Natural Killer Cell Killing of Acute Myelogenous Leukemia and Acute Lymphoblastic Leukemia Blasts by Killer Cell Immunoglobulin-Like Receptor–Negative Natural Killer Cells after NKG2A and LIR-1 Blockade

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Although the study of natural killer (NK) cell alloreactivity has been dominated by studies of killer cell immunoglobulin-like receptors (KIRs), we hypothesized that NKG2A and LIR-1, present on 53% ± 13% and 36% ± 18% of normal NK cells, respectively, play roles in the NK cell killing of primary leukemia targets. KIR⁻ cells, which compose nearly half of the circulating NK cell population, exhibit tolerance to primary leukemia targets, suggesting signaling through other inhibitory receptors. Both acute myelogenous leukemia and acute lymphoblastic leukemia targets were rendered susceptible to lysis by fresh resting KIR⁻ NK cells when inhibitory receptor–major histocompatibility class I interactions were blocked by pan-HLA antibodies, demonstrating that these cells are functionally competent. Blockade of a single inhibitory receptor resulted in slightly increased killing, whereas combined LIR-1 and NKG2A blockade consistently resulted in increased NK cell cytotoxicity. Dual blockade of NKG2A and LIR-1 led to significant killing of targets by resting KIR⁻ NK cells, demonstrating that this population is not hyporesponsive. Together these results suggest that alloreactivity of a significant fraction of KIR⁻ NK cells is mediated by NKG2A and LIR-1. Thus strategies to interrupt NKG2A and LIR-1 in combination with anti-KIR blockade hold promise for exploiting NK cell therapy in acute leukemias.

INTRODUCTION

Human natural killer (NK) cells express several families of inhibitory NK cell receptors that recognize "self" human leukocyte antigen (HLA) class I ligands. These receptors act through several mechanisms to determine whether or not a target will be susceptible to NK cell–mediated lysis. Recognition of HLA class I by inhibitory receptors leads to self-tolerance by preventing cytolysis of normal cells [1-4]. Although somewhat paradoxical, the same self-receptors that lead to tolerance also play a role in the acquisition of functional competence, a process termed NK cell education or licensing [5-7]. Transient interruption of NK cell inhibitory receptor signaling on educated NK cells may be a therapeutic strategy for augmenting antitumor activity.

Three main inhibitory receptor families recognize major histocompatibility class (MHC) class I molecules: killer cell immunoglobulin-like receptors (KIRs), CD94/NKG2A, and leukocyte immunoglobulin-like receptor 1 (LIR-1). KIRs display specificity for allele-specific variable regions of the alpha chain of classical HLA class I (ie, HLA- A, -B, and -C). CD94/NKG2A receptors recognize mainly nonclassical HLA-E, whereas LIR-1 receptors recognize a broad spectrum of classical HLA-A, -B, and -C and nonclassical HLA-E, -F, and -G by binding to conserved regions of the alpha 3 domain [1,2,8-10]. Although 2 studies investigating the inhibitory potential of LIR-1 on primary peripheral blood (PB) NK cells found that NK cell inhibition is largely attributed to HLA-G recognition [11,12], the functional role of LIR-1 interactions with primary leukemia cells remains poorly defined.

NK cells are the first immune cells to reconstitute after hematopoietic cell transplantation (HCT),

representing the predominant lymphocyte population with the potential to control leukemia relapse in the months preceding T cell reconstitution [13-15]. Although NK cell alloreactivity has been reported for acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) after KIR ligand–mismatched HCT [16-20], not all reports are in agreement with this [21-24], and the mechanism of apparent resistance in some studies is unclear. We hypothesize that NK cell receptors other than KIRs may explain these clinical results.

MATERIALS AND METHODS

Cell Isolation and Cell Culture

All human samples were obtained after informed consent was obtained following guidelines approved by the University of Minnesota's Committee on the Use of Human Subjects in Research and in accordance with the Declaration of Helsinki. Primary cells from 18 patients were collected by leukapheresis (AML, n = 5; pre–B-ALL, n = 3; T-ALL, n = 1) and bone marrow aspiration (AML, n = 5; pre–B-ALL, n = 4). Blasts composed >80% of each sample. After thawing, necrotic blasts were removed by density-gradient centrifugation using Ficoll-Histopaque (Sigma-Aldrich, St Louis, MO) and kept in Ham/F12 (Cellgro, Manassas, VA), basal medium supplemented with 20% human AB serum (Valley Biomedical, Winchester, VA) for 12 hours. NK cells were isolated from PB mononuclear cells (PBMCs) obtained from 42 healthy donors through depletion of other cells by immunomagnetic beads (NK Cell Isolation Kit; Miltenyi Biotech, Auburn, CA). KIR⁺ NK cells were positively separated by staining with phycoerythrin (PE)-conjugated antibodies against CD158a (HP-3E4), CD158b (CH-L), CD158e (DX9) (all from BD Biosciences, San Jose, CA), and CD158i (FES172; Beckman Coulter, Fullerton, CA), and subsequent selection using anti-PE beads (Miltenyi Biotech). KIR⁻ NK cells were isolated by positive depletion of KIR⁺ NK cells. In addition, NK cells from unseparated blood mononuclear cells 100 days after transplantation (autologous, n = 1; umbilical cord blood, n = 2; unrelated adult donor, n = 2) were tested.

KIR Ligand Typing, Reverse-Transcriptase Polymerase Chain Reaction for HLA-G, and Western Blot Analysis

KIR ligand typing was performed by pyrosequencing as described by Yun et al. [25]. HLA-G transcripts were amplified over 35 cycles of reverse-transcriptase polymerase chain reaction (RT-PCR) using Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) with published pan–HLA-G primer sets G.257F and

G.1004R, as described previously [26]. As an internal control, β-actin gene amplification was carried out for each sample. HLA-E and HLA-G expression of primary targets was determined by Western blotting with the anti–HLA-E clone MEM–E/02 and anti–HLA-G clone MEM–G/01 (Abcam, Cambridge, MA), as described previously [27]. The choriocarcinoma cell line JEG-3 was used as a positive control, and the mouse cell line EL08-1D2 was used as a negative control [28].

Flow Cytometry

Immunophenotypic analysis of cells was performed using 4-color analysis on a FACSCalibur analyzer with CELLQuest Pro software (BD Biosciences). Cells were stained with the following monoclonal antibodies (mAbs), as indicated: fluorescein isothiocyanate (FITC)- or PE-conjugated DX9 (anti-CD158e), HP-3E4 (anti-CD158a/h), and CH-L (158b); peridinin chlorophyll A protein (PerCP)-conjugated SK7 (anti-CD3); allophycocyanin (APC)-conjugated NCAM16.2 (anti-CD56) (BD Biosciences); and PE-conjugated Z199 (anti-NKG2A) and HP-F1 (anti–LIR-1) (Beckman Coulter). HLA expression was analyzed by flow cytometry using PE-conjugated pan–anti-HLA class 1 mAbs W6/32 (Abcam, Cambridge, MA) and HP-1F7 (kindly provided by M. Lopez-Botet, Universitat Pompeu Fabra, Barcelona, Spain).

Determination of NK Cell–Mediated Cytotoxicity

Flow cytometric detection of NK cell–mediated cytotoxicity was performed as described previously [29-31]. In brief, tumor target cells were labeled with 3 mMol (dissolved in DMSO) green lipophylic fluorescent dye DIOC18 (3,3'-dioctadecylox-acarbo cyanine perchlorate; Sigma-Aldrich, St Louis, MO). Effector cells and target cells were coincubated at various ratios for 4 hours, after which propidium iodide was added at a final concentration of 5 μg/mL for 5 minutes to determine the proportion of dead cells. The proportion of propidium iodide–positive cells was determined by flow cytometry in the FL3 channel. The HLA class I–deficient cell line K562 was used as a positive control. Primary targets that exhibited >10% specific cytolysis were considered sensitive to killing.

Degranulation Assays

Degranulation was determined as described by Betts et al. [32] and Rubio et al. [33]. Compared with cytotoxicity assays, degranulation assays have the advantage of being applicable to batch analysis of frozen samples and provide additional data on the killing potential of NK cell subsets. In brief, isolated resting or interleukin (IL)-2–activated CD56⁺/CD3⁻ cells were coincubated with target cells at an effector:target (E:T) ratio of 1:2

under conditions identical to the cytotoxicity assays. Anti–CD107a-PE or anti-CD107a,b FITC antibody clone H4A3 and clone H4B4 (BD Biosciences) were added during the entire incubation period. After 1 hour of incubation, monensin (Sigma-Aldrich) was added to a final concentration of 10 ug/mL. After an additional 5 hours, cells were washed and stained with anti-CD56 mAb. CD107 expression on NK cells was analyzed by flow cytometry.

Antibody-Blocking Experiments

KIR blocking was performed with mAb IgG4 clone 1-7F9 (blocking KIR2DL1/2DL2/2DL3), and NKG2A blocking was performed with mAb clone Z270 (both from Novo Nordisk, Copenhagen, Denmark). LIR-1 blocking was performed with mouse mAb IgG2b clone 292319 (R&D Systems). Blocking experiments were performed in medium supplemented with 20% human AB serum. Mouse mAb IgG1 clone HP-1F7 (kindly provided by M. Lopez Botet) was used as a positive control for pan-HLA blocking. The mAb clone HP-1F7 effectively binds to the alpha chain of HLA-A, -B, -C, -E, and -G, and blocks all NK cell inhibitory receptor engagement [10,34,35]. NK cells were preincubated for 30 minutes with NK receptor–blocking antibodies to a final concentration of 20 μg/mL, and target cells were preincubated for 30 minutes with anti-HLA mAb to a final concentration of 20 μg/mL, which was at least 10-fold higher than the maximal saturating concentration. Mouse anti-hNCAM/CD56 IgG2b clone 301040 and IgG2b clone 20116.11 (R&D Systems) were used as isotype controls.

Statistical Analysis

The Student t test was applied for statistical evaluation of differences between groups.

RESULTS

A High Frequency of KIR⁻ Blood NK Cells Express NKG2A and LIR-1

NK cells receptors from 3 families—KIR, NKG2A, and LIR-1—recognize MHC class I molecules. The surface expression of these receptors on KIR⁺ and KIR⁻ peripheral blood NK cells from 42 healthy donors was measured by flow cytometry. Almost half of the NK cells (45% ± 11%) lacked KIR expression, although there was variability among donors (range, 28%-79%). NKG2A and LIR-1 were expressed on 53% ± 13% (range, 20%-81%) and 36% ± 18% (range 5%-80%) of all NK cells, respectively. The likelihood of NKG2A and LIR-1 coexpression varied among the KIR subsets (Table 1). NKG2A was more commonly expressed on KIR⁻ cells (KIR⁺/NKG2A⁺:KIR⁻/

NKG2A⁺ ratio of 1:2.1; P <.0001), whereas LIR-1 was more likely coexpressed on KIR⁺ NK cells (KIR⁺/LIR-1⁺:KIR⁻/LIR-1⁺ ratio of 2.3:1; P <.0001). Some individuals displayed an inhibitory receptor pattern comprising predominantly KIR, whereas others expressed a decreased percentage of KIR⁺ cells and an NKG2A-dominant pattern (Figure 1A).

Primary Acute Leukemia Blasts Express NKG2A and LIR-1 Ligands

The cognate ligands for NKG2A and LIR-1 were measured on primary AML (n = 5) and primary ALL blasts (n = 4). All targets expressed HLA class I molecules on the surface, as detected by a pan-HLA antibody recognizing HLA-A, -B, -C, -E, and -G. Class I expression was consistently higher on ALL blasts compared with AML blasts (mean fluorescence intensity, 244 ± 52 [n = 4] vs 70 ± 30 [n = 5]; P = .0004), suggesting that more inhibition by HLA class I molecules may explain the relative resistance of ALL to NK cell–mediated killing [36]. All leukemia blasts also expressed HLA-E, the ligand for NKG2A, as measured by Western blot analysis [21] (data not shown). LIR-1 ligands are less definitively characterized but are thought to include HLA-G and other classical MHC ligands. Although the MHC is variably expressed by leukemia, the expression of HLA-G on primary leukemia remains unclear [37,38]. Western blot analysis detected no HLA-G protein in any of the primary leukemia samples used in this study. Because the sensitivity of the anti–HLA-G antibody is not well documented and the specificity of anti–HLA-G antibodies is uncertain [39], we also used RT-PCR to measure HLA-G transcripts in the samples. No HLA-G transcripts were found in 5 of 9 samples tested, supporting the conclusion that they were truly HLA-G negative (Figure 1C). The low levels of HLA-G1 (770 bp) and HLA-G2/-G4 (490 bp) transcripts detected in 4 of 9 leukemia samples did not result in expression of detectable HLA-G protein and likely represent low-level HLA-G transcripts that have been found in normal PB [40].

KIR, NKG2A, and LIR-1 Contribute to Alloreactivity against Primary AML and ALL Blasts

To characterize the contributions of KIR, NKG2A, and LIR-1 blockade to NK cell–mediated alloreactivity against leukemia, we tested the ability of polyclonal allogeneic NK cells to kill primary AML and ALL blasts in the presence and absence of blocking mAbs. Cytotoxicity and degranulation were tested using fresh resting NK cells without confounding effects of exogenous cytokines. For pan-HLA blockade, we selected the antibody clone HP-1F7 because it binds to the alpha chain of HLA-A, -B, -C, -E, and -G to functionally block multiple HLA interactions

Table 1. Inhibitory NK Receptor Expression on KIR⁺ and KIR⁻ NK Cells

Receptor	KIR⁺ Population	KIR⁻ Population	n	P Value
NKG2A⁺*	35% ± 13%	75% ± 12%	42	<.0001
LIR-1⁺*	44% ± 20%	27% ± 14%	42	<.0001
NKG2A⁺LIR-1⁺*	11.2% ± 7.4%	7.6% ± 6.1%	7	NS
NKG2A⁺LIR-1⁻*	16.9% ± 9.4%	44% ± 15%	7	<.0001
NKG2A⁻LIR-1⁺*	30% ± 19%	13% ± 12%	7	.01
NKG2A⁻LIR-1⁻*	41% ± 18%	35% ± 7.2%	7	NS
KIR⁺†	87% ± 7.6%	7.8% ± 5.3%	15	<.0001
KIR⁻NKG2A⁻LIR-1⁻	16% ± 2.7% of total NK cells (n = 7)			

NS indicates not significant.
*Mean percentage (± SD) of each receptor expressed on gated KIR⁺ and KIR⁻ NK cells determined by flow cytometry before bead separation.
†Percentage of each receptor on KIR⁺ and KIR⁻ NK cells after bead separation.

[10,34,35]. For anti-KIR blockade, no single reagent can block all KIR, and so we focused on an antibody currently in clinical deevelopment (anti-2DL1/2DL2/2DL3), which interacts with all HLA-C1 and HLA-C2 ligands. Other blocking antibodies were used individually or in combinations as indicated.

Fresh polyclonal NK cells potently lysed HLA class I–negative K562 cell targets used as a control for their broad cytotoxicity. Lysis of primary leukemia targets was always significantly less. Pan-HLA block-ade resulted in increased target lysis by resting NK cells for both AML targets (∼3-fold; n = 2;

P <.0005) and ALL targets (∼6-fold; n = 2; P <.0005) compared with no blockade (Figure 2A and Supplemental Figure 1A). Clinically achievable KIR blockade using an anti-KIR reagent increased the level of cytotoxicity against AML and ALL blasts slightly, but not to the level of pan-HLA blockade, suggesting that other class I–recognizing receptors were functionally operant. AML blasts (AML3) lacking 2 KIR ligands (HLA-C2 and HLA-Bw4) were slightly more sensitive to killing without blockade by allogeneic NK cell donors mismatched in both ligands (n = 3) compared with completely matched donors (n = 3)
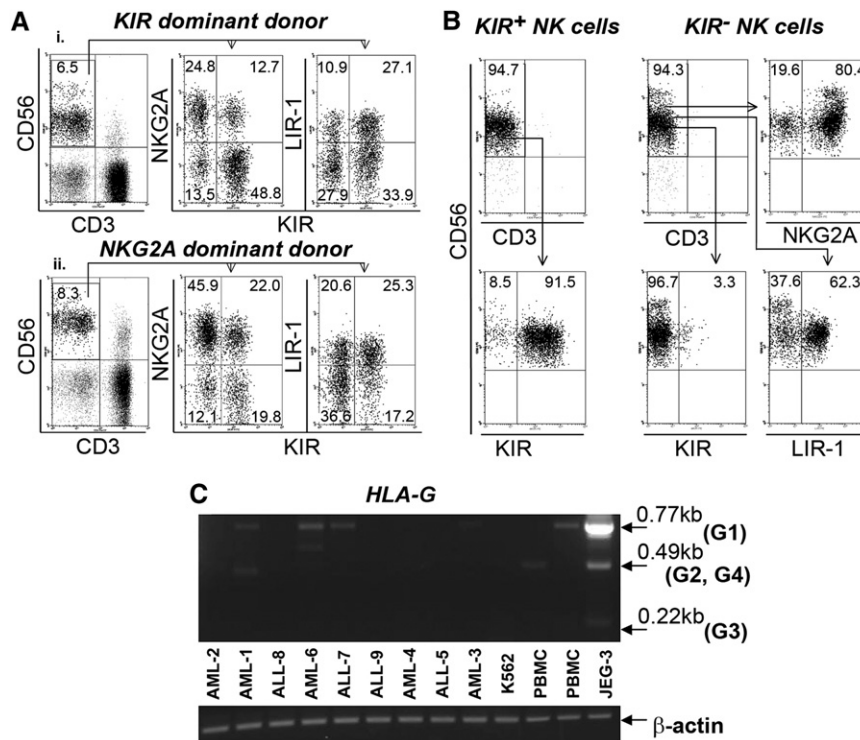

Figure 1. NK cells express KIR, NKG2A, and LIR-1, and leukemia targets minimally express HLA-G transcripts. (A) NK cells were evaluated by flow cytometry for the expression of class I–recognizing inhibitory receptors (n = 42). Each CD56⁺/CD3⁻ NK cell population was gated to evaluate the expression of NKG2A and LIR-1 on KIR-expressing cells (using a cocktail recognizing KIR2DL1/S1, KIR2DL2/L3/S2, and KIR3DL1). Shown are representative examples from a donor with a KIR-dominant phenotype and an NKG2A-dominant phenotype. (B) NK cells were then enriched into KIR⁺ and KIR⁻ populations and KIR⁻ population using anti-PE–conjugated immunomagnetic beads recognizing a cocktail of anti-KIR mAbs. Representative examples of KIR⁺ and KIR⁻ populations are shown. KIR⁻ NK cells coexpress a high proportion of NKG2A and less LIR-1. (C) RT-PCR and Southern blot analysis were used to test for alternatively spliced HLA-G on 9 leukemia samples, the erythroleukemia cell line K562, 2 normal PBMC populations, and a choriocarcinoma cell line (JEG-3) as a positive control. β-actin was used as an internal control.
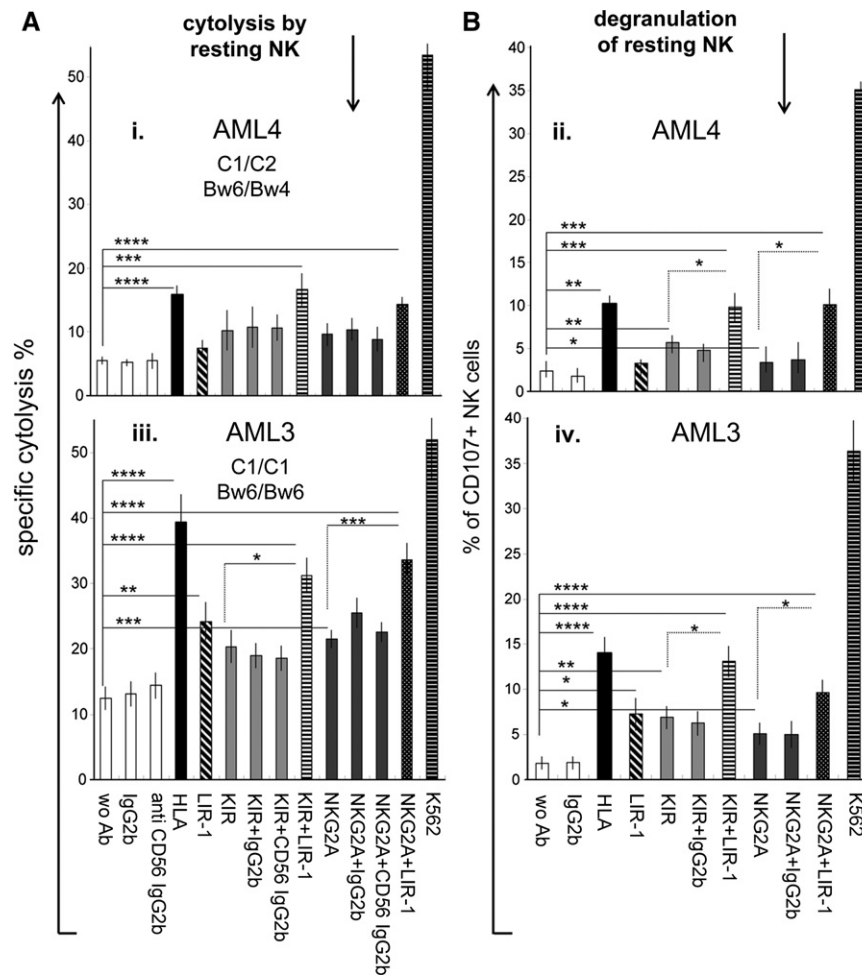
Figure 2. KIR, NKG2A, and LIR-1 blockade all contribute to cytotoxicity against primary AML. (A) Primary AML blasts from 2 patients, AML4 (i, ii) (tested with 5 allogeneic NK cell donors) and AML3 (iii, iv) (tested with 6 allogeneic donors), were investigated for their susceptibility to cytolysis mediated by resting NK cells (i, iii) at an E:T ratio of 5:1. (B) Degranulation of resting NK cells (ii, iv) was determined by CD107a expression following coincubation with AML4 blasts (ii) (3 NK cell donors) and AML3 blasts (iv) (6 NK cell donors). Mean ± standard error of the mean from different NK cell donors are shown. Pan-HLA blockade was compared with blockade of KIR, NKG2A, and LIR-1 individually and in combinations. Statistically significant differences between groups are noted (*P <05; **P <001; ***P <005; ****P <0005). HLA-B and -C ligand status of leukemia blasts is shown. There was KIR ligand match in 3 donors and mismatch in 2 KIR ligands in 3 donors used for AML3.

(15.6% ± 2.1% vs 9.4% ±1.1%; P = .06). The effect of NKG2A blockade was similar to that of KIR blockade. The combination of LIR-1 blockade with either KIR or NKG2A blockade resulted in strongly increased killing of both AML and ALL targets. These results indicate that all 3 receptors have the potential to inhibit NK cell cytotoxicity.

In samples negative for HLA-G transcripts and protein, indicating a lack of HLA-G expression, LIR-1 blockade enhanced killing of blasts from these patients. This suggests that the engagement of classical and/or nonclassical HLA class I molecules other than HLA-G on primary blasts can inhibit NK cell killing through LIR-1. Measurement of the degranulation response by CD107a yielded similar results (Figure 2B and Supplemental Figure 1B). Dual blockade of KIR and LIR-1 or of NKG2A and LIR-1 resulted in an increased proportion of degranulating NK cells, comparable to that achieved with pan-HLA blockade.

KIR⁻ NK Cells Exhibit Significant Cytotoxicity against Primary Leukemia Blasts

To further study the differential function of non-KIR receptors against primary leukemia targets, KIR⁺ and KIR⁻ NK cells were separated from 15 healthy donors using a cocktail of anti-KIR mAbs (Figure 1B and Table 1). Both populations lysed class I–negative K562 targets; however KIR⁻ NK cells exhibited less killing than KIR⁺ cells (56% ± 8% [n = 5] vs 39% ± 16% [n = 11] at an E:T ratio of 5:1; P = .02). Cytotoxicity assays were then performed against primary AML and ALL blasts (Figures 3A and 4A). Pan-HLA blockade of both AML and ALL blasts resulted in potent cytotoxicity by resting KIR⁺ and KIR⁻ NK cells against both targets. Similar to the results obtained using unsorted NK cells, blockade of both NKG2A and LIR-1 in the KIR⁻ NK cell population resulted in a 3.1-fold (P <.01) and 2.3-fold (P <.01) increase in cytotoxicity, respectively, against
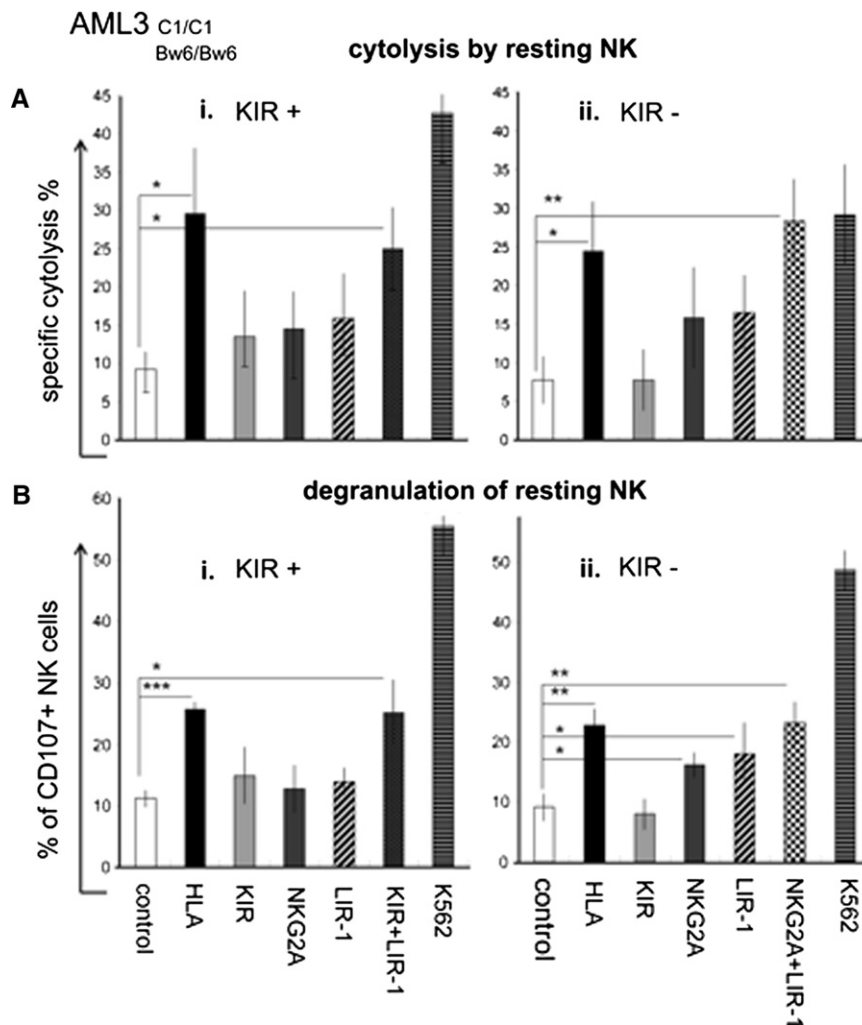
Figure 3. KIR⁻ NK cells exhibit significant alloreactivity against primary AML blasts. Primary AML blasts (AML3) were investigated for their susceptibility to KIR-enriched (KIR⁺) and KIR⁻-resting (4 NK cell donors) NK cells in cytotoxicity (A) and degranulation (B) assays. Lysis or degranulation are shown with NK cells alone against leukemia targets (control) and the presence of the indicated blocking antibodies, or against K562 targets. Statistically significant differences between groups are marked (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$).

both AML and ALL blasts compared with no blockade. Similar results were obtained using CD107a degranulation (Figures 3B and 4B).

Maximal Killing of Primary AML and ALL Blasts Requires NKG2A and LIR-1 Blockade along with KIR Blockade

The aforementioned studies demonstrated that KIR⁻ NK cells are not hyporesponsive and are capable of lysing targets. To better characterize the contribution of antileukemic killing by the 3 families of inhibitory receptors, we measured the sensitivity of additional primary AML and ALL blasts to killing by polyclonal resting NK cells (Supplemental Figure 2). One of 9 AML samples and 1 of 7 ALL samples were completely resistant to NK cell–mediated lysis. Consistent with the cytotoxicity findings, neither resistant sample induced degranulation of NK cells (data not shown). We then characterized the sensitivity to alloreactive NK cell–mediated killing by setting a threshold of at least

a 10% increment in specific lysis with pan-HLA blockade. Based on this definition, 2 AML samples that were killed by resting NK cells at baseline were not sensitive to NK alloreactivity after pan-HLA blockade or combined blockade of inhibitory receptors. Six of 9 AML samples and 6 of 7 ALL samples (including 1 T-ALL sample) demonstrated sensitivity to alloreactive NK cells, with pan-HLA blockade resulting in increased killing. Individual blockade of KIR, NKG2A, or LIR-1 resulted in modest increases in leukemia lysis, but the effects were significantly less that those seen with pan-HLA blockade in AML (23.9% ± 3.7% vs 7.8% ± 2.3%; n = 6; $P < .005$) and in ALL (24.7% ± 3.9% vs 8.21% ± 2.3%; n = 6; $P < .005$). Blockade of 2 or more receptors resulted in increased leukemia killing. Furthermore, blockade of KIR, NKG2A, and LIR-1 achieved the same level of killing obtained with the pan-HLA antibody, suggesting that the 3 receptor families account for most of the MHC class I interactions between bulk NK cells and leukemia blasts.
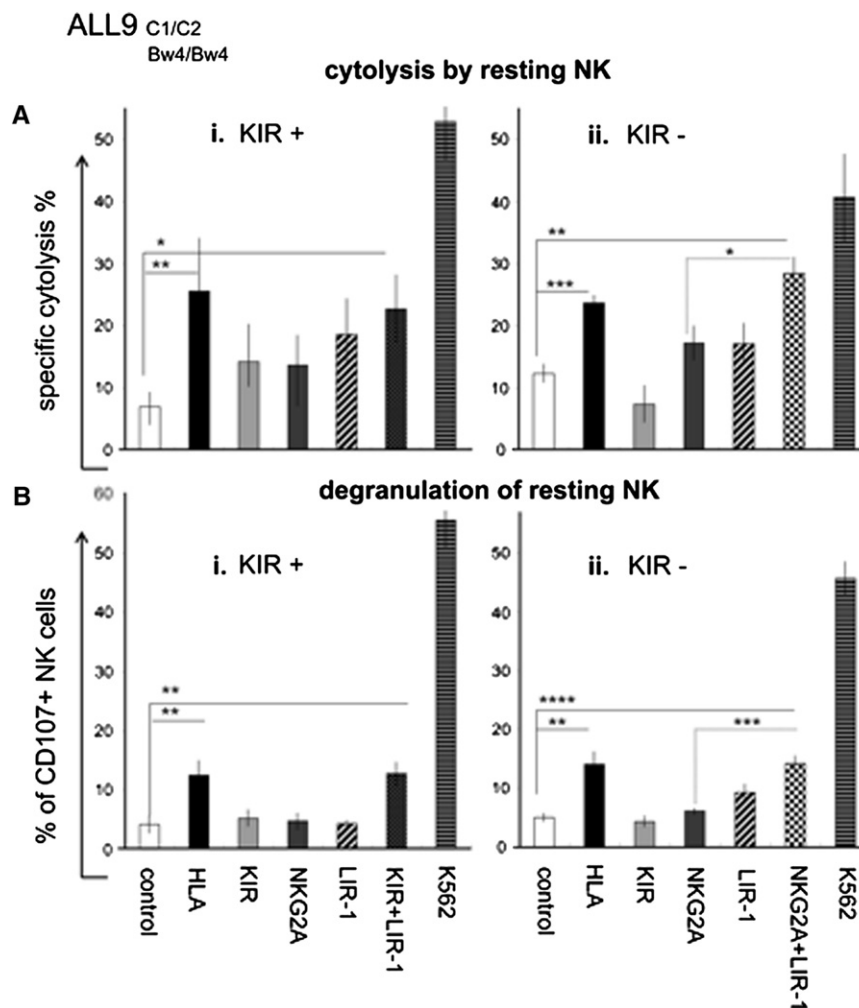
Figure 4. KIR⁻ NK cells exhibit significant alloreactivity against primary ALL blasts. Primary ALL blasts (ALL9) were investigated for their susceptibility to KIR-enriched (KIR⁺) and KIR⁻-resting (4 NK cell donors) NK cells in cytotoxicity (A) and degranulation (B) assays.

Augmented Killing by Day 100 Posttransplantation NK Cells with Inhibitory Receptor Blockade

We have previously shown that despite a 4-fold increase in the percentage of NK cells reconstituting in the PB 100 days after transplantation, the NK cell receptor repertoire in recipients of T cell–replete transplants is perturbed. KIRs are diminished, and NKG2A is the dominant inhibitory receptor (expressed on > 80% NK cells) [15]. We found that LIR-1 expression was diminished, occurring on only ∼20% of NK cells at this time point posttransplantation (data not shown). These results suggest that NKG2A and LIR-1 might be ideal for inducing posttransplantation NK cell reactivity. Five patients were studied at ∼100 days posttransplantation to explore whether NK cells are functional and to determine their tolerance mechanism against primary allogeneic leukemia blasts. AML or ALL targets known to be sensitive to enhanced killing after interruption of HLA class I interactions between normal donor NK cells and targets were used. Given

the unscheduled nature of receipt of patient samples, PBMCs were incubated in 5 U/mL of IL-2 for 48 hours before testing, to allow thawing and preparation of targets. This concentration and duration of IL-2 exposure did not alter the expression of KIR, NKG2A, or LIR-1 (n = 5; data not shown). In this setting, KIR blockade had no effect on killing compared with control conditions; however, the addition of NKG2A, LIR-1, or a combination of all 3 blocking antibodies significantly increased killing, demonstrating that NK cells are functional in a setting in which non-KIR class I recognizing receptors dominate (Figure 5).

DISCUSSION

Tolerance to allogeneic NK cell killing is generally attributed to the inhibitory effects of KIRs. Our findings suggest that simply overcoming KIR inhibition may be insufficient to maximize killing of primary leukemia targets. In fact, in some settings in which KIR expression is low, this tolerance
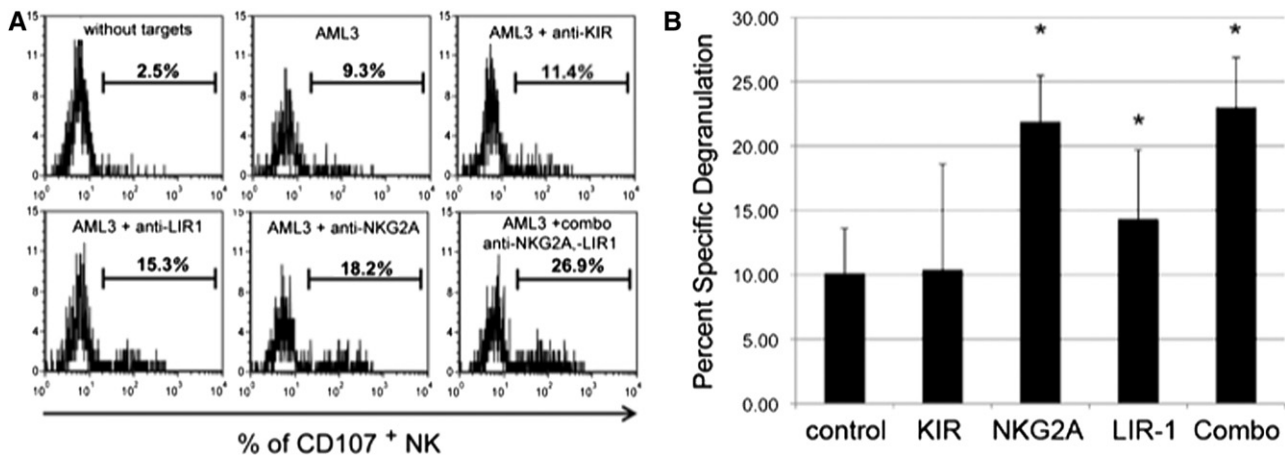
Figure 5. Enhanced killing by NK cells 100 days after allogeneic HCT by NKG2A and LIR-1 blockade. PBMCs from patients 100 days posttransplantation were tested against NK cell–susceptible AML (n = 3) and ALL (n = 2) primary blasts alone or with anti-KIR, LIR-1, or NKG2A blockade or a combination (combo). Representative (A) and aggregate (B) data are shown. *P <.05.

mechanism may be minor. Although a humanized anti-KIR reagent (binding to KIR2DL1, L2, and L3) was recently reported to effectively enhance killing of leukemia targets, it does not block all inhibitory KIRs, because KIR3DL1 is not bound [41]. But, nearly half of our AML targets were Bw4-negative, supporting the notion that KIR3DL1 interactions cannot explain the lack of response to KIR blockade seen in that study. This finding prompted us to assess the importance of other inhibitory NK cell receptors in the recognition and killing of acute leukemia cells. In the present study, we have demonstrated that 2 other families of inhibitory receptors—NKG2A and LIR-1—are present on both KIR⁺ and KIR⁻ NK cells and are implicated in NK cell tolerance of primary AML and ALL blasts.

Our findings indicate that LIR-1 interactions can occur in the absence of HLA-G (a high-affinity ligand), supporting the observation that LIR-1 recognizes other HLA class I molecules [9,10,35,42]. This is in contrast to findings reported by Riteau and coworkers [11,12] that a "dim" level of LIR-1 expression on primary PB NK cells is incapable of mediating significant inhibitory signals by HLA class I molecules. The KIR⁻/NKG2A⁺ NK cell population is dominant in NK cells residing in lymphoid tissue, in the PB of some healthy individuals, and in most patients posttransplantation [15,43,44]. All AML and ALL samples that we tested expressed the NKG2A ligand HLA-E. Our findings demonstrate that resting polyclonal KIR⁻/NKG2A⁺ NK cell populations from donors exhibit potent cytotoxicity against primary blasts, in agreement with the findings of an NK cell clone study by Yawata et al. [45] using HLA class I–deficient targets. Our KIR⁻ NK cell results may be particularly important in patients reconstituting early after T cell–replete transplantation, where KIR⁻ NK cells predominate. The results may be different in a T cell–

depleted setting. Yu et al. [46] recently reported that tolerance to self may be broken early after transplantation, but reestablished later. Although further kinetic studies with various graft sources (eg, adult donor, umbilical cord blood) are needed to fully explore NK cell function in vivo, our data nonetheless support the notion that NKG2A and LIR-1 blockade may be more important posttransplantation given the perturbed KIR repertoire in that setting.

Our results demonstrate that primary AML and ALL blasts effectively protect themselves against cytolysis from KIR⁻ NK cells through MHC interactions with NKG2A and LIR-1. This resistance can be diminished by combined blockade of these inhibitory receptors. The tight correlation between cytotoxicity and degranulation will allow us to measure NK cell subsets responsible for this activity to better understand this process. We acknowledge that other factors also are important in determining tumor kill. NK cell function is determined by the net sum of inhibitory signals, but activating receptor signals are also required for NK cell killing. Studies of activating receptors and how they reconstitute after HCT are needed to fully elucidate the role of NK cells in a graft-versus-leukemia effect. Although the functional competence of KIR⁻/NKG2A⁺ NK cell reconstitution after HCT could possibly be adversely affected by posttransplantation immunosuppression, our data support the concept that inhibitory receptor blockade enhances leukemia killing. A comprehensive analysis of NK cell function after transplantation has been initiated to explore whether combining inhibitory receptor blockade with other strategies, such as IL-2 or IL-15 administration, might improve transplantation outcomes, and whether in vitro function correlates with clinical efficacy.

Our work also provides insight into ALL resistance to NK alloreactivity. ALL blasts exhibit substantially

higher HLA class I expression compared with myelogenous blasts, as shown here. Importantly, the resistance of ALL blasts to NK cell–mediated killing can be overcome by pan-HLA blockade. The blockade of multiple MHC class I–recognizing receptors can mediate robust NK cytotoxicity against ALL blasts, similar to that achieved in AML, supporting the therapeutic potential of inhibitory NK cell receptor blockade in patients with ALL.

In summary, our results show that inhibitory receptors other than KIR endow polyclonal bulk NK cells with self-tolerance mechanisms through NKG2A and LIR-1. The patterns of KIR, NKG2A, and LIR-1 expression on NK cells and the expressions of their cognate ligands on leukemia blasts determine the magnitude of potential inhibition by self MHC class I. Screening of donor NK cells against patients' leukemia blasts may be used to exclude patients who exhibit a primary NK cell resistance pattern from adoptive NK cell therapy trials." Simultaneous blockade of more than one inhibitory receptor family significantly increases the frequency of alloreactive NK cells. Our findings warrant clinical testing of KIR, NKG2A, and LIR-1 blockade (alone and in combination) to increase the efficacy of NK cell–based therapies in the treatment of leukemia and other class I–expressing malignancies, especially in patients undergoing HCT, in which non-KIR mechanisms may dominate.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbmt.2010.01.019.

REFERENCES

1. Lopez-Botet M, Llano M, Navarro F, et al. NK cell recognition of non-classical HLA class I molecules. *Semin Immunol*. 2000;12:109-119.
2. Parham P. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol*. 2005;5:201-214.
3. Caligiuri MA. Human natural killer cells. *Blood*. 2008;112:461-469.
4. Lanier LL. Evolutionary struggles between NK cells and viruses. *Nat Rev Immunol*. 2008;8:259-268.
5. Kim S, Poursine-Laurent J, Truscott SM, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature*. 2005;436:709-713.
6. Raulet DH, Vance RE. Self-tolerance of natural killer cells. *Nat Rev Immunol*. 2006;6:520-531.
7. Anfossi N, Andre P, Guia S, et al. Human NK cell education by inhibitory receptors for MHC class I. *Immunity*. 2006;25:331-342.
8. Lee N, Llano M, Carretero M, et al. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc Natl Acad Sci USA*. 1998;95:5199-5204.
9. Cosman D, Fanger N, Borges L, et al. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity*. 1997;7:273-282.
10. Colonna M, Navarro F, Bellon T, et al. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J Exp Med*. 1997;186:1809-1818.
11. Riteau B, Menier C, Khalil-Daher I, et al. HLA-G1 co-expression boosts the HLA class I–mediated NK lysis inhibition. *Int Immunol*. 2001;13:193-201.
12. Riteau B, Rouas-Freiss N, Menier C, et al. HLA-G2, -G3, and -G4 isoforms expressed as nonmature cell surface glycoproteins inhibit NK and antigen-specific CTL cytolysis. *J Immunol*. 2001;166:5018-5026.
13. Shilling HG, McQueen KL, Cheng NW, et al. Reconstitution of NK cell receptor repertoire following HLA-matched hematopoietic cell transplantation. *Blood*. 2003;101:3730-3740.
14. Vitale C, Chiossone L, Morreale G, et al. Analysis of the activating receptors and cytolytic function of human natural killer cells undergoing in vivo differentiation after allogeneic bone marrow transplantation. *Eur J Immunol*. 2004;34:455-460.
15. Cooley S, McCullar V, Wangen R, et al. KIR reconstitution is altered by T cells in the graft and correlates with clinical outcomes after unrelated donor transplantation. *Blood*. 2005;106:4370-4376.
16. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295:2097-2100.
17. Miller JS, Cooley S, Parham P, et al. Missing KIR ligands are associated with less relapse and increased graft-versus-host disease (GVHD) following unrelated donor allogeneic HCT. *Blood*. 2007;109:5058-5061.
18. Leung W, Iyengar R, Turner V, et al. Determinants of antileukemia effects of allogeneic NK cells. *J Immunol*. 2004;172:644-650.
19. Hsu KC, Keever-Taylor CA, Wilton A, et al. Improved outcome in HLA-identical sibling hematopoietic stem cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes. *Blood*. 2005;105:4878-4884.
20. Giebel S, Locatelli F, Lamparelli T, et al. Survival advantage with KIR ligand incompatibility in hematopoietic stem cell transplantation from unrelated donors. *Blood*. 2003;102:814-819.
21. Nguyen S, Dhedin N, Vernant JP, et al. NK cell reconstitution after haploidentical hematopoietic stem cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. *Blood*. 2005;105:4135-4142.
22. Vago L, Forno B, Sormani MP, et al. Temporal, quantitative, and functional characteristics of single-KIR–positive alloreactive natural killer cell recovery account for impaired graft-versus-leukemia activity after haploidentical hematopoietic stem cell transplantation. *Blood*. 2008;112:3488-3499.
23. Farag SS, Bacigalupo A, Eapen M, et al. The effect of KIR ligand incompatibility on the outcome of unrelated donor transplantation: a report from the Center for International Blood and Marrow Transplant Research, the European Blood and Marrow Transplant Registry, and the Dutch Registry. *Biol Blood Marrow Transplant*. 2006;12:876-884.
24. Malmberg KJ, Schaffer M, Ringden O, et al. KIR-ligand mismatch in allogeneic hematopoietic stem cell transplantation. *Mol Immunol*. 2005;42:531-534.
25. Yun G, Tolar J, Yerich AK, et al. A novel method for KIR-ligand typing by pyrosequencing to predict NK cell alloreactivity. *Clin Immunol*. 2007;123:272-280.
26. Paul P, Rouas-Freiss N, Moreau P, et al. HLA-G, -E, -F preworkshop: tools and protocols for analysis of non-classical

class I gene transcription and protein expression. *Hum Immunol*. 2000;61:1177-1195.

27. Menier C, Saez B, Horejsi V, et al. Characterization of monoclonal antibodies recognizing HLA-G or HLA-E: new tools to analyze the expression of nonclassical HLA class I molecules. *Hum Immunol*. 2003;64:315-326.

28. McCullar V, Oostendorp R, Panoskaltsis-Mortari A, et al. Mouse fetal and embryonic liver cells differentiate human umbilical cord blood progenitors into CD56-negative natural killer cell precursors in the absence of interleukin-15. *Exp Hematol*. 2008;36:598-608.

29. Piriou L, Chilmonczyk S, Genetet N, et al. Design of a flow cytometric assay for the determination of natural killer and cytotoxic T-lymphocyte activity in human and in different animal species. *Cytometry*. 2000;41:289-297.

30. Pozarowski P, Grabarek J, Darzynkiewicz Z. Flow cytometry of apoptosis. *Curr Protoc Cell Biol*. 2004; doi:10.1002/0471143030. cb1808s21. Chap 18:Unit 18 18.

31. Godal R, Keilholz U, Uharek L, et al. Lymphomas are sensitive to perforin-dependent cytotoxic pathways despite expression of PI-9 and overexpression of bcl-2. *Blood*. 2006;107:3205-3211.

32. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8⁺ T cells by a flow cytometric assay for degranulation. *J Immunol Methods*. 2003;281:65-78.

33. Rubio V, Stuge TB, Singh N, et al. Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. *Nat Med*. 2003;9: 1377-1382.

34. Perez-Villar JJ, Melero I, Navarro F, et al. The CD94/NKG2-A inhibitory receptor complex is involved in natural killer cell–mediated recognition of cells expressing HLA-G1. *J Immunol*. 1997;158:5736-5743.

35. Navarro F, Llano M, Bellon T, et al. The ILT2(LIR1) and CD94/NKG2A NK cell receptors respectively recognize HLA-G1 and HLA-E molecules co-expressed on target cells. *Eur J Immunol*. 1999;29:277-283.

36. Pende D, Spaggiari GM, Marcenaro S, et al. Analysis of the receptor–ligand interactions in the natural killer–mediated lysis of freshly isolated myeloid or lymphoblastic leukemias: evidence

for the involvement of the poliovirus receptor (CD155) and nectin-2 (CD112). *Blood*. 2005;105:2066-2073.

37. Polakova K, Krcova M, Kuba D, et al. Analysis of HLA-G expression in malignant hematopoetic cells from leukemia patients. *Leuk Res*. 2003;27:643-648.

38. Yan WH, Lin A, Chen BG, et al. Unfavourable clinical implications for HLA-G expression in acute myeloid leukaemia. *J Cell Mol Med*. 2008;12:889-898.

39. Apps R, Gardner L, Moffett A. A critical look at HLA-G. *Trends Immunol*. 2008;29:313-321.

40. Moreau P, Adrian-Cabestre F, Menier C, et al. IL-10 selectively induces HLA-G expression in human trophoblasts and monocytes. *Int Immunol*. 1999;11:803-811.

41. Romagne F, Andre P, Spee P, et al. Pre-clinical characterization of 1-7F9, a novel human anti-KIR therapeutic antibody that augments NK-mediated killing of tumor cells. *Blood*. 2009;114: 2667-2677.

42. Vitale M, Castriconi R, Parolini S, et al. The leukocyte Ig-like receptor (LIR)-1 for the cytomegalovirus UL18 protein displays a broad specificity for different HLA class I alleles: analysis of LIR-1⁺ NK cell clones. *Int Immunol*. 1999;11: 29-35.

43. Freud AG, Becknell B, Roychowdhury S, et al. A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. *Immunity*. 2005;22:295-304.

44. Freud AG, Yokohama A, Becknell B, et al. Evidence for discrete stages of human natural killer cell differentiation in vivo. *J Exp Med*. 2006;203:1033-1043.

45. Yawata M, Yawata N, Draghi M, et al. MHC class I–specific inhibitory receptors and their ligands structure diverse human NK-cell repertoires toward a balance of missing self-response. *Blood*. 2008;112:2369-2380.

46. Yu J, Venstrom JM, Liu XR, et al. Breaking tolerance to self, circulating natural killer cells expressing inhibitory KIR for non-self HLA exhibit effector function after T cell–depleted allogeneic hematopoietic cell transplantation. *Blood*. 2009;113: 3875-3884.