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SHORT COMMUNICATION

Expression of KIR2DS1 does not significantly contribute to NK cell cytotoxicity in HLA-C1/C2 heterozygous haplotype B donors

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Abstract

NK cells are functionally controlled by the killer immunoglobulin-like receptor (KIR) family that comprises inhibitory (iKIR) and activating (aKIR) members. Genetic association studies suggest that donors expressing aKIRs next to iKIRs will be superior donors in the setting of hematopoietic stem cell transplantation of patients with leukemia. However, contrary evidence states that aKIR expression may be irrelevant or even detrimental. Using a complex methodology incorporating KIR-Q-PCR, double fluorescence and viSNE analysis, we characterized subset distribution patterns and functionality in haplotype A donors which lack aKIRs and haplotype B donors that express a variety of B-specific genes. Here, we show that the alloreactive KIR2DS1$^+$ NK cell subset in HLA-C1/C2 donors is highly responsive towards C2-expressing targets but quantitatively small and as such does not significantly contribute to cytotoxicity. Thus, we fail to find a direct link between haplotype allocation status and NK cell cytotoxicity at least in HLA-C1/C2 heterozygous donors.

Keywords: graft-versus-leukemia effect, killer immunoglobulin-like receptor, natural killer cell

Introduction

NK cells are functionally controlled by the HLA class I-restricted killer immunoglobulin-like receptor (KIR) family that comprises both inhibitory (iKIR) and activating (aKIR) family members which recognize allotypic variants of HLA class I alleles (KIRL). Depending on the absence or presence of aKIRs, two haplotypes are distinguished: haplotype A donors express a canonical iKIR gene content while haplotype B donors have a variable gene content with one or more of the B-specific genes (KIR2DS1, 2, 3, 5, KIR3DS1, KIR2DL2 and/or KIR2DL5) (1). Genetic association studies suggest that a donor who provides an iKIR-KIRL mismatch towards the recipient (2), but who also possesses aKIRs (3, 4) and who ideally expresses multiple aKIRs (3, 5), will be the potentially 'optimal' donor in the setting of hematopoietic stem cell transplantation (HSCT) to treat patients with leukemia as NK cell-mediated graft-versus-leukemia (GvL) effects are maximally exploited.

However, controversy exists about the usefulness to select a donor based on genetically predicted determinants of NK cell alloreactivity. This may in part be explained by the fact that the prediction of alloreactivity by HLA/KIR genotyping alone does not incorporate information on the actual size of the alloreactive subset which differs considerably from donor to donor (6) and the circumstance that discrepancies may exist between KIR genotype and protein expression (7). Moreover, only few studies have been able to directly document a binding of aKIRs to HLA class I molecules, presumably as a result of weak or sometimes even non-existent binding. So far, KIR2DS1 is the only aKIR known to play a clinically relevant role in the context of alloreactivity and tolerance. In this regard, it has been shown that KIR2DS1 is able to recognize HLA-C2-expressing targets and to mediate alloresponses (8–10); however, its binding affinity is distinctly lower than that of its inhibitory counterpart KIR2DL1 (11). In addition, aKIRs are assumed to be a subject of education to prevent autoimmunity and, in line with this, it has been demonstrated in HLA-C2 homozygous hosts that KIR2DS1$^+$iKIR$^-$NKG2A$^-$ NK cells are hyporesponsive (12, 13).

Thus, we face the situation in which genetic association studies suggest a clinical benefit of 'multiple aKIR' expression;

however, a sound scientific basis to document this is missing. Using expanded NK cell preparations of HLA-C1/C2 heterozygous donors as effectors and the HLA-C2-transfected B lymphoblastoid L721.221-cw*0602 cell line as a target, we here apply a complex methodology incorporating KIR-Q-PCR, double fluorescence analysis and viSNE analysis to pursue the question of how large the alloreactive KIR2DS1$^+$ NK cell subset actually is and to what extent it contributes to NK cell cytotoxicity.

Methods

NK cell expansion procedure

PBMCs from volunteer HLA-C1/C2 donors were subjected to sequence-based four-digit resolution [sequence-based typing (SBT)] typing in accordance with the Declaration of Helsinki (Supplementary Table 1, available at *International Immunology* Online). NK cells were activated and expanded as described before using the HLA-null K562-mbIL15-41BBL transfectant (14, 15). Prior to experiments, these expanded NK cells [NK cell activation and expansion (NKAES)] were cultured overnight in the presence of 100 IU ml^{-1} IL-2 for *in vitro* cytotoxicity assays or characterization experiments and 200 IU ml^{-1} IL-2 for functional response staining.

Cell lines

K562-mbIL15-41BBL cells (14) and L721.221-cw*0602 (16) were maintained at 0.1–0.5 × 10^6 cells ml^{-1} in RPMI-1640 containing 10% FCS, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 2 mM L-glutamine or rather RPMI-1640 containing 10% FCS, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2 mM L-glutamine and 1 mM pyruvate and selected by 0.4 mg ml^{-1} hygromycin B resistance.

KIR genotyping and haplotype group assignment

KIR genotyping was performed as previously described (15). The B content score, the KIR genotype group assignment and the centromeric and telomeric gene content motif assignment were assessed as previously described (3).

Flow cytometry

Live, vital cells were selected and doublets excluded based on scatter characteristics and low (auto-)fluorescence intensities after incubation with ARD- or Pacific Blue-Succinimidyl Ester (Thermo Fischer Scientific). Antibodies and their corresponding isotype controls were purchased from BD Bioscience, Biolegend, Beckman Coulter, Miltenyi Biotec, R&D Systems and Abcam PLC. The following antibody clones were used: anti-NKp30 (clone Z25), anti-NKp44 (clone Z231), anti-NKp46 (clone 9-E2), anti-NKG2A (clone Z199), anti-NKG2D (clone BAT221), anti-CD16 (clone 3G8), anti-CD25 (clone 2A3), anti-CD69 (clone L78), anti-HLA-A/B/C (clone W6/32), anti-HLA-E (clone MEM-E/08), anti-CD112 (clone TX31), anti-CD155 (clone SKII.4) and anti-pan-NKG2D-L (clone AMO1, BMO1, AUMO3, BUMO1 and CUMO3)—provided by A. Steinle, Frankfurt, Germany (17). For functional response staining, the following mAbs were used: anti-CD107a (clone H4A3), anti-CD56 (clone NCAM16.2), anti-CD3 (clone UCHT1), anti-CD94/NKG2A (clone 131411), anti-KIR2DL1/S5 (clone 143211; abbreviated to KIR2DL1 as 2DS5 is considered to be 'uneducated'), anti-KIR2DL1/S1 (clone EB6B), anti-KIR2DL2/L3/S2 (clone GL183), anti-KIR2DS3/S4 (clone JJC11.6), anti-KIR3DL1 (clone DX9) and anti-KIR3DL2 (clone Q66)—provided by D. Pende, Genoa, Italy. Note that anti-KIR2DL1/S1 mAb was added 15 min after addition of the other antibodies to allow the analysis of all KIR2DS1$^+$ NK cells (12, 18).

Determination of in vitro cytotoxicity

The target cell line L721.221-cw*0602 was labeled with 0.5 µM CFSE (Vybrant CFDA SE Cell Tracer Kit®, Invitrogen) 1 day prior to use. NK cells and L721.221-cw*0602 cells were co-incubated for 5 h. Subsequently, cells were stained with Pacific Blue-Succinimidyl Ester (Thermo Fischer Scientific) and analyzed by flow cytometry. To correct for spontaneously occurring cell death, target cell monoculture controls were included in every experiment. The percentage of specific cytotoxicity was calculated as follows: (%CFSE$^+$PB$^+$ dead targets – %CFSE$^+$PB$^+$ spontaneously dead targets)/(100 – %CFSE$^+$PB$^+$ spontaneously dead targets) × 100%.

Functional NK cell response staining

NK cells were co-cultured for 6 h with L721.221-cw*0602 cells (E:T ratio 1:3) in the presence of anti-CD107a (clone H4A3) and Golgi-Plug (BD Bioscience). Subsequently, NK cells were stained with the indicated surface antibodies, permeabilized with Fix&Perm (Nordic-MUbio) and stained with perforin-bv510 (clone dG9). The identification and functional characterization of the respective alloreactive NK cell subset were achieved using Boolean gating strategy (Supplementary Figure S1, available at *International Immunology* Online). Percentages of the respective NK cell subpopulation were normalized to the baseline levels of NKAES cells cultured in control medium only.

viSNE analysis

viSNE maps (bh-SNE1/bh-SNE2) were calculated with the default settings (Barnes-Hut approximation, using no_dims = 2, perplexity = 30.000000, and theta = 0.500000) of the interactive visualization tool *cyt* (19) from Dana Pe'er's lab (http://www.c2b2.columbia.edu/danapeerlab/html/cyt-download.html, last accessed October 18, 2017) which was run on Matlab (9.0.0.341360). Viable singlet CD56$^+$CD3$^-$NKG2A$^-$CD107a$^+$ or perforin$^+$ cells from six haplotype A and six haplotype B donors were gated in FACSDiva (BD, Heidelberg, Germany) and exported as FCS 2.0 files. Each file was subsampled with *cyt* and a sample size of ~10 000 or 4881 events per donor was included for the viSNE analysis. All 12 donors were run in the same viSNE analysis to enable direct comparison between the donor groups. The following parameters were included: KIR2DL1/S5, KIR2DL1/S1, KIR2DL2/L3/S2, KIR2DS3/S4, KIR3DL1, KIR3DL2, perforin and CD107a. Scale of X and Y axis: fixed from –40 to 40 or –50 to 50; color scale: 0 (blue) to 600 (red).

Statistics

Statistical evaluation was performed using GraphPad Prism version 6 (La Jolla, CA, USA), applying the two-tailed

Table 1. KIR-KIRL repertoire constellations of the haplotype A and B donors included in this study in reference to L721.221-cw*0602 cells

Haplotype	Donor ID	KIRL	Donor education of KIRs		Phenotype of the presumably alloreactive NK cell subset in response to L721.221-cw*0602 (C2)[b]
			Educated	Uneducated	
A	SNK18U	ABw4 Bw4/Bw4 C1/C2	2DL1, 2DL3, 3DL1	3DL2, 3DL3[a]	3DL1
	SNK24B	Bw4/Bw4 C1/C2	2DL1, 2DL3, 3DL1	3DL2, 3DL3[a]	3DL1
	SNK49S	A3 ABw4 Bw4/Bw4 C1/C2	2DL1, 2DL3, 3DL1, 3DL2	3DL3[a]	3DL1, 3DL2
	SNK65W	Bw6/Bw4 C1/C2	2DL1, 2DL3, 3DL1	3DL2, 3DL3[a]	3DL1
	SNK86W	ABw4 Bw6/Bw6 C1/C2	2DL1, 2DL3, 3DL1	3DL2, 3DL3[a]	3DL1
	SNK87J	Bw6/Bw4 C1/C2	2DL1, 2DL3, 3DL1	3DL2, 3DL3[a]	3DL1
	SNK17K	Bw4/Bw6 C1/C2	2DL1, 2DL3, 3DL1, 2DS1, 3DS1	2DL5[a], 3DL2, 3DL3[a], 2DS3[a]	3DL1, 2DS1
	SNK22B	Bw6/Bw6 C1/C2	2DL1, 2DL2, 2DL3, 2DS1	2DL5[a], 3DL1, 3DL2, 3DL3[a], 2DS2[a], 2DS5[a]	3DL1, 2DS1
B	SNK32R	A3 Bw6/Bw6 C1/C2	2DL1, 2DL3, 3DL2, 2DS1	2DL5[a], 3DL3[a], 2DS5[a], 3DS1	3DL2, 2DS1
	SNK33S	ABw4 Bw6/Bw6 C1/C2	2DL1, 2DL3, 3DL1, 2DS1	3DL2, 3DL3[a], 2DL5[a], 2DS5[a], 3DS1	3DL1, 2DS1
	SNK41K	Bw4/Bw6 C1/C2	2DL1, 2DL3, 3DL1, 2DS1, 3DS1	2DL5[a], 3DL2, 3DL3[a], 2DS5[a]	3DL1, 2DS1
	SNK94B	Bw4/Bw4 C1/C2	2DL1, 2DL3, 3DL1, 2DS1, 3DS1	2DL3[a], 2DL5[a], 3DL2, 3DL3[a], 2DS5[a]	3DL1, 2DS1

Given is the internal de-identification code of the NK cell donors (SNK), the donor-specific KIR repertoire [as determined by (Q)-PCR] relevant for characterization of the alloreactive subset (38, 39) and the identification of the educated KIRs that are specific for the missing KIRL in the target cells. The following KIRs have been excluded from this table: KIR2DL4, which is expressed by all donors (data not shown) and which recognizes HLA-G; KIR3DS1, which is not considered as 'classical' alloreactive KIR; and KIR2DS4, which is to a large extent a soluble receptor that does not recognize cw*0602.

[a]Grouped as 'uneducated' as the ligand is not defined (10, 38, 39).

[b]C2 (cw*0602) is only recognized directly by KIR2DL1 and 2DS1 (9–11, 21). Note, that the weak binding properties of HLA-C2 (cw*0602) to KIR2DL2 and 2DL3 (10, 40, 41) have been left aside.

Mann–Whitney test for comparison of the two haplotype groups of non-paired data. Significant values were defined as $P \leq 0.05$.

Results and discussion

To answer the question of to what extent aKIRs contribute to NK cell cytotoxicity, we selected six HLA-C1/C2 heterozygous haplotype A and B donors (Supplementary Table 1, available at *International Immunology* Online), knowing that KIR2DS1 is subject to education only in HLA-C2 homozygous but not heterozygous donors (12, 13). Given that the activating KIR2DS1 is able to directly recognize C2 epitopes (8–11, 20, 21), we chose only haplotype B donors with geno- and phenotypically detectable KIR2DS1 (Supplementary Table 2 and Figure S1, available at *International Immunology* Online) and selected L721.221-cw*0602 cells as the target cell line which expresses a HLA-C2 epitope but notably also other important NK cell receptor ligands (Supplementary Figure S2A and B, available at *International Immunology* Online). Knowing that unlicensed and licensed NK cells maintain their KIR-KIRL-determined licensing status upon *in vitro* expansion with the HLA-null K562-mbIL15-41BBL cell line (22), we predicted the specificity of the alloreactive NK cell subset pool that should exert alloreactivity in response to L721.221-cw*0602 cells, i.e. NKG2A$^-$KIR2DL1$^-$KIR3DL1$^+$ and/or KIR3DL2$^+$ NK cells in any donor and additionally KIR2DS1$^+$ NK cells in haplotype B donors (Table 1, last column). In addition, we verified that the receptor expression of important activating and inhibitory receptors other than KIRs did not significantly differ between haplotype A and B donors (Supplementary Figure S3A, available at *International Immunology* Online) and that the ability to exert cytotoxicity towards the null-mutant 721.221 or K562 was comparable (Supplementary Figure S3B, available at *International Immunology* Online).

Interestingly, *in vitro* cytotoxicity assays evidenced that haplotype A and B donors have a comparable ability for cytotoxicity (Fig. 1A). Analysis of the subset size revealed a comparable pool of alloreactive KIR3DL1$^+$, 3DL2$^+$ and/or KIR2DS1$^+$ NKG2A$^-$KIR2DL1$^-$ NK cells in haplotype A (16.76 ± 7.2%) and B (16.11 ± 7.1%) donors (Fig. 1B and C). However, the size of the KIR2DS1 single-positive NKG2A$^-$KIR2DL1$^-$ NK cell subset was very small in haplotype B donors (1.41 ± 0.85%) and the frequency of double-positive KIR2DS1$^+$3DL1$^+$ or KIR2DS1$^+$3DL2$^+$ NK cells was negligible (data not shown). To further delineate the relative contribution of KIR2DS1 expression to overall NK cell functionality, we performed viSNE analysis gating on the CD107a$^+$NKG2A$^-$ (Fig. 2A and B) or perforin$^+$NKG2A$^-$ (Supplementary Figure S4, available at *International Immunology* Online) NK cell

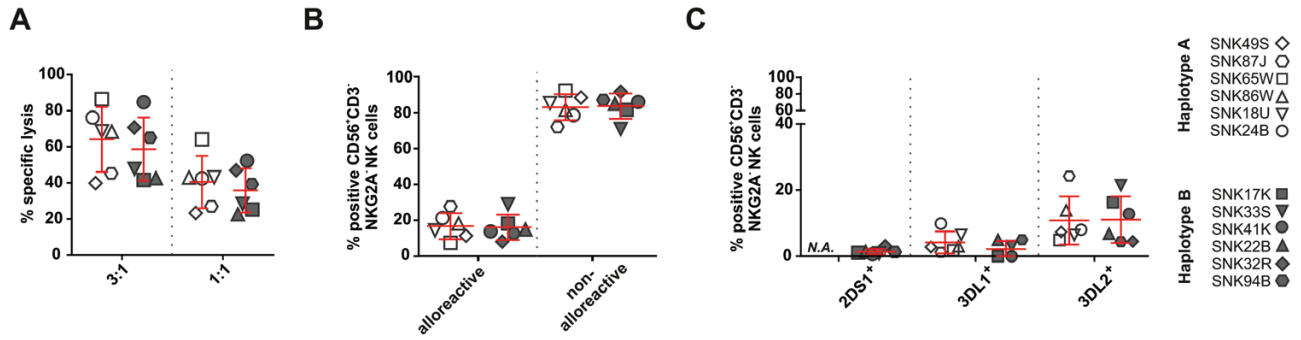

Fig. 1. Haplotype A and B donors exhibit comparable NK cell cytotoxicity. (A) *In vitro* cytotoxicity assays. NK cells of six haplotype A (open) and B (filled) donors were tested with respect to phenotype or cytotoxic functionality in co-culture with L721.221-cw*0602 cells. (B, C) The size of the alloreactive NK cell pool is comparable in HLA-C1/C2 heterozygous haplotype A and B donors. Size of the single-, double- and triple-positive alloreactive NK cell pool (B) and size of the 2DS1$^+$, 3DL1$^+$ and 3DL2$^+$ single-positive NK cell subset (C) as determined by double fluorescence analysis. Pooled data of each scatter plot showing mean ± SD. Note, that cells have been gated on NKG2A$^-$KIR2DL1$^-$ NK cell fraction. There is no statistical difference between haplotype A and B donors as determined by two-tailed Mann–Whitney *U*-test. N.A., not applicable.

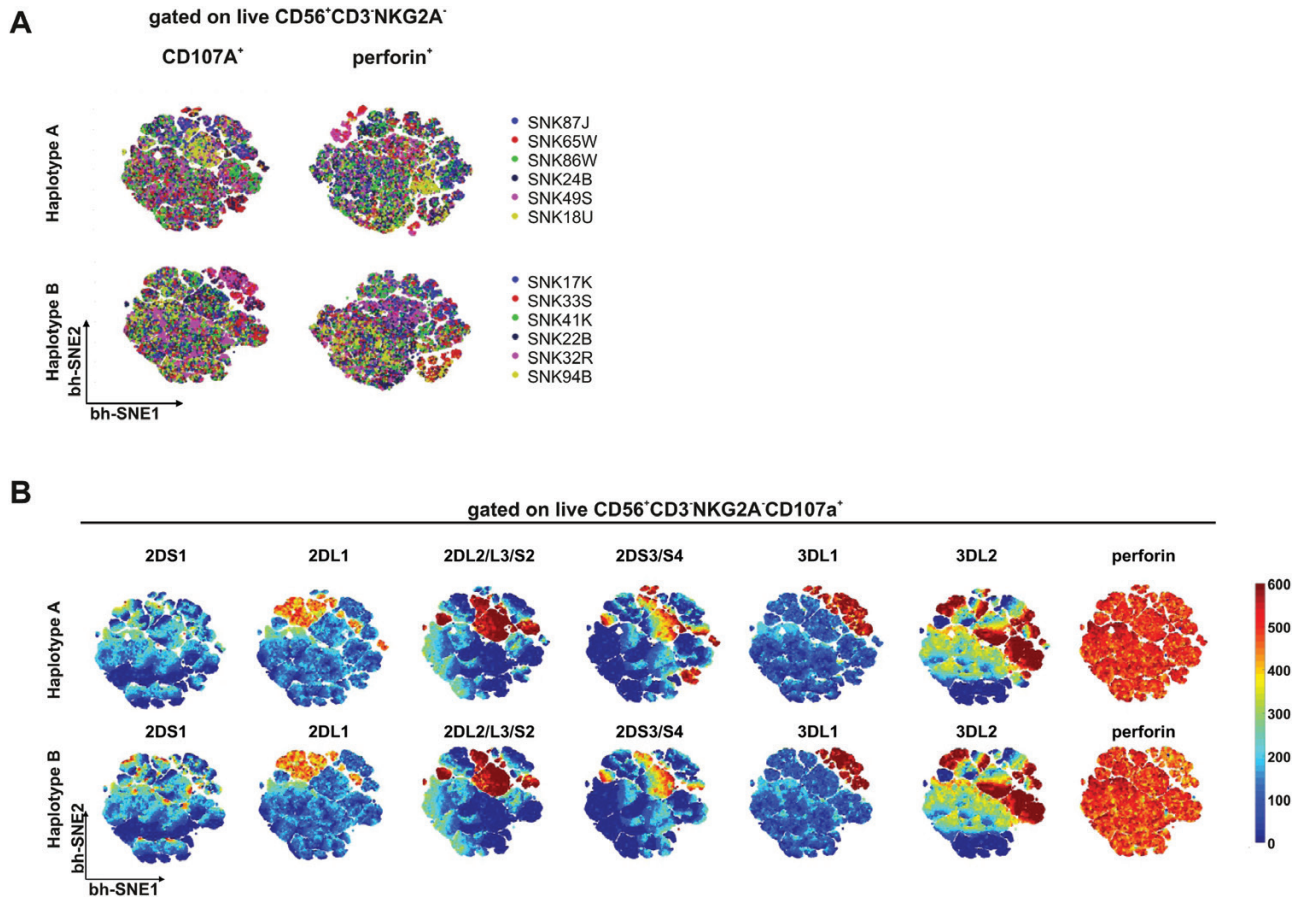

Fig. 2. viSNE analysis shows similar clustering of CD107a$^+$NKG2A$^-$ NK cells in haplotype A and B donors. viSNE maps (bh-SNE1/bh-SNE2) of six haplotype A (top) and B donors (bottom). In the third dimension (color scale), the relative expression level of the respective antigen is shown. (A) Identification of each donor by individual color coding. (B) viSNE analysis of CD107a$^+$ NK cells after co-culture with L721.221-cw*0602 cells. Note that haplotype B donors express a minute KIR2DS1$^+$NKG2A$^-$ NK cell subset which is distinctly CD107a$^+$.

subset. As expected, CD107a$^+$ or perforin$^+$ NK cells clustered similarly in haplotype A and B donors but only haplotype B donors expressed minute KIR2DS1$^+$NKG2A$^-$ NK cell subsets (Fig. 2B; Supplementary Figure S4, available at *International Immunology* Online). Functional response staining showed that a large proportion of this KIR2DS1$^+$ single-positive NK cell subset indeed expressed CD107a (mean 41.4 ± 16.74%) or degranulated to a significant extent (mean Δ: −40.25 ± 23.13%) (Fig. 3A). However, when analyzing the bulk alloreactive NK cell pool (Fig. 3B), the alloreactive

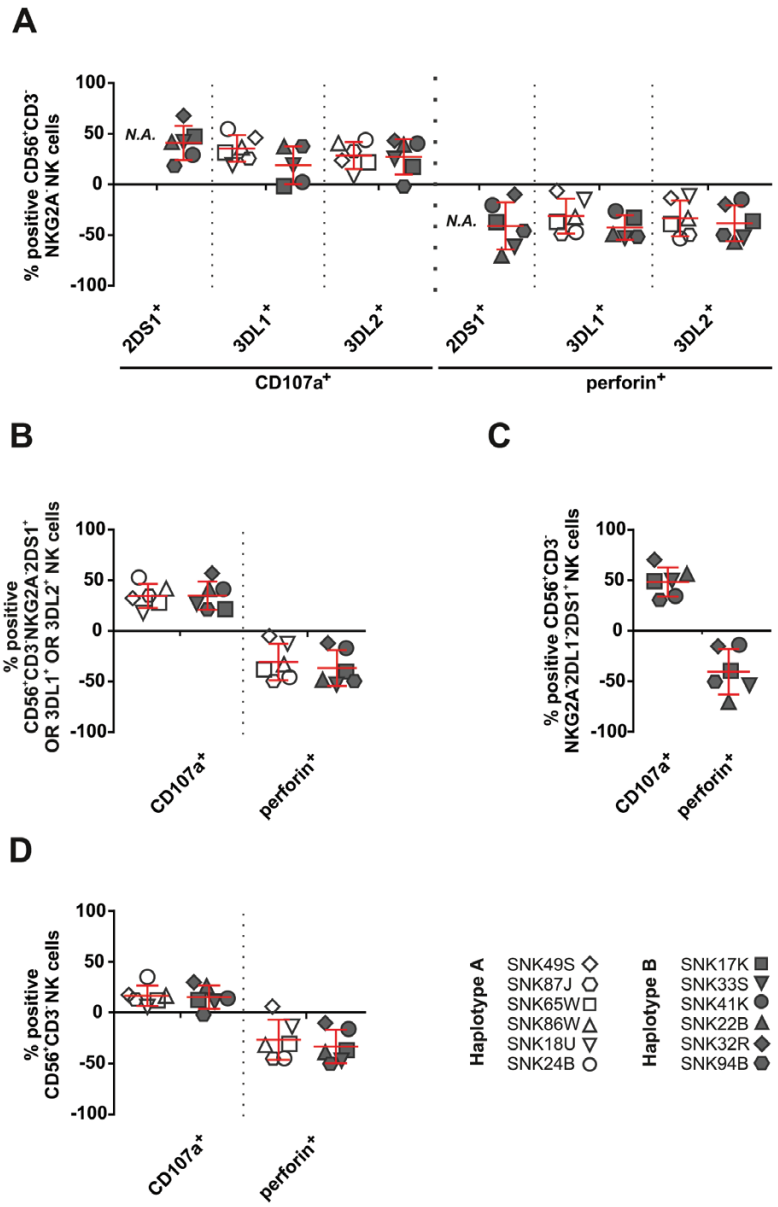

Fig. 3. The alloreactive KIR2DS1⁺NKG2A⁻KIR2DL1⁻ NK cell subset in HLA-C1/C2 donors is functionally highly responsive but quantitatively too small to contribute to overall NK cell cytotoxicity. (A) Size of CD107a- or perforin-expressing single-positive 2DS1⁺, 3DL1⁺ or 3DL2⁺ NKG2A⁻KIR2DL1⁻ NK cells. Note, that the 2DS1⁺ single-positive NK cell subset is to a large extent CD107a⁺ or secretes perforin. (B) Size of the CD107a- or perforin-expressing total alloreactive NK cell pool and (C) alloreactive KIR2DS1⁺KIR2DL1⁻ subset that may express other KIRs next to KIR2DS1. (D) Size of the CD107a- or perforin-expressing total CD56⁺CD3⁻ NK cell pool. Note, that despite the high CD107a expression of the KIR2DS1⁺ alloreactive NK cell subset, the overall capacity of haplotype B donor NK cells for degranulation and perforin secretion is comparable to haplotype A donors. Percentages in A–D are normalized to the corresponding baseline levels of expanded NK cells cultured in control medium only. As NK cell degranulation is accompanied with a decline in perforin levels, the numbers are negative.

KIR2DS1⁺KIR2DL1⁻ subset which may express other KIRs next to KIR2DS1 (Fig. 3C) or the total CD56⁺CD3⁻ NK cell pool (Fig. 3D), we did not see any differences whatsoever in the ability for degranulation or perforin synthesis upon co-culture with L721.221-cw*0602 cells. Thus, we show that the alloreactive KIR2DS1⁺NKG2A⁻KIR2DL1⁻ NK cell subset in HLA-C1/C2 donors is functionally highly responsive but quantitatively very small. In this, we fail to obtain a proof for the biological basis of aKIR functionality in HLA-C1/C2 donors but instead demonstrate that alloreactive NK cells of haplotype A and B donors do not differ in their net ability to exert cytotoxicity towards a C2-expressing target cell line. Considering the substantial evidence that MHC class I expression is partially or completely lost in various tumor types, we at this point assume that the alloreactivity of the KIR2DS1⁺ NK cell subset will even be lower when studied towards primary tumor specimens.

Without doubt, there is sound epidemiologic evidence for a beneficial effect of aKIR expression obtained in large cohort studies (3–5). However, contrary evidence also exists that

aKIR expression may either be irrelevant or even detrimental (23–27). To make the matter even more complicated, aKIRs do not only mediate GvL effects but also contribute to the prevention of cytomegalovirus (CMV) re-activation (28, 29) and may reduce the incidence of graft-versus-host disease (GvHD) (23, 30). Thus, we have to consider the possibility that the benefits associated with the haplotype B status are not necessarily attributable to aKIR-mediated GvL effects. As CMV infection itself stably imprints the KIR repertoire (31) and as such promotes the clonal expansion of an NKG2C$^+$aKIR$^+$ NK cell compartment (32, 33), it is conceivable that such an augmented alloreactive NK cell pool will indeed exert clinically relevant 'secondary or induced' GvL effects. In line with these thoughts, transplantation from 69 KIR2DS1$^+$ and/or KIR3DS1-expressing donors was associated with a reduced risk of non-relapse mortality which was largely infection-related (34). With respect to the pathophysiologic origin of GvHD occurrence, it has been speculated that the improved clinical outcome in haplo-HSCT with KIR2DS1-expressing donors may to a lesser degree be a result of improved NK cell cytotoxicity but rather a result of increased NK cell-mediated killing of C2/C2 or C1/C2 dendritic cells (DCs) and allogeneic T cells (20). In addition, the acquisition of the chemokine receptor CCR7 by NK cells is KIR2DS1-dependent and essentially results in increased migratory properties of NK cells to recipient lymph nodes which may prevent priming of recipient's T cells (35).

With this publication, we do not want to question the genetic association data obtained in large cohort studies; however, we here fail to find a direct link between KIR2DS1 expression and NK cell-mediated GvL effects at least in HLA-C1/C2 heterozygous donors. Given the strong linkage disequilibrium of KIR2DS1 to KIR3DS1 (36) but also KIR3DL1, KIR2DL4 and KIR2DL5 (37) and evidence that the activation threshold of NK cells may be modulated via the co-expression of an aKIR [as shown for KIR2DL2/3$^+$ NK cells which may recognize C2 targets in the presence of KIR2DS1 (10)], further clinical studies are urgently needed that incorporate a thorough phenotypical and functional NK cell characterization into analysis—not only on the clonal NK cell population level but also on the entire pool of alloreactive NK cells—to delineate the various effects of aKIRs in recognizing and targeting leukemia. As aKIR-HLA-class I interactions are determined by variable tissue-specific HLA-class I expression, by varying affinities of KIRs to their cognate HLA-class I ligands (that remain partially undefined) and by varying peptide expression on those HLA-class I molecules (that have to a large extent not yet been identified), it is still a long way to go to understand the biological basis of aKIR functionality and to be able to answer the question of whether 'more' is really 'better'.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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