeutic strategies (27). We investigated the determinants of NK cell cytotoxicity toward these leukemia cells to develop a new treatment approach.

The cytotoxicity of NK cells against the MLL cell lines was also assessed in vivo using an nonobese diabetic/SCID mouse model. Control mice received only leukemia cells (20 × 10⁶) via tail vein injections; treated mice received leukemia cells and NK cells in a 1:1 cell ratio (20 × 10⁶ each). When leukemia cell burden was the outcome of interest, an NK cell-treated mouse was sacrificed on the same day that a control mouse was moribund with signs of leukemia (or vice versa if an NK-treated mouse was sick first). The numbers of cells in the spleen and bone marrow of the two femurs were counted (Z1 cell counter; Coulter Diagnostics, Miami, FL), and the percentages of leukemia cells were determined by flow cytometry using mAbs specific for the leukemia cells that had been injected into the mouse. When survival was the outcome of interest, each mouse was sacrificed on the day when it became moribund, and the cells from their marrow and spleen were examined by flow cytometry to confirm the cause of illness was leukemia.

Results

Risk of leukemia relapse is best predicted by the receptor-ligand model

We followed prospectively the outcomes of 36 consecutive pediatric patients with high risk hematologic malignancy who received an HLA-haploidentical peripheral blood stem cell transplant from a family member. We used three models to dichotomize a priori the risk of primary disease relapse for each patient into high and low risk categories. HLA testing revealed that 28 of the 36 donor/recipient pairs were KIR ligand-ligand compatible and were therefore classified according to the ligand-ligand model as being at high risk for relapse. Analysis of the KIR expression on the donor’s NK cells and HLA testing of the recipient demonstrated that 13 recipients had appropriate KIR ligands for all KIRs on their NK cells. This group of patients were classed according to the ligand-ligand model as being at high risk for relapse.
Relationship between donor KIR repertoire and self HLA

A concurrent laboratory investigation was performed to evaluate the ligand-ligand model and receptor-ligand model by studying the KIR repertoire of 50 normal donors to determine the relationship between KIR repertoire and self HLA. CD158a, CD158b1/b2, or CD158e1 was found in the repertoire of many donors (24–36%) with no corresponding ligand, whereas the HLA-B<sup>Bw4</sup>, HLA-C<sup>dr<sub>8</sub></sup>, or HLA-C<sup>A*80</sup> allotype was present in a few donors (0–8%) with no corresponding KIR (Fig. 4). The former observation provided an explanation for why the KIR ligand-ligand model might misclassify many ligand-ligand-compatible patients who were not at high risk of relapse, and the latter observation provided an explanation for why the ligand-ligand model was quite accurate for the ligand-ligand-incompatible patients in its low risk category. Misclassification may involve either CD158a, CD158b, or CD158e1.

KIR expression is donor specific, but not related to the donor or recipient HLA

To further investigate whether the donor or recipient HLA affects the level or frequency of expression of each KIR on NK cells, we assessed KIR expression on the donor’s NK cells before and after transplantation. Among the 50 potential donors, we observed no significant relationship between KIR expression and self HLA allelic groups. The presence or the absence of a group of ligands did not predictably alter the mean fluorescent intensity (MFI) or the frequency of expression of its corresponding KIR on the NK cells. The expression of one KIR also did not affect the MFI or frequency of expression of another KIR on the NK cells (Fig. 5). Subsets of NK cells in some of our donors did not express any inhibitory KIRs for any self classical-HLA molecules.

KIR expression was monitored in the developing NK cells of our patients after they received a highly purified CD34<sup>+</sup> cell graft from an HLA-haploidentical donor. This was a unique setting for the biological study of KIR development in an HLA-disparate recipient, because the stem cell graft contained <0.01% contamination of mature CD56<sup>+</sup> or CD3<sup>+</sup> cells, and the patients did not receive any immunosuppressants for GvHD prophylaxis and did not develop GvHD. Within 3 mo of transplantation, the KIR expression pattern of all 22 study patients resumed a donor-specific pattern recognized in the donor NK cells before transplantation (Fig. 6). Thereafter, KIR expression was stable in all samples examined for up to 12 mo and did not appear to be affected by the recipient’s HLA groups (data not shown).

Sensitivity of leukemia cells depends on their KIR ligand repertoire

To further characterize the receptor-ligand model, we investigated whether a larger number of receptor-ligand mismatch pairs would...
result in greater antileukemia effects in vitro and in vivo in a mouse model. NK cells from donors who expressed all the inhibitory KIRs (CD158a, CD158b1/b2, and CD158e1) were used. In the in vitro NK cell cytotoxicity assay (Fig. 7a), the sensitivity of MLL-1 leukemia cells expressing the HLA-C^{Asn80} ligand only (with two receptor-ligand mismatch pairs) was similar to that of LCL cells transfected with Cw03 (HLA-C^{Asn80}) and was lower than that of K562 cells that expressed a very low level of HLA class I molecules (equivalent to three receptor-ligand mismatch pairs in this assay). In MLL-2 cells the expression of HLA-CLys80 in addition to HLA-C^{Asn80} resulted in only one receptor-ligand mismatch pair and provided additional protection against NK cell cytotoxicity, lysis that was less than that of LCL cells transfected with either Cw03 (HLA-C^{Asn80}) or Cw04 (HLA-C^{Lys80}) alone. The MLL-3 cells that expressed all three KIR ligands (HLA-C^{Asn80}, HLA-C^{Lys80}, and HLA-B^{Bw4}) with no receptor-ligand mismatch pairs were most resistant to NK cell-mediated lysis (MLL-1 vs MLL-2, \( p = 0.004 \); MLL-2 vs MLL-3, \( p = 0.05 \)). The susceptibility of these cell lines to NK cell cytotoxicity was independent of their expression of adhesion molecules, including members of the \( \beta_1 \) integrin family (CD49d), \( \beta_2 \) integrin family (CD11a,b,c), L-selectin family (CD62L), and Ig superfamily (CD54, CD58; data not shown).

Using an in vivo mouse system (Fig. 7b), leukemia burden in bone marrow and spleen of mice transplanted with NK cells and MLL cells (in a 1:1 cell ratio) was lowest in mice injected with MLL-1 cells, higher in those injected with MLL-2 cells, and highest in those injected with MLL-3 cells. Subsequent experiments demonstrated prolongation of survival (median, 7 days) in the mice injected with NK cells and MLL-1 cells (1:1 cell ratio), but not in those injected with NK cells and MLL-3 cells (Fig. 7c).

**Resistance of leukemia cells that express all KIR-ligands may be overcome by cytokines**

Leukemias such as MLL-3 cells that express all groups of ligands will probably not be cured with unmanipulated NK cell therapy, because they are inherently resistant to NK cell cytotoxicity regardless of the donor KIR repertoire. We investigated whether this inherent resistance could be overcome by prestimulating the NK cells with IL-12 and IL18 and whether these cytokines changed the expression of KIR. These two NK-stimulatory cytokines were selected for study, because they might paradoxically reduce GvHD and therefore be useful in the clinic (28, 29). We found that these two cytokines or high dose IL-12 alone overcame the inherent resistance of MLL-3 cells to NK cell-mediated lysis (Fig. 8), but did not change the expression of inhibitory or activating KIRs, CD94, and CD161 (data not shown).

**Discussion**

The missing self hypothesis of the antilymphoma effects of NK cells was proposed by Karre et al. (30) over a decade ago. Subsequent murine and clinical studies of adult leukemias conducted...
by Ruggeri and Velardi et al. (6, 7) led to the novel concept of perfect mismatch to deliberately search for a donor with an HLA mismatch that will induce favorable NK cell-mediated antileukemic effects (2). In this study we showed that 1) the accuracy of the prediction of relapse could be improved by directly evaluating the KIR repertoire of the donor; 2) the pattern of KIR expression was donor-specific, even after stem cell transplantation into an HLA-disparate recipient; 3) the potency of the antileukemia effects increased with an increasing number of receptor-ligand mismatch pairs; and 4) the resistance of leukemia cells to lysis in the setting of no receptor-ligand mismatch could be overcome by prestimulating the NK cells with IL-12 and IL-18.

In agreement with the findings of Velardi et al. (6, 7), we found that the KIR ligand-ligand prediction model was quite accurate for the patients in its low risk category, an accuracy comparable to that of the receptor-ligand model (Fig. 4). Moreover, we observed that the NK cells derived from highly purified CD34⁺ cells (<0.01% contamination of CD56⁻ or CD3⁺ cells) always acquired a donor-specific pattern of KIR expression independent of self HLA within the first 3 mo of transplantation. Thus, during this period of receptor acquisition, subsets of NK cells may express only one of the KIRs and could, therefore, be alloreactive if the corresponding ligand was absent in the recipient’s cells. This response appears to occur only during the first few months after transplantation; alloreactive clones could not be generated beyond 3 mo after transplantation even when their generation was possible earlier (6). This latter finding may explain why autoimmunity is not found in our adult donors with a specific KIR but no corresponding ligand. At present, the molecular mechanism is still poorly understood for the apparent presence of a potent antileukemia effect immediately after transplantation but the lack of a long term, NK cell-mediated autoimmunity in our patients.
with receptor-ligand mismatch pairs, although a dominant activity of ligand-negative cells in inducing tolerance has been demonstrated by neighboring hemopoietic cells and nonhemopoietic stromal cells during NK cell development in mice (31–33). Nonetheless, our finding that the KIR expression on NK cells derived from donor’s CD34+ cells always adopts a donor-specific pattern before the end of the 3-mo window suggests that a perfect mismatch donor could be selected on the basis of a single evaluation of the donor’s KIR repertoire before transplantation.

CD158k was not considered a priori in both the ligand-ligand model and the receptor-ligand model, because its specificity is uncertain. First, NK cells expressing CD158k were inhibited by both HLA-A3 and A11 in one study (34), but not by HLA-A11 in another study (35). Second, if HLA-A3 is the only ligand for the ubiquitously expressed CD158k, one would predict an unrealistically low relapse rate (~10%), because the overall frequency of HLA-A3 is only ~10% in the general population (36). In fact, both the ligand-ligand model and the receptor-ligand model were found to be less accurate if the CD158k and its ligand (HLA-A3 with or without A11) were included in our prediction models (data not shown). Taken together, these findings suggest that CD158k may have additional ligands not yet identified. When the complete set of ligands is known, incorporation of CD158k-ligand matching may perhaps be useful for donor selection.

Prior investigations in adult patients have demonstrated the NK cell-mediated antileukemia effect toward AML, but not ALL (6, 7). In this pediatric study we confirmed the effect on myeloid leukemia, but also extended the observation to lymphoid malignancy (Fig. 3). Unlike adult ALL that frequently do not express adhesion molecules that are essential for NK target conjugation and activation (6), blasts from all cases of childhood ALL have been consistently found to express at a high rate adhesion molecules such as members of the β₂ integrin family (CD29, CD49d), the β₂ integrin family (LFA-1), and the Ig superfamily (ICAM-1, LFA-3) (37).

The KIR repertoire in a normal individual who did not receive transplantation had been shown to be primarily genetically regulated and not linked to self HLA (20, 38, 39). In 18 patients who had received routine immunosuppression for GvHD prophylaxis after transplantation from an HLA-matched sibling or unrelated donor, Shilling et al. (39, 40) reported idiosyncratic patterns of KIR reconstitution associated with clinical acute or chronic GvHD. Our transplantation of highly purified CD34+ cells from an HLA-haploidentical donor allowed for the first time a biological study of KIR expression in NK cells developing in HLA-disparate hosts, without interference by prophylaxis or treatment for GvHD. Similar to (donor) self HLA, we found that the ligand repertoire of the HLA-disparate recipient did not appear to correlate with the expression of KIRs in the NK cells derived from the donor’s stem cells, a finding that is not compatible with the Ly49 receptor calibration model in mice (41). The expression of one KIR also did not appear to affect the frequency or level of expression of another KIR, a finding incompatible with the Ly49 selection or sequential models in mice (31, 42, 43). However, for all 50 donors in this study we always observed subsets of NK cells expressing at least one inhibitory KIR that recognized at least one of the self HLA groups, in agreement with the “at least one” hypothesis (5, 31). As the presence of a KIR ligand almost always correlates with the presence of its corresponding receptor in the NK cell receptor repertoire (Fig. 4), all our clinical donors had at least one inhibitory KIR in their receptor repertoire that recognized one of the HLA groups expressed in the recipient (the haplotype that is shared by the donor and recipient). Although the “at least one” hypothesis applied to our data at the repertoire level, four-color flow cytometric analyses demonstrated that subsets of NK cells in some of our donors did not express any inhibitory KIRs for any self or recipient classical HLA molecules at the individual cell level. A recent study demonstrated that Ly49 receptors in subsets of murine NK cells can inhibit killing both by those cells and, via a cell-cell interaction, by other subsets of NK cells that do not express that inhibitory receptor (44). This finding suggests that “at least one” match at the repertoire level may translate functionally to the cellular level in vivo. In the clinical perspective, one has to be cautious when selecting a donor with complete receptor-ligand mismatch (not “at least one”) in an attempt to maximize antileukemia activity, because the safety of such a transplant is not yet known, although autoimmunity was not observed in mice with complete absence of MHC class I ligands (45–47).

For the patients who have more than one potential donor, we asked whether a larger number of receptor-ligand mismatch pairs increases the antileukemia effects of the donor’s NK cells. We selected three cell lines with MLL rearrangement common in infants that have one, two, or three groups of KIR-ligands and tested their susceptibility to NK cells with all three corresponding KIRs in their repertoire. The results from our in vitro and in vivo assays suggest that the larger the number of receptor-ligand mismatch pairs, the higher the susceptibility of MLL cells to NK cell-mediated lysis. This finding leads to the speculation that a donor with a larger number of mismatch pairs may be a better donor, provided that the NK cells of the donor have at least one KIR in their repertoire that recognizes one of the recipient’s HLA, as discussed above.

Can patients with all three groups of KIR ligands still benefit from NK cell therapy? We found that the MLL-3 cell line, which had all three ligands, was very resistant to NK cell-mediated lysis, and this type of leukemia will probably not be cured with unmaintained NK cell therapy. However, we found that prestimulation of NK cells with IL-12 and IL-18 overcome the leukemia cells’ resistance to lysis. Besides the known effects of IL-12 and IL-18 on cytokine production by NK cells (48), our results demonstrated for the first time that this cytokine-augmented natural cytotoxicity was KIR independent, thus suggesting that patients with all three groups of KIR ligands may still benefit from NK cell therapy. IL-12 and IL-18 are convenient and attractive candidates for ex vivo stimulation of NK cells, because they have been shown to paradoxically reduce T cell-mediated GvH disease in mice (28, 29), although their efficacy and toxicity in human are unknown (49). High dose IL-12 may also be used for the augmentation of NK cell cytotoxicity toward leukemia cells with no receptor-ligand mismatch.

In summary, this work provides additional fundamental insights into the antileukemia response of allogeneic NK cells. These results may be useful for the development of strategies for NK cell therapy in the 70% of patients who do not have an HLA-identical sibling donor.

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