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Estimation of the Size of the Alloreactive NK Cell Repertoire: Studies in Individuals Homozygous for the Group A *KIR* Haplotype[1]

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Stem cell transplantation across HLA barriers may trigger NK cell-mediated graft-vs-leukemia effects leading to improved survival for patients with hematological malignancies. However, the genetic algorithm based on killer cell Ig-like receptor (KIR) and HLA genes used to predict NK cell alloreactivity have yielded discrepant results. Accordingly, it has been difficult to define transplantation settings that favor NK cell alloreactivity. In this study, we have used multiparameter flow cytometry to simultaneously analyze the cell surface expression of all four major inhibitory KIR and CD94/NKG2A to determine the size of the alloreactive NK cell repertoires in 31 individuals homozygous for the group A *KIR* haplotype. We observed a vast variability in the frequencies of cells with an alloreactive potential, ranging from 0 to 62% of the total NK cell population depending on which, and how many, KIR ligands were missing in theoretical recipients. This analysis required a functional examination of KIR3DL2-single positive NK cells, showing that this subset was hyporesponsive in individuals harboring the cognate ligands HLA-A3/A11. The results provide new insights into the variability of the functional alloreactive NK cell repertoire and have implications for donor selection in hematopoietic stem cell transplantation and adoptive NK cell-based immunotherapy. ***The Journal of Immunology,*** **2008, 181: 6010–6019.**

Natural killer cells are lymphocytes of the innate immune system with a capacity to eliminate virus-infected cells and tumor cells (1, 2). Because of their ability to kill transformed cells, there is significant interest in exploring the potential of NK cells in treatment of human cancer (3). NK cell function is regulated by integrated signaling through multiple activation and inhibitory receptors (4, 5). In humans, inhibition is largely mediated via interaction of HLA class I molecules with killer cell Ig-like receptors (KIR)[4] and/or CD94/NKG2A (6, 7). Inhibitory KIR with four specificities for polymorphic HLA class I have been defined. These specificities correspond to HLA-C allotypes with asparagine 80 (Cw3-related alleles referred to as "C1," KIR2DL2/3), HLA-C allotypes with lysine 80 (Cw4-related alleles referred to as "C2," KIR2DL1), HLA-A and HLA-B alleles with Bw4 motifs at positions 77−83 (KIR3DL1), and HLA-A3/A11 (KIR3DL2) (8). The ligands for the lectin-like CD94:NKG2A/C/E receptors are complexes of HLA-E bound to peptides derived from the leader sequences of other HLA class I molecules (9).

The "missing-self" hypothesis (10) and the identification of inhibitory receptors for self HLA class I molecules (6, 11) have led to the assumption that transplantation across *HLA* barriers can trigger graft-vs-leukemia (GVL) effects mediated by a repertoire of alloreactive NK cells (12, 13). In haploidentical stem cell transplantation (SCT), an algorithm consisting of genetic analysis of donor and recipient KIR ligands has been used to demonstrate a dramatic impact on survival associated with the presence of an alloreactive NK cell repertoire in the donor (13). This observation triggered a cascade of similar analyses in different settings of allogeneic SCT for several types of hematological malignancies (14–29). Some of these studies have used modified algorithms to analyze the impact of donor-recipient *KIR* haplotypes as well as individual combinations of activating and inhibitory *KIR* genes and specific *HLA* alleles on several end-points including leukemia relapse, graft-vs-host disease (GVHD), and survival. To date, however, no general conclusions can be drawn from those studies with respect to a beneficial effect of genetically predicted NK cell alloreactivity for patients undergoing this treatment. The complexity of the transplantation procedure and the large number of confounding parameters make it difficult to pinpoint biological factors that favor or counteract NK cell-mediated alloreactivity. Furthermore, prediction of alloreactivity by *HLA* and *KIR* genotyping does not take into account the actual frequency of functionally mature alloreactive NK cells in the donor (30). Indeed, the existence of NK

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[4] Abbreviations used in this paper: KIR, killer cell Ig-like receptor; GVL, graft-versus-leukemia; SCT, stem cell transplantation; GVHD, graft-vs-host disease.

cells lacking inhibitory receptors or expressing receptors for non-self HLA molecules has been observed and reflects discrepancies between genotype and protein expression (30, 31). New insights into the regulation of the NK cell receptor repertoire (32, 33), and the requirements for a functional maturation by interactions with self HLA class I molecules (31, 34–37), indicate a need to complement the genetic analysis of donors and recipients with an extensive phenotypic and functional characterization of the actual repertoire of NK cells that mediate the GVL effect. Indeed, Ruggeri et al. performed a functional analysis of donor-derived NK cell clones (13), but few other centers have adopted this somewhat laborious procedure.

Recent improvements in flow cytometry with respect to instrumentation and available reagents now permit high resolution phenotypic and functional analyses of small lymphocyte subsets (38). In this study, we have analyzed the size of the alloreactive NK cell repertoire in a cohort of 31 individuals homozygous for the group A *KIR* haplotype, using eight-color flow cytometry and a panel of mAbs specific for the four major inhibitory KIR and CD94/NKG2A. To allow a complete estimation of the alloreactive NK cell subset, we first examined the function of KIR3DL2-single positive NK cells, revealing that this subset was hyporesponsive, and not fully educated in individuals harboring the cognate ligands HLA-A3/A11. We observed a vast variability in the frequency of alloreactive NK cells in donors classified as KIR ligand mismatched by the genetic algorithm. The type of analysis performed in this study may be used to identify donors with an optimal NK cell repertoire capable of mediating GVL effects in allogeneic SCT.

Materials and Methods

Human subjects, blood samples, and cell lines

This study was approved by the regional ethics committee (Stockholm, Sweden, approval number 2006/229-31/3). Buffy coats were prepared from peripheral blood of healthy donors and separated by density gradient centrifugation (Ficoll-Hypaque; GE Healthcare). The human erythroleukemia cell line K562 (American Type Culture Collection) was maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 1 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

KIR *and* HLA *genotyping*

Genomic DNA was isolated from 100 μl of buffy coats by using the DNeasy Blood and Tissue kit (Qiagen). *KIR* genotyping was performed as described previously using PCR- sequence-specific primer technology and *KIR* typing kit (Olerup-SSP; Ref. 39). KIR ligands were determined using the *KIR HLA* ligand kit (Olerup-SSP) for detecting the *-Bw4*, *-Cw3* (C1), and *-Cw4* (C2) motifs. For analysis of *HLA-A3/A11*, complementary *HLA* genotyping was performed with the *HLA-A* low-resolution kit (Olerup-SSP).

Abs and flow cytometry

The following conjugated mAbs were used: anti-KIR3DL1 FITC (DX9), anti-CD56 PE-Cy7 (NCAM 16.2), anti-CD14 allophycocyanin-Cy7 (MϕP9), and anti-CD107a FITC (H4A3) were obtained from BD Biosciences. Anti-KIR2DL1/S1 PE (EB6) and anti-KIR2DL2/3/S2 allophycocyanin (GL183) were purchased from Beckman Coulter. Anti-CD3 Cascade Yellow (CY; UCHT1) was obtained from DakoCytomation. Anti-NKG2A (z199), purchased from Beckman Coulter, was conjugated with Pacific Blue using a mAb labeling kit (Invitrogen). The hybridoma-secreting anti-KIR3DL2 (DX31) was provided by Dr. J. Philips (DNAX Research Institute, Palo Alto, CA). Biotinylation of anti-KIR3DL2 was performed by Mabtech. Streptavidin Qdot605, purchased from Invitrogen, was used to detect bound biotinylated anti-KIR3DL2 mAbs. For some experiments the anti-KIR3DL2 mAb (DX31) was directly conjugated to Qdot605 with a QD605 labeling kit (Invitrogen). LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Invitrogen) was used to exclude dead cells from the analyses. Data were acquired on a CyAn ADP LX nine-color flow cytometer (DakoCytomation), equipped with a 405-nm laser, a 488-nm laser, and a 635-nm laser. The detailed specifications of the instrument, including filters, has been published previously (38). Anti-mouse IgGk beads (BD Biosciences) were stained with each of the fluorochrome-conjugated Abs separately and were used as compensation controls. Acquired data were compensated with software-based compensation, using the compensation platform in FlowJo 8.6 (Tree Star) and analyzed with FlowJo 8.6. Graphics were generated with the aid of SPICE software, version 4.1 (obtained from M. Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD).

Functional assay

PBMC (2×10^6cells/ml) were added to target cells at a ratio of 10:1 in a final volume of 200 μl in V-bottom 96-well plates (5). Target and effector cells were mixed by pipetting and were centrifuged at 300 rpm for 3 min, followed by 6 h of incubation at 37°C and 5% CO_2. After incubation, cells were centrifuged at 1400 rpm for 4 min at 4°C. Pellets were resuspended in cold FACS buffer (PBS supplemented with 2% FBS and 2 mM EDTA). The surface staining was performed on ice for 20 min and included NK cell markers (e.g., CD56, KIR, NKG2A) and CD107a as a functional readout. Cells were washed three times, resuspended in FACS buffer, and then analyzed by flow cytometry.

Statistical analysis

Data were analyzed for deviations from Gaussian distribution using the Kolmogorov-Smirnov test. For data populations that were Gaussian, a one-way ANOVA was applied. For non-Gaussian populations either Mann-Whitney or Kruskal-Wallis nonparametric tests were performed, depending on the number of groups compared. In figures, *** indicates $p < 0.001$; ** indicates $p < 0.01$; and * indicates $p < 0.05$. All statistical analyses were performed using GraphPad Software.

Results

Design of an anti-KIR Ab-panel for eight-color flow cytometry, covering all four major inhibitory KIR and NKG2A

The "missing-self" hypothesis predicts that a prerequisite for NK cell alloreactivity in clinical SCT is that the recipient lacks one or more HLA class I alleles present in the donor (12). However, because inhibitory KIR are clonally distributed in the NK cell population and their expression frequencies are highly variable between different individuals (33, 40), the repertoire of NK cells that do not express any inhibitory KIR for HLA class I molecules present on recipient cells may be limited. In this study, we set out to determine the size of the alloreactive NK cell repertoires. To allow a stringent analysis of KIR repertoires and avoid confounding effects by cross-reactivity of anti-KIR mAbs binding to activating KIR (41), we limited the study to individuals homozygous for the group A *KIR* haplotype. Group A haplotypes contain seven genes, most with inhibitory function, whereas group B haplotypes have a variable number of *KIR* genes, including those with activating function (42, 43). We combined Abs to inhibitory KIR with specificities for the four defined polymorphic HLA class I motifs and NKG2A in an eight-color flow cytometry analysis (Fig. 1*A*). The analysis was restricted to $CD56^{dim}$ NK cells, because $CD56^{bright}$ NK cells lack inhibitory KIR (44). To assure the quality of the analysis, we conformed to the recently described standard operating procedures for multiparameter flow cytometry and included a "dump" channel and a dead cell discriminator to eliminate nonspecific binding of Abs to $CD14^+$ subsets and by dead cells (38, 45). Using this protocol, the intra- and interexperimental variation was <5% (data not shown).

Identification of NK cell subsets expressing single inhibitory KIR and combinations thereof

We compiled the data obtained from 31 individuals and used Boolean gating to calculate the frequencies of NKG2A-negative NK cells expressing a single KIR and all combinations thereof (Fig. 1*B*). Despite significant donor variability in the frequencies of NK cell subsets, the following observations were made. First, most NKG2A-negative NK cells expressed either one or two inhibitory KIR at the cell surface (Fig. 1*B*, *inset*). More rarely, NK cells coexpressed three or all four inhibitory KIR. Second, ~20% of the

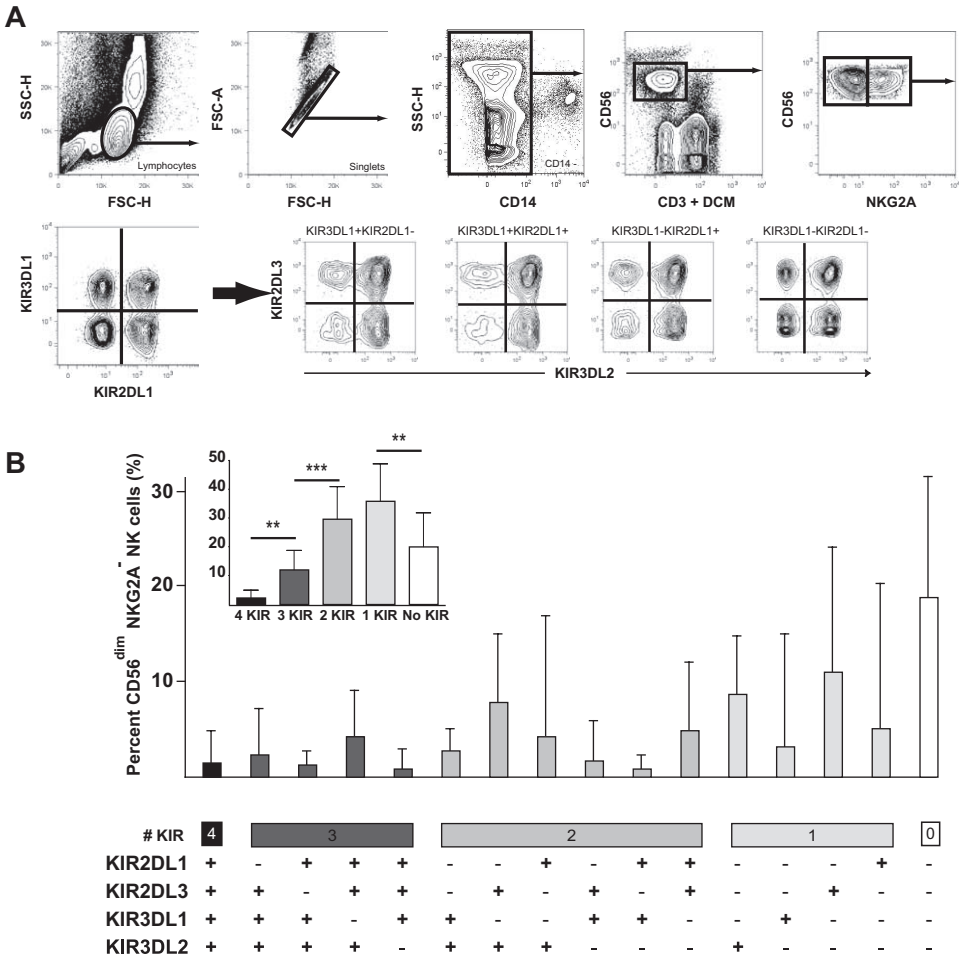

FIGURE 1. Identification of NK cell subsets expressing discrete combinations of inhibitory KIR. *A*, Gating scheme to identify NKG2A-negative CD56^{dim} NK cells expressing single inhibitory KIR and combinations thereof. Doublet cells and CD14^{+} cells were excluded based on a forward scatter (FSC)-area (A) vs FSC-height (H) gate and CD14 expression, respectively. Gates were set on live CD3^{-} cells as determined by staining with a dead cell marker and anti-CD3. NK cells expressing all combinations of KIR3DL1 and KIR2DL1 or neither of these receptors were monitored for expression of KIR2DL3 and KIR3DL2. Data from one representative donor is shown. *B*, Boolean gating was performed to illustrate the mean frequency of the NK cell subsets identified using the gating scheme described above for all 31 donors homozygous for the group A *KIR* haplotype. Error bars represent the SD. The inset shows the frequency of NKG2A-negative CD56^{dim} NK cells expressing 0–4 KIR. SSC, side scatter.

NKG2A-negative NK cells lacked all four inhibitory KIR, thus representing the previously described hyporesponsive and functionally immature NK cell subset (Fig. 1*B*, *inset*; Refs. 31, 46). Finally, the KIR2DL3-single positive NK cell subset was slightly larger than subsets expressing other KIR, whereas KIR3DL1-single positive cells were significantly less frequent ($p < 0.01$; Fig. 1*B*). The increased frequency of KIR2DL3-single positive NK cells, as determined by the mAb GL183, was not a consequence of the binding of mAb to both KIR2DL2 and KIR2DL3, because haplotype A donors lack the KIR2DL2 gene (33; www.ebi.ac.uk/ipd/kir/introduction.html). Our genetic analyses confirmed that none of the donors in our cohort expressed the KIR2DL2 gene (data not shown). Of note, four donors also completely lacked KIR3DL1-positive NK cells. These donors all had the *KIR3DL1*004* allele that is not expressed at the cell surface and were, therefore, excluded from the statistical analysis of KIR3DL1-expressing cells (47). These results show, on a population level, that some inhibitory KIR are more commonly used as single receptors.

Coexpression of NKG2A is more common on KIR3DL2-single positive NK cells and correlates inversely with the number of coexpressed KIR

It has previously been shown that there is an inverse correlation between NKG2A expression and the total frequency of NK cells expressing inhibitory KIR (33, 40). Coordinated expression of the two types of inhibitory receptors in an individual may serve to maintain tolerance to self (40). However, how and if these two receptors are complementarily expressed on a single cell level is not clear, nor is the possibility that NKG2A expression is affected by the number of coexpressed KIR on a particular NK cell. In this study, we examined whether NKG2A expression differs among NK cells expressing one, two, three, or four inhibitory KIR. Indeed, NKG2A expression was significantly more frequent on NK cells lacking all KIR, and the frequency of NKG2A expression gradually decreased as NK cells expressed additional KIR (Fig. 2*A*). Notably, NK cells expressing only one KIR displayed a considerable variation in the frequency of NKG2A expression (median 36%; range 1–75%). To dissect this finding further, we plotted the NKG2A expression in NK cell subsets expressing individual inhibitory KIR (Fig. 2*B*). In this cohort, KIR3DL2-single positive NK cells more frequently expressed NKG2A compared with KIR2DL3-single positive and KIR2DL1-single positive NK cells (Fig. 2*B*). Thus, NKG2A expression correlated inversely not only with the total frequency of cells expressing KIR, but also with the number of KIR that were coexpressed on the cell surface. Moreover, coexpression of NKG2A varied among single KIR-positive NK cells, being most prominent on NK cells expressing KIR3DL2 as their only inhibitory KIR.

KIR3DL2-single positive NK cells are hyporesponsive in HLA-A3/A11-positive individuals

Recent data indicate that mouse and human NK cells have to undergo an education process, also referred to as licensing or arming, to become functionally competent (34, 35, 48). Acquisition of full effector function is dependent on the interaction with self MHC class I molecules (35), which has important consequences for the functionality of the NK cell repertoire in individuals with different

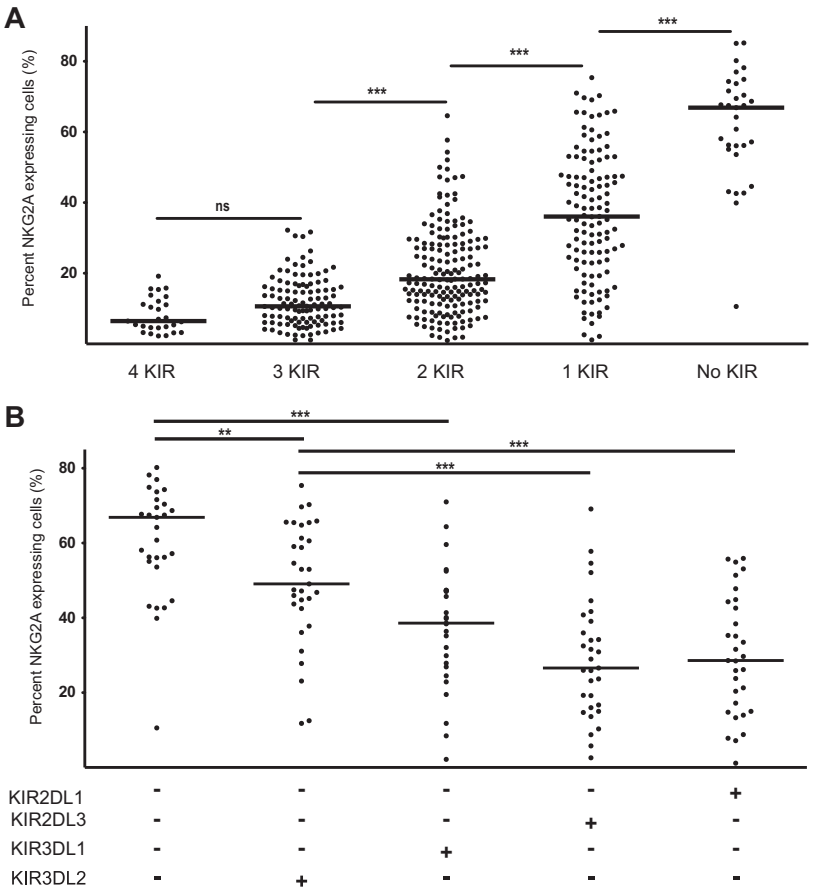

FIGURE 2. NKG2A expression is inversely correlated with the number of coexpressed KIR and is more frequent on KIR3DL2-single positive NK cells. *A*, Gating of CD56^{dim} NK cells was performed as shown in Fig. 1*A*. NKG2A expression was monitored on NK cell subsets expressing 0 to 4 KIR. Among the 16 different NK cell subsets, one subset expressed all four KIR (number of donors $n = 31$, accumulated number of data points $n = 31$), four subsets expressed combinations of three KIR ($n = 31$, $n = 128$); six subsets expressed combinations of two KIR ($n = 31$, $n = 192$); four subsets expressed only one KIR ($n = 31$, $n = 128$), and one subset expressed no KIR ($n = 31$, $n = 31$). *B*, Percentage of NKG2A-positive cells is shown on CD56^{dim} NK cells expressing one single KIR. Data obtained from 31 group A *KIR* haplotype donors are shown. Bars represent median.

HLA backgrounds (31, 36, 37). This phenomenon has been described for the education of KIR2DL2/3-, KIR2DL1-, and KIR3DL1-single positive NK cells by their respective ligands (HLA-C1, -C2, -Bw4; Ref. 31, 36, 37). To determine the total repertoire of educated NK cells, we first assessed whether similar rules apply for the education of KIR3DL2-single positive NK cells by the cognate ligands HLA-A3/A11 and how coexpression of NKG2A modulates the function of this subset of NK cells. To this end, the full inhibitory KIR Ab panel was complemented with a functional readout of the ability of NK cells to degranulate upon incubation with K562 cells (Fig. 3; Refs. 49, 50). Intriguingly, NK cells expressing KIR3DL2 as their only inhibitory receptor were hyporesponsive in individuals harboring the cognate ligands HLA-A3, -A11, or both (Fig. 3*A*). As control, NK cell subsets expressing KIR2DL3 were fully responsive in these HLA-C1-positive individuals (Fig. 3*B*). One should bear in mind that hyporesponsiveness in this context has been defined as fewer responding cells and not as diminished response by all cells within a particular subset (31, 34). To address how coexpression of additional KIR for nonself HLA molecules affected NK cell education, NK cell responses were assessed in four donors that shared KIR ligands, carrying HLA-C1 and HLA-A3 but lacking HLA-Bw4 and HLA-C2. NK cells expressing a self KIR, e.g., KIR2DL3 alone or in combination with other KIR, responded equally well to stimulation by K562 cells (Fig. 3*B*). In contrast, NK cells expressing only nonself KIR, i.e., KIR with specificity for HLA class I molecules lacking in these individuals (KIR2DL1 and/or KIR3DL1, as well as KIR-negative NK cells), were hyporesponsive to stimulation by K562 cells (Fig. 3*B*). NK cells expressing KIR3DL2 were only responsive if they coexpressed KIR2DL3. The relative hyporesponsiveness of KIR3DL2-single positive NK cells was confirmed in eight HLA-A3-positive donors and three HLA-A11-positive donors (Fig. 3*C*). The functionality of KIR3DL2-single positive NK cells in these individuals was compared with that of KIR2DL3-single positive NK cells in HLA-C1 or HLA-C2 homozygous donors (Fig. 3*C*). We next tested whether coexpression of NKG2A would render the otherwise hyporesponsive KIR3DL2-single positive NK cell subset permissive to stimulation by K562 cells. One previous study showed that KIR$^-$NKG2A$^+$ NK cells are fully functional (46); yet another study reported only a modest effect of NKG2A on the function of KIR$^-$ NK cells (37). In this study, NKG2A$^+$ KIR$^-$ NK cells and NKG2A$^+$ nonself KIR2DL3-single positive NK cells responded equally well to K562 as fully educated self KIR2DL3-single positive NK cells (Fig. 3*D*). Interestingly, NKG2A$^+$KIR3DL2-single positive NK cells also displayed full functional competence, suggesting that coexpression of NKG2A can compensate for the lack of education in KIR3DL2-single positive NK cells (Fig. 3*D*). Moreover, coexpression of NKG2A increased the responsiveness of NK cells expressing KIR2DL3 also in individuals homozygous for the HLA-C1 motif, indicating an additive effect mediated by NKG2A. Together, these data suggest that KIR3DL2-single positive NK cells are not educated by HLA-A3/A11 alleles and are thus not functionally competent.

Determining the repertoire of educated NK cells as predicted by the presence of cognate HLA class I molecules

To gauge the size of the alloreactive NK cell subset, we first needed to determine the repertoire of NK cells predicted to be educated based on the presence/absence of cognate HLA class I molecules. By combining data on the relative frequencies of various KIR-expressing NK cell subsets and presence/absence of the four major inhibitory HLA class I motifs, the total repertoire of

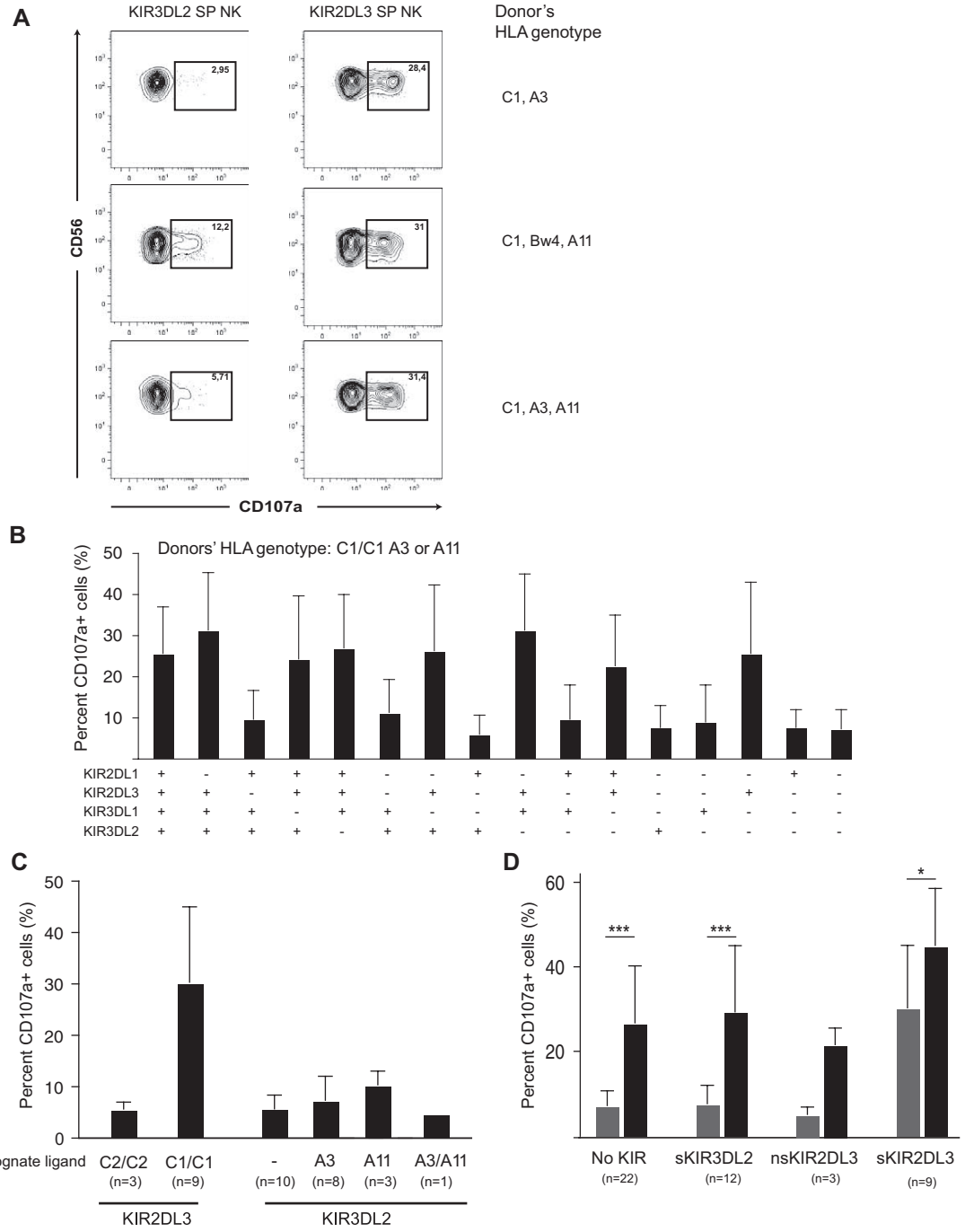

FIGURE 3. Influence of cognate HLA on the functionality of NK cells expressing one single KIR. PBMC from group A *KIR* haplotype donors were incubated with or without K562 and CD56^{dim} NKG2A^{-} KIR-single positive (KIR-SP) NK cells were analyzed for expression of CD107a by flow cytometry. *A*, Responsiveness of KIR3DL2-SP (*left column*) was assessed in donors with expression of HLA-A3 (*top*), -A11 (*middle*), or both (*bottom*). As an internal control, responsiveness of KIR2DL3-SP NK cells educated by HLA-C1 in each donor was assessed in parallel (*right column*). *B*, Percentage of responding cells within subsets of NK cells expressing combinations of inhibitory KIR. Four donors sharing the same KIR ligands (HLA-C1 and HLA-A3) are shown. *C*, Summary of experiments showing the frequency of responding (CD107a^{+}) KIR2DL3-SP or KIR3DL2-SP NK cells in donors homozygous for HLA-C1 or -C2, and those positive for HLA-A3, -A11, or -A3/-A11, respectively. Mean values and SD are shown. *D*, Impact of NKG2A coexpression (filled bars) on the responsiveness of CD56^{dim} NK cells expressing no KIR (KIR^{-}), sKIR3DL2 (A3 donors), nsKIR2DL3 (C2/C2 donors), and sKIR2DL3 (C1/C1 donors). Error bars represent the SD.

educated NK cells could be evaluated in each individual (Table I). Importantly, the values in Table I represent frequencies of NK cells predicted to be functionally competent based on their education by cognate HLA class I molecules, not the actual frequencies of responding NK cells. Based on previous studies (37) and the results shown in Fig. 3, NK cells expressing at least one KIR for self HLA class I molecules were considered educated regardless of their expression of additional KIR for nonself ligands. Because recent data have shown that some HLA-A alleles bearing the Bw4 motif also serve as ligands for KIR3DL1 (51, 52), KIR3DL1-positive NK cells in donors carrying such HLA-A alleles (Bw4) were also included in our calculations of the educated repertoire. In contrast, KIR3DL2-single positive NK cells, shown here to be hyporesponsive in HLA-A3/A11-positive individuals (Fig. 3, *B* and

Table I. *Prediction of the educated NKG2A$^-$ NK cell repertoire*[a]

	HLA-C1	HLA-C2	HLA-BW4	HLA-A(BW4)	HLA-A3	HLA-A11		4 KIR	3 KIR				2 KIR						1 KIR				No KIR	At least 1 sKIR*
KIR2DL1								+	-	+	+	+	-	-	+	-	+	+	-	-	-	+	-	
KIR2DL3								+	+	-	+	+	-	+	-	+	-	+	-	-	+	-	-	
KIR3DL1								+	+	+	-	+	+	-	-	+	+	-	-	+	-	-	-	
KIR2DL2								+	+	+	+	-	+	+	+	-	-	-	+	-	-	-	-	
BC158	X			X				**3,4**	**2,9**	**2,3**	**5,2**	**3,2**	**2,3**	**5,5**	2,7	**3,8**	**2,5**	**7,1**	3,9	**3,8**	**10,8**	3,9	13,1	**52,8**
BC164	X	X						**0,0**	**0,0**	**0,0**	**7,3**	**0,0**	0,0	**8,1**	**4,0**	**0,0**	**0,0**	**2,7**	4,8	0,0	**5,6**	**2,3**	7,0	**30,0**
BC167	X	X						**0,3**	**0,6**	**0,3**	**1,7**	**0,6**	0,5	**2,6**	**1,9**	**1,4**	**0,8**	**4,0**	1,8	1,8	**9,0**	**6,0**	9,5	**29,2**
BC229		X	X	X				**0,7**	**1,4**	**0,7**	**2,1**	**1,5**	**1,8**	2,2	**3,1**	**2,5**	**2,2**	**5,3**	4,0	**4,5**	4,5	**11,0**	16,3	**36,8**
BC249	X				X			**1,7**	**5,6**	0,6	**0,9**	**3,0**	2,3	**3,1**	0,4	**8,8**	1,6	**2,1**	1,8	6,5	**8,9**	1,0	12,3	**34,1**
BC250	X					X		**3,9**	**3,5**	1,1	**4,5**	**1,6**	1,3	**6,2**	1,1	**1,9**	0,7	**2,2**	1,5	1,0	**35,1**	0,9	2,7	**58,9**
BC252	X			X				**1,6**	**1,7**	**1,7**	**4,5**	**0,6**	**1,9**	**6,6**	5,7	**0,8**	**0,9**	**2,4**	10,2	**1,2**	**4,2**	4,0	12,8	**28,1**
BC255	X				X			**0,2**	**0,3**	0,3	**1,6**	**0,7**	4,4	**0,5**	2,5	**1,8**	0,7	**4,3**	0,7	38,8	**2,9**	3,2	4,7	**12,3**
BC275	X	X	X			X		**0,6**	**3,5**	**1,4**	**2,0**	**0,3**	**2,2**	**2,6**	**39,0**	**1,4**	**0,8**	**1,3**	4,3	**3,3**	**3,7**	**4,0**	8,7	**66,1**
BC278	X	X	X					**0,8**	**1,3**	**1,3**	**4,0**	**0,6**	**2,1**	**8,2**	**5,9**	**1,1**	**1,1**	**3,6**	6,4	**3,2**	**9,1**	**8,3**	16,8	**50,6**
BC280		X	X					**0,5**	**1,3**	**0,5**	**0,9**	**1,3**	**1,4**	1,3	**1,0**	**1,5**	**0,9**	**2,0**	2,5	**2,9**	2,4	**3,0**	9,2	**17,2**
BC287	X				X	X		**3,7**	**5,4**	2,3	**2,9**	**4,7**	3,6	**4,0**	1,3	**8,4**	3,3	**4,3**	2,1	7,3	**9,6**	2,5	7,7	**43,0**
BC290	X							**3,8**	**4,4**	2,4	**4,0**	**1,3**	2,9	**6,2**	3,0	**1,8**	1,1	**1,6**	7,3	2,9	**5,4**	2,5	12,8	**28,5**
BC294	X	X	X		X			**0,5**	**2,2**	**0,4**	**2,6**	**0,2**	**1,8**	**8,1**	**2,5**	**0,8**	**0,2**	**1,4**	6,3	**1,2**	**4,8**	**1,7**	8,0	**28,4**
BC300		X	X					**0,2**	**0,5**	**0,2**	**1,4**	**0,1**	**0,7**	1,6	**2,5**	**0,4**	**0,2**	**1,6**	3,3	**1,0**	3,0	**3,6**	13,1	**12,4**
BC304	X			X	X			**0,9**	**2,6**	**0,8**	**1,6**	**0,3**	**2,7**	**8,0**	2,0	**1,2**	**0,4**	**1,5**	9,6	**1,9**	**7,9**	1,5	15,8	**29,8**
BC306	X		X					**0,0**	**0,0**	**0,0**	**10,9**	**0,0**	**0,0**	**16,3**	2,1	**0,0**	**0,0**	**9,2**	3,7	**0,0**	**16,8**	2,5	9,0	**53,2**
BC308		X	X					**0,5**	**1,2**	**0,6**	**2,3**	**0,4**	**1,7**	4,3	**2,2**	**0,6**	**0,6**	**1,9**	13,1	**3,1**	4,8	**3,0**	44,6	**18,1**
BC309	X				X			**0,0**	**0,0**	0,0	**2,3**	**0,0**	0,0	**2,9**	20,2	**0,0**	0,0	**5,3**	14,7	0,0	**4,4**	19,4	13,7	**14,9**
BC312	X		X					**0,1**	**0,4**	**1,0**	**0,7**	**0,3**	**0,6**	**2,3**	2,6	**0,6**	**2,3**	**0,9**	1,9	**1,8**	**1,4**	44,2	5,9	**12,4**
BC316	X		X	X		X		**0,7**	**2,1**	**1,1**	**2,0**	**0,2**	**3,7**	**7,8**	2,9	**0,4**	**0,4**	**0,8**	10,9	**1,0**	**3,9**	1,0	4,4	**24,1**
BC320	X	X	X					**2,0**	**4,0**	**1,0**	**2,0**	**0,6**	**2,4**	**4,0**	**1,6**	**0,7**	**0,4**	**0,5**	5,8	**0,7**	**1,1**	**1,0**	7,6	**22,0**
BC322		X	X	X				**0,1**	**0,9**	**0,2**	**0,8**	**0,3**	**1,9**	2,9	**1,5**	**1,1**	**0,4**	**1,8**	8,4	**3,2**	6,6	**4,1**	25,3	**16,3**
BC327	X							**1,1**	**0,9**	0,9	**3,0**	**1,4**	0,7	**3,4**	3,3	**1,7**	1,7	**5,9**	4,3	2,7	**10,1**	9,6	22,6	**27,5**
BC328	X				X			**8,8**	**9,7**	4,6	**7,4**	**1,3**	5,6	**10,6**	3,9	**2,2**	0,9	**1,6**	7,1	1,5	**3,5**	1,1	5,4	**45,1**
BC355	X	X		X		X		**0,0**	**0,0**	**0,0**	**5,8**	**0,0**	**0,0**	**6,7**	**3,9**	**0,0**	**0,0**	**9,8**	5,1	**0,0**	**7,6**	**4,9**	15,3	**38,7**
BC356		X	X					**1,5**	**0,8**	**0,7**	**4,5**	**0,6**	**0,6**	2,1	**23,2**	**0,6**	**0,5**	**25,3**	2,3	**0,7**	2,2	**7,5**	4,0	**66,5**
BC364	X		X					**1,1**	**1,8**	**0,9**	**2,5**	**0,3**	**1,3**	**16,8**	0,8	**0,7**	**0,2**	**0,7**	2,8	**0,7**	**40,6**	0,5	4,1	**67,6**
BC368	X	X	X					**2,9**	**4,2**	**0,9**	**3,3**	**1,7**	**1,5**	**4,2**	**1,1**	**3,1**	**0,5**	**3,2**	1,5	**1,9**	**4,9**	**1,7**	4,1	**35,1**
BC369	X							**2,1**	**3,7**	1,6	**3,6**	**0,7**	2,8	**6,9**	2,3	**1,9**	0,6	**1,9**	7,5	1,8	**6,2**	1,9	10,7	**27,0**
BC373		X	X	X				**0,2**	**0,3**	**0,2**	**1,2**	**0,2**	**0,5**	2,0	**1,4**	**0,7**	**0,3**	**2,4**	3,8	**1,7**	5,9	**4,9**	17,7	**14,0**
Mean								1,4	2,2	1,0	3,2	0,9	1,8	5,4	4,9	1,7	0,8	3,8	5,3	3,3	8,0	5,4	11,8	**33,6**

[a] sKIR (*) indicates self KIR and refers to NK cells expressing inhibitory KIR for self HLA class I molecules. NK cells expressing KIR3DL2 were not included in the predicted educated repertoire even in the presence of HLA-A3/A11 because such cells were hyporesponsive. Numbers in boldface represent NK cells expressing at least one inhibitory KIR for self HLA class I molecules and represent educated NK cell subsets. BC, buffy coat.

C), were not considered as educated despite the presence of HLA-A3/A11. In many hematological malignancies, including acute myeloid leukemia, the affected cell clone expresses abundant levels of HLA-E (53), and although NKG2A-positive NK cells are fully functional (Fig. 3*D*), such NK cells may not confer GVL effects in allogeneic SCT or during NK cell therapy. Therefore, the data in Table I depict the total repertoire of educated NKG2A-negative NK cells. The analysis revealed significant variations in the frequencies of educated NKG2A-negative NK cells in different donors ranging from only 12 to ~68% of the total CD56^{dim} NK cell population (mean 33%). Differences in the repertoire of educated, and therefore functional, NK cells among individuals may have consequences for their antiviral and antitumor immunity. It should be stressed that the total repertoire of educated NK cells, including those educated by NKG2A, was larger, ranging from 36 to 97% (mean 72%). Indeed, there was a significant inverse correlation between the frequency of educated NKG2A-negative NK cells and the overall frequency of NKG2A-positive NK cells (data not shown). To explore how these results may influence the interpretation of NK cell-mediated GVL effects in clinical SCT, we next calculated the size of the alloreactive NK cell subset for different donor-recipient combinations.

The size of the alloreactive NK cell repertoire

The educated NK cell repertoires calculated in Table I represent the total pool of potentially alloreactive NK cells contingent on which and how many KIR ligands a tentative recipient lacks. Previous analysis in a limited set of donors demonstrated that the size of the alloreactive repertoire is directly proportional to the killing of acute myeloid leukemia blasts (54). The data in Table I were used to calculate the size of the alloreactive NK cell subset given that one or more KIR ligands are missing (Fig. 4). On average, the repertoire of functionally mature NK cells that sensed missing

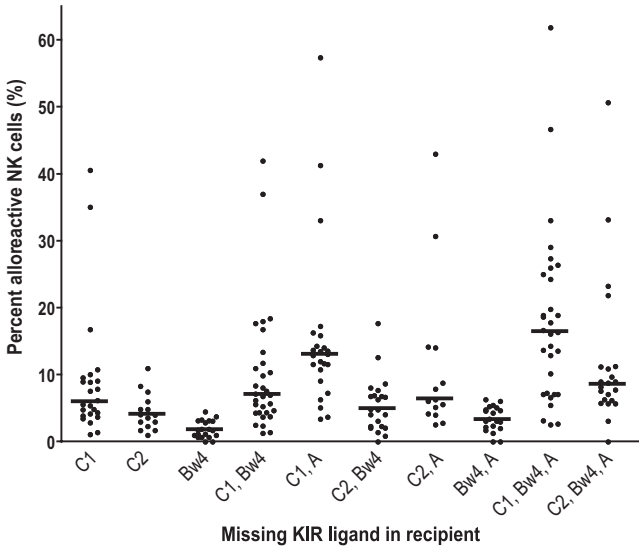

FIGURE 4. The size of the alloreactive NK cell repertoire. The percentage of educated NKG2A-negative CD56^{dim} NK cells alloreactive to theoretical recipients lacking the indicated HLA alleles or combinations thereof are shown. Values were obtained by summarizing frequencies of relevant subsets from Table I for each individual donor. Bars represent the median frequencies of alloreactive NK cells for the 31 individuals with the group A *KIR* haplotype included in the study.

Table II. *The size of the alloreactive NK cell repertoire*[a]

	C1	C2	Bw4	C1,Bw4	C1,A	C2,Bw4	C2,A	Bw4,A	C1,Bw4,A	C2,Bw4,A
BC158	10,8		3,8	18,4	16,3	6,3		6,1	29.1	10,9
BC164	5,6	2,3		5,6	13,7	2,3	6,3		13,7	6.3
BC167	9	6,0		10,4	11,6	6,8	7,9		13.6	9
BC229		11,0	4,5	7		17,7	14,1	6,3	10,2	23.3
BC249	8,9			17,7	12				26.4	
BC250	35,1			37	41.3				46,7	
BC252	4,2		1,2	6,2	10,8	2,1		3,1	16,4	5,7
BC255	2,9			4,7	3.4				5.5	
BC275	3,7	4,0	3,3	8,4	6.3	8,1	43	5,5	16.7	50.7
BC278	9,1	8,3	3,2	13,4	17,3	12,6	14,2	5,3	25	21,9
BC280		3,0	2,9	4,4		6,8	4	4,3	7,1	9,7
BC287	9,6			18	13.6				27.4	
BC290	5,4			7,2	11,6				17,8	
BC294	4,8	1,7	1,2	6,8	12.9	3,1	4.2	3	18.9	7.8
BC300		3,6	1	1,4		4,8	6,1	1,7	2,6	8,2
BC304	7,9		1,9	11	15.9	2.3		4,6	24.3	5.8
BC306	16,8		0	16,8	33,1	0		0	33,1	0
BC308		3	3,1	3,7		6,7	5,2	4,8	6,6	11,2
BC309	4,4			4,4	7.3				7.3	
BC312	1,4		1,8	3,8	3,7	4,1		2,4	7,1	5,7
BC316	3,9		1	5,3	11.7	1,4		4,7	18.9	6,2
BC320	1,1	1,0	0,7	2,5	5,1	2,1	2,6	3,1	12,9	7,1
BC322		4,1	3,2	4,3		7,7	5,6	5,1	7,1	11,3
BC327	10,1			11,8	13,5				16,1	
BC328	3,5			5,7	14.1				26	
BC355	7,6	4,9	0	7,6	14.3	4,9	8.8	0	14.3	8.8
BC356		7,5	0,7	1,3		8,7	30,7	1,3	2,7	33,2
BC364	40,6		0,7	42	57,4	0,9		2	61,9	3,1
BC368	4,9	1,7	1,9	9,9	9,1	4,1	2,8	3,4	19,8	7,6
BC369	6,2			8,1	13,1				18,7	
BC373		4,9	1,7	2,4		6,9	6,3	2,2	3,2	9
Avg.	9,1	4,5	1,9	9,9	16,3	5,6	11,4	3,4	17,7	10,0

[a] The size of the alloreactive repertoire was calculated by pooling educated NK cell subsets lacking NKG2A and KIR for the indicated HLA class I ligands. Gray indicates no available educated NK cells; Avg, average; A, HLA-A3 and/or HLA-A11.

HLA-C1 was the largest, followed by NK cell subsets sensing missing HLA-C2 and HLA-Bw4 ($p < 0.001$). Because NK cells expressing only KIR3DL2 were hyporesponsive, it was not meaningful to calculate the frequency of cells sensing missing HLA-A3/A11. Nevertheless, functional NK cells (educated by other KIR) coexpressing KIR3DL2 may be inhibited by HLA-A3/A11 (55–57). Thus for the present analysis, assuming that the KIR3DL2-HLA-A3/A11 interaction is inhibitory, we did not consider NK cells coexpressing, for example, KIR2DL3 and KIR3DL2, as alloreactive for recipients lacking HLA-C1 if they harbored either of the HLA-A3/A11 motifs. As expected, the alloreactive NK cell subset was larger for recipients lacking more than one KIR ligand, because it included NK cells expressing combinations of one or more self KIR with and without additional nonself KIR to HLA ligands missing in the recipient (Fig. 4 and Table II). In particular, missing HLA-C1 in combination with HLA-A3/A11 was associated with the most prominent alloreactive NK cell repertoire, and that likelihood was further increased when including cells sensing missing HLA-Bw4 ($p < 0.001$; Fig. 4 and Table II). All theoretically possible donor-recipient combinations were evaluated and are depicted in Fig. 4. To enable identification of the alloreactive NK cell subset in each individual donor and for all possible *HLA* genotypes in the recipient, we compiled all data in Table II. Altogether, this analysis revealed that the size of alloreactive repertoire ranged from 0 to 62% of the total NK cell repertoire in donor-recipient pairs, all of whom were considered KIR ligand mismatched as determined by the genetic algorithm.

Discussion

In this study, we report data obtained using a full inhibitory KIR Ab panel covering the four major KIR and CD94/NKG2A in 31 individuals homozygous for the group A *KIR* haplotype. The data enabled us to calculate frequencies of NK cells predicted to be functionally mature based on their education by cognate HLA class I ligands and to determine the size of the alloreactive NK cell repertoire given that one or more KIR ligands are missing in a tentative recipient. Grafts from donors with few alloreactive NK cells may not provide beneficial GVL effects in clinical SCT despite a genetically predicted NK cell alloreactivity.

New insights into the regulation of NK cell function have led to an increased interest in exploring their potential in clinical therapies against human cancer (3). One notable possibility is that the GVL effects are mediated by a repertoire of alloreactive NK cells following transplantation over *HLA* barriers. To date, the factors favoring such NK cell-mediated GVL effects in hematopoietic SCT remain elusive. Most likely, several differences in the transplantation protocols explain discrepancies in the results at different transplantation centers. These include the stem cell dose, the degree of T cell depletion, the posttransplantation immune suppression, and the GVHD prophylaxis (58, 59). In particular, the

presence of T cells in the hematopoietic graft seems to antagonize the recovery of potentially alloreactive KIR-expressing NK cells (60). We reasoned that differences in the repertoire of alloreactive NK cells among donors may provide an additional explanation for the failure of the genetic algorithm to predict NK cell-mediated GVL effect in some settings. Because inhibitory KIR are clonally distributed in the NK cell repertoire, the frequency of NK cells expressing one single KIR and combinations of multiple KIR varies from donor to donor (40, 61). Thus, although a particular donor is mismatched to the recipient on a genetic level with respect to *KIR* and *HLA* genes, the frequency of alloreactive NK cells may be very low. In this study, by using a multiparameter flow cytometry and an Ab panel covering the four major KIR and NKG2A, we have identified the educated NK cell repertoire in 31 donors homozygous for the group A *KIR* haplotype. Our results show that the size of their alloreactive repertoires varied substantially, i.e., from 0 to 62% of the total NK cell repertoire. The alloreactive NK cell repertoires sensing missing HLA-C1 were the largest, followed consecutively by those sensing missing HLA-C2 and HLA-Bw4. In haploidentical SCT or in therapies based on adoptive NK cell transfer from haploidentical donors, a recipient may lack up to three inhibitory KIR motifs. Donor-recipient combinations in which three KIR ligands were missing naturally yielded a larger alloreactive NK cell subset, albeit only ~2-fold for recipients missing only one KIR ligand. On a population level, these data could theoretically translate into GVL effects being more prominent in transplantation from HLA-C1/C2 heterozygous donors to HLA-C2 homozygous recipients compared with other donor-recipient combinations and in situations when more than one KIR ligand is missing. However, individual assessment of each donor-recipient pair is required, and interpretation of these data in a population-based context should be done with caution. Yet, differences in the size of the alloreactive repertoire are likely to influence the ability of NK cells to eradicate leukemic cells and may therefore affect the outcome of the treatment. Indeed, results from experiments performed in vitro suggest that donors with higher percentages of alloreactive NK cells had higher levels of cytolytic activity against leukemic cells (54). Thus, we believe that it will be important to perform a full phenotypic and functional characterization of the alloreactive NK cell repertoire to gain a better understanding of the ability of NK cells to mediate GVL effects and to pinpoint the transplantation settings that favor such events. Similarly, the type of analysis performed in this study may be included in algorithms used to select donors for adoptive NK cell-based immunotherapy.

Most mAbs to KIR molecules fail to discriminate inhibitory KIR from their activating counterparts; therefore, to ensure stringent analysis of the alloreactive repertoire, this study was restricted to donors who were homozygous for the group A *KIR* haplotype. The methods used in this study enabled us to calculate frequencies of NK cells predicted to be functionally mature based on their education by cognate HLA class I ligands and to determine the size of the alloreactive repertoire given that one or more KIR ligands were missing in a tentative recipient. Because the frequency of donors homozygous for the group A *KIR* haplotype varies among ethnicities (43, 62), some transplantation centers may have limited numbers of donor-recipient pairs available for this type of analysis. For example, to study 31 individuals homozygous for the group A *KIR* haplotype, we screened 114 donors, equivalent to a frequency of 27% group A *KIR* haplotype donors in the Swedish population. This should be compared with 80% in the Japanese population where this haplotype dominates (62, 63). Assessing the size of the alloreactive NK cell repertoire will also be important in donors with the haplotype B *KIR* genotype. Although the present study was restricted to individuals homozygous for the group A *KIR* haplotype, we believe this should be possible and have made efforts to design Ab panels using only mAbs with a more restricted specificity for inhibitory KIR (data not shown). Future studies should also take into account effects mediated by activating KIR. Outcomes from several studies have suggested an epistatic interaction between activating KIR and different HLA alleles that affect key outcomes in transplantation including GVHD, relapse, and survival (16, 20, 24, 27, 29). To date, no clear consensus exists for how activating KIR influence the result of clinical transplantation, and the biological basis for such events remains unknown. Such studies await the development of more specific reagents for activating KIR.

To estimate the size of the alloreactive NK cell subset, we had to consider the repertoire of educated NK cells in each individual based on knowledge of their *HLA* background. We now know that NK cells must undergo an education process to become functionally competent (31, 34–37). NK cells lacking inhibitory receptors or expressing only inhibitory KIR for nonself HLA class I molecules are hyporesponsive (31, 35–37). In this study, we have shown that KIR3DL2-single positive NK cells are hyporesponsive even in donors that harbor the cognate ligands (HLA-A3/A11). Based on these results, KIR3DL2-single positive NK cells were excluded from the repertoire of educated NK cells that formed the basis for calculations of the size of the alloreactive NK cell subset. Thus, these data give experimental support for ignoring KIR3DL2 HLA-A3/A11 interactions, as is currently being done by most genetic algorithms, for the identification of donors with an alloreactive NK cell repertoire in allogeneic SCT. The interaction between KIR3DL2 and HLA-A3/A11 was previously found to depend on the peptides bound by the HLA-A3/A11 molecules (56), thus in part solving the controversy about whether this interaction is inhibitory (55, 57) or not (40). In particular, it was shown that EBV-derived peptides conferred recognition of HLA-A3/A11 by KIR3DL2. In that study it could not be excluded that a limited set of self-peptides would allow recognition of the HLA-A3/A11 alleles by KIR3DL2 (56). The observation that KIR3DL2-single positive NK cells were nonfunctional in individuals expressing HLA-A3/A11 could suggest that no such self-peptides were available in these donors at sufficient levels to mediate education through a functional interaction between KIR3DL2 and HLA-A3/A11. It is conceivable that the interaction between KIR3DL2 and HLA-A3/A11 primarily modulates the host response to pathogens such as EBV and/or tumors including EBV-induced lymphomas. In this study, we have considered the KIR3DL2 HLA-A3/A11 interaction to be inhibitory at the target cell level, albeit that it did not confer significant education of KIR3DL2-single positive NK cells. For situations in which this interaction is predicted to be noninhibitory, the size of the alloreactive repertoires may be slightly larger.

KIR3DL2-single positive NK cells were found to more frequently coexpress NKG2A compared with NK cells expressing any of the other three inhibitory KIR. Moreover, a gradual decrease in NKG2A expression was observed, being most frequent on NK cells lacking all KIR and least frequent on NK cells coexpressing all four inhibitory KIR. Thus, NKG2A expression correlated inversely, not only with the total frequency of KIR-expressing cells in an individual (33), but also with the number of KIR coexpressed by specific NK cell subsets. Taken together, these observations can be interpreted in at least two ways. First, if the interaction between HLA-A3/A11 and KIR3DL2 is nonfunctional, there may be developmental pressure for KIR3DL2-single positive NK cells to coexpress NKG2A, similar to KIR-negative NK cells

for the preservation of tolerance to self. However, such complementary actions of KIR and NKG2A fit rather poorly in this context, because KIR3DL2-single positive NK cells are hyporesponsive and thus have little need to express alternative inhibitory receptors. A second possibility is that the frequent coexpression of NKG2A and KIR3DL2 is a consequence of the functional maturation of NK cells rather than a result of mechanisms striving to preserve tolerance to self. As shown previously and confirmed in this study, NK cells expressing only NKG2A are fully functional (Fig. 3*D*; Ref. 46). Because NKG2A expression was less frequent on NK cells coexpressing greater numbers of KIR, such cells may represent later stages of NK cell differentiation. NK cells expressing no KIR or KIR that fails to engage in functional interactions with cognate HLA would, hypothetically, require additional maturation steps before they become functional and thus gradually lose their NKG2A expression. In this context, one may speculate that the frequent coexpression of KIR3DL2-single positive NK cells reflects the fact that these cells have not been educated by HLA-A3/A11 and remain functionally immature. This latter possibility is compatible with studies on NK cell development showing that immature NK cells, at an early stage, start to express NKG2A followed sequentially by the stochastic expression of various KIR (64).

The complex regulation of NK cell receptor repertoire formation and development of tolerance clearly demonstrate that algorithms used to identify donors with an alloreactive NK cell repertoire and those without, based solely on *KIR* and *HLA* genotyping, is an oversimplification that risks blurring the interpretation of results. In this study, we have used a full inhibitory KIR panel and multicolor flow cytometry to calculate the frequency of educated NK cells that could contribute to NK cell alloreactivity in SCT. The analysis clearly demonstrates that the size of the alloreactive NK cell subset varies from donor to donor and often represents only a minor fraction of the total NK cell repertoire. Because the KIR repertoire of the recipient is primarily determined by the *KIR* genotype of the donor (32), the variability in the size of donor-alloreactive NK cell subsets may provide one additional explanation for the failure of genetically predicted NK cell alloreactivity to translate into clinically visible GVL effects. The analysis performed in this study can serve as a platform to complement the genetic analysis of *KIR* and *HLA* genotypes and allow better interpretation of the role of alloreactive NK cells in settings of allogeneic SCT. Future prospective studies of the size of the alloreactive repertoire in the donor and the functional maturation of this subset during immune reconstitution may shed light on which components of the transplantation procedure favor NK cell-mediated GVL effects.

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Disclosures

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