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ORIGINAL ARTICLE

Partial break in tolerance of NKG2A$^-$/LIR-1$^-$ single KIR$^+$ NK cells early in the course of HLA-matched, KIR-mismatched hematopoietic cell transplantation

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Natural killer (NK) cell subpopulations from 8 HLA-matched but killer cell immunoglobulin-like receptor (KIR)/HLA-ligand-mismatched patient–donor pairs were analyzed in the course of allogeneic hematopoietic stem cell transplantation (HCT). The patients' post-transplantation NKG2A$^-$/LIR-1$^-$ NK cells, which expressed only inhibitory KIRs for which the patient had no HLA class I ligands, showed higher cytotoxic capacity than the NKG2A$^-$/LIR-1$^-$ NK cells lacking any inhibitory KIRs that remained tolerant throughout the course of HCT. The NKG2A$^+$ NK cell subpopulations displayed the highest levels of cytotoxic activation, which appeared to be significantly enhanced in comparison with that in allogeneic graft's donors. LIR-1$^-$ NK cells were much more frequent after HCT than LIR-1$^+$ NK cells and LIR-1 expression on NKG2A$^+$ or NKG2A$^-$ NK cells was associated with significantly lower cytotoxic activities. Thus NKG2A$^-$/LIR-1$^-$ NK cells expressing only HLA-mismatched KIRs show a partial break in tolerance in the first year following HCT. The failure to exclude LIR-1$^+$ cells within the NKG2A$^-$ NK cell subset in previous studies could explain the earlier conflicting results. Thus systemic immune activation in patients following HCT augments the GvL effect through both increasing overall NK cell activities and partially breaking tolerance of unlicensed NK cells.

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INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) is a potentially curative therapy for adult patients with AMLs, myelodysplastic syndromes and aggressive leukemias/lymphomas. Several studies suggest that patients who have received grafts from killer cell immunoglobulin-like receptor (KIR)/HLA-ligand-mismatched donors have a reduced risk of relapse and improved survival compared with patients transplanted from KIR/HLA-matched donors.[1–3] This natural killer (NK)-mediated GvL effect is likely caused by NK cells that are activated by as yet unknown ligands expressed at higher levels on leukemic cells compared with normal cells.[4] An explanation for this phenomenon is that these activated NK cells are less effectively repressed by signals from inhibitory NK receptors in KIR/HLA class I-mismatched patients than in KIR/HLA class I-matched patients. Inhibitory NK receptors in humans can be divided into two functional groups. The first group includes inhibitory KIRs that recognize products of individual groups of *HLA class I* alleles that are the *B* and *C*, and more rarely *A* alleles, while the second group consists of the 'generalized' inhibitory receptors CD94/NKG2A and LIR-1 (LILRB1, CD85J), that show a broad specificity for most HLA class I allotypes.[5] In contrast to NKG2A that is found almost exclusively on NK cells, LIR-1 is also expressed on CD8$^+$ T cells, B cells, monocytes and dendritic cells.[6,7] Interestingly, LIR-1 has an about a 1000-fold higher affinity for the cytomegalovirus (CMV) protein UL18 than for natural HLA class I molecules[8] and LIR-1 expression on lymphocytes may be induced by CMV infection.[6,9] Expression of KIRs is controlled epigenetically at the levels of DNA methylation, histone modification and promoter-derived transcriptional regulation, while cytokines (such as interleukin-12, interleukin-15) and other factors may further modulate KIR expression.[10–13] However, the mechanism that determines which combinations of inhibitory and activating KIRs are expressed by individual NK cells[12,13] remains controversial. Andersson *et al.*[14] proposed a stochastic mechanism, independent of selection by self-HLA class I ligands, where the probability that KIRs in a given genotype are simultaneously expressed by an NK cell equals the product of the individual frequencies of these KIRs in the respective NK cell pool. Alternatively, more recent data favor a ligand-instructed model of NK cell education,[15,16] in which recognition of HLA class I by an inhibitory receptor, such as KIR2DL2, suppresses subsequent expression of a second receptor of related specificity, such as KIR2DL1. In order to avoid autoreactivity, each mature NK cell should express 'at least one' inhibitory receptor for self-HLA. NK cells that fail to conform to this rule are considered 'unlicensed'.[17,18] There is disagreement whether in the period following HCT this 'at least one' rule might be over-ruled or softened, particularly in the situation of KIR/HLA

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Table 1. Characteristics of KIR/HLA-mismatched patients

#	Age (years)	Sex	Diagnosis	Disease status at HCT	Donor type	KIR ligands[a] (donor)	KIR ligands (patient)	Inhibitory KIR (donor)	Activating KIR (donor)	KIR locus	KIR–ligand mismatch	GvHD Prophylaxis	GvHD (acute)	GvHD (chronic)	Outcome
15	64	M	MDS	NT	FD	C1	C1	2DL1,2DL2/3,3DL1	2DS2	A/B	KIR2DL1–HLA-C2/ KIR3DL1–HLA-Bw4	CyA/MTX	G1	Mild	CR (+66 months) alive
21	57	F	MDS	NT	UD	C1,C2	C2[b]	2DL1,2DL2/3,3DL1	2DS2/3	A/B	KIR2DL2/3–HLA-C1 /KIR3DL1–HLA-Bw4	CyA/ Campath20	G1	Mild	CR (+52 months) REL at +53 months alive
28	52	M	MDS	NT	UD	C1,C2	C1,C2	2DL1,2DL3,3DL1	No	A/A	KIR3DL1–HLA-Bw4	CyA/MTX/ ATG30	G4	Sev	CR (+66 months) alive
30	67	M	MDS	NT	UD	Bw4,C1	Bw4,C1	2DL1,2DL3,3DL1	2DS1,3DS1	A/B	KIR2DL1–HLA-C2	CyA/ Campath20	G2	No	CR (+66 months) alive
33	57	M	AML	REF	UD	Bw4,C1,C2	Bw4,C1[b]	2DL1,2DL2/3,3DL1	2DS2	A/B	KIR2DL1–HLA-C2	CyA/ Campath20	G3	Mod	CR (+65 months) alive
34	66	M	MDS	NT	UD	C1,C2	C1,C2	2DL1,2DL2/3,3DL1	2DS1,2DS2/3,3DS1	A/B	KIR3DL1–HLA-Bw4	CyA/ Campath20	No	No	CR (+65 months) alive
35	62	M	MDS	REF	UD	C1,C2	C1,C2	2DL1,2DL3,3DL1	No	A/A	KIR3DL1–HLA-Bw4	CyA/ Campath10	No	Mild	CR (+64 months) alive
38	44	M	T-ALL	CR	UD	C1	C1	2DL1,2DL3,3DL1	No	A/A	KIR2DL1–HLA-C2 /KIR3DL1–HLA-Bw4	CyA/MTX	G1	Mod	CR (+65 months) alive

Abbreviations: F = female; FD = family donor; G = grade; KIR = killer cell immunoglobulin-like receptor; M = male; MDS = myelodysplastic syndrome; Mod = moderate; NT = not treated; REF = refractory; REL = relapse; Sev = severe; UD = unrelated donor. [a]C1 and C2 KIR ligands are products of *HLA C* alleles with an asparagine (N) or lysine (K) at position 80, respectively. Products of most Bw4+ *HLA B* alleles and certain rare *HLA A* alleles serve as the Bw4 KIR ligands.[27–29] Additional details about KIR ligands can be found in the IPD-KIR database (http://www.ebi.ac.uk/ipd/kir/ligand.html). [b]HLA-C mismatch between the patient and the donor.

ligand-mismatched transplants.[19–21] In hematopoietic transplant recipients, donor NK cells that do not express a sufficient quantity of inhibitory receptors might be primarily non-reactive ('unlicensed') or might become tolerant toward HLA class I non-expressing target cells in the period following HCT.[22,23] Here we asked whether such 'unlicensed' NK cells, expressing only KIRs for HLA ligands missing in the leukemia patient, but not broadly reactive NK receptors, remain tolerant toward self after HCT or whether tolerance may be broken. We characterized diverse NK cell subpopulations of eight KIR/HLA class I-mismatched donor–patient pairs in terms of the course, cytotoxic capacity and expression of KIRs, NKG2A, LIR-1 and the activating NK receptors NKp46 and NKG2D during the first year after HCT.

SUBJECTS AND METHODS

Patients

Eight adult recipients of allogeneic HCT were studied. Inclusion criteria were that the patients received an allogeneic HCT for myelodysplastic syndrome, AML or ALL, that they were mostly HLA class I-matched and that there was at least one KIR-HLA ligand mismatch in GvH direction. The study was exploratory without a prespecified effect size and was approved by the University of Freiburg Hospital Ethics Committee. All clinical investigations were conducted according to the Declaration of Helsinki Ethical Principles and the patients provided informed written consent prior to their inclusion in the study. Each patient received T cell containing G-CSF-mobilized peripheral blood progenitor cells from her/his donor and was treated with Cyclosporine A for GvHD prophylaxis. Six patients underwent HCT by HLA A-, B- and C-locus-matched donors; two received transplants from HLA A- and B-locus-matched but C-locus-mismatched donors (assigned to different HLA-C allele groups C1 or C2). Using high-resolution HLA typing, all patients were assigned to C1, C2 or C1/C2 groups (HLA-C) and to the Bw4 group (HLA-B). In addition, donors' peripheral blood mononuclear cells (PBMCs) were examined by multicolor flow cytometry for NK cells expressing KIR2DL1/S1 (C2-specific), KIR2DL2/DL3/S2 (C1-specific) or KIR3DL1/S1 (Bw4-specific). According to the 'receptor–ligand',[24] that is 'KIR/HLA-ligand mismatch', model (donor NK cells expressing KIR for which the patient had no HLA-C or Bw4 ligand), all patients were treated by KIR/HLA-ligand-mismatched HCT—for details, see Table 1.

Sample collection and preparation

Blood samples were drawn from donors and recipients before and after transplantation. The blood samples obtained following HCT (40 ml) were collected around day 29 (±3), day 115 (±5), day 343 (±19) and day 500 (±30) of HCT. PBMCs isolated on Ficoll-gradients (LSM 1077, PAA, Pasching, Austria) were frozen in 90% FCS (HyClone, South Logan, UT, USA) supplemented with 10% DMSO (AppliChem, Darmstadt, Germany) and stored in liquid nitrogen until use. For testing, frozen samples were thawed and used immediately without further culture for the experiments described below.

KIR-PCR genotyping

Donor DNA samples were extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). KIR genotyping determining the KIR haplotypes and distinguishing inhibitory KIRs from activating KIRs (KIR2DL1/S1, KIR2DL2/S2, KIR2DL3/S3, KIR2DL5, KIR3DL1/S1, KIR3DL2) was performed (with minor modifications) as described previously.[25]

Monoclonal antibodies

For multicolor flow cytometry and the CD107a assay (Figure 1), mouse anti-human monoclonal antibodies (mAbs) anti-CD3-FITC (UCHT1; A07746), anti-NKG2D-PE (ON72; A08934), anti-NKp46-PE (BAB281; IM3711) and anti-CD158a,h-APC (EB6.B; A22332) were obtained from Beckman Coulter, Krefeld, Germany. The mAbs anti-NKG2A-PE-Cy7 (Z199), anti-LIR-1 (CD85j-PE-Cy7 and CD85j-PE (HP-F1)), anti-CD56-APC-AlexaFluor700 (N901, NKH-1), anti-CD158e-ECD (Z27), anti-CD158b,j-APC-AlexaFluor750 (GL183) and anti-CD107a-PE-Cy5.5 (H4A3) were purchased as custom-conjugates (Beckman Coulter, Custom Design Service).

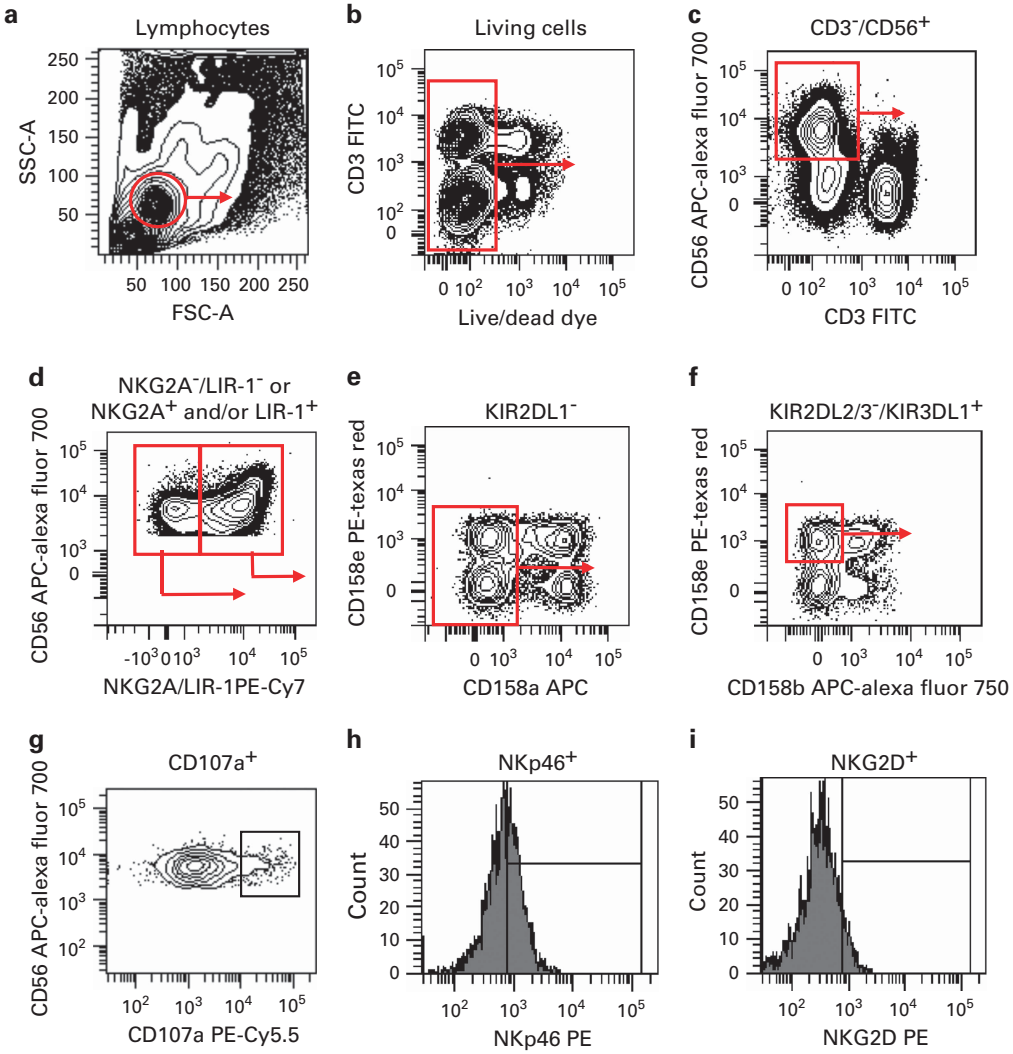

Figure 1. Gating strategy in analyzing the CD107a, NKp46 and NKG2D expression on diverse NK cell subpopulations. Initially, NK cells were identified in thawed cryopreserved samples by gating on FSC and SSC (**a**), as well as live cells using LIVE/DEAD yellow fluorescent reactive dye in order to exclude dead or dying cells (**b**). Subsequent gating was on NK cells, the $CD3^{-}/CD56^{+}$ lymphocyte subpopulation (**c**) that expressed NKG2A and/or LIR-1 and NK cells that expressed neither NKG2A nor LIR-1 ($NKG2A^{-}/LIR\text{-}1^{-}$) (**d**). Subsequently, NK cell subpopulations expressing different combinations of KIR2DL1, KIR2DL2/3 and/or KIR3DL1 were identified (**e**, **f**). Within these subpopulations, the expression levels of CD107a (**g**) and activating NK receptors NKp46 or NKG2D (**h**, **i**) were analyzed in separate tubes.

CD107a assay and flow cytometry

The CD107a assay was performed in order to determine the cytotoxic potential of the NK cell subpopulations.[26] PBMCs were incubated in complete medium (Iscove's Modified Dulbecco's Medium, supplemented with L-Arginine, L-Asparagine, L-Glutamine, penicillin, streptomycin (all supplements purchased from Gibco, Invitrogen, Karlsruhe, Germany) and heat-inactivated 10% male human serum (PAN-Biotech, Aidenbach, Germany) for 4 h at 37 °C in the presence of monensin (BD GolgiStop, BD Bioscieces, Heidelberg, Germany), fluorescent CD107 mAb and K562 cells (50 000 K562/150 000 PBMCs). Subsequently, the cells were washed (phosphate-buffered saline+1% human serum) and stained with LIVE/DEAD yellow fluorescent reactive dye (30 min) (Invitrogen, Karlsruhe, Germany) to eliminate apoptotic cells by live-cell gating. The cells were washed again and stained for surface markers (30 min). Following incubation with mAbs, the cells were washed, resuspended in 250 µl IOTest 3 Fixative solution (Beckman Coulter) and analyzed on an LSR II cytometer using automated compensation with the Diva software (BD Biosciences) or the Kaluza software (Beckman Coulter). The CD107a stimulation index (SI) was defined as the percentage of CD107a-positive cells following stimulation by K562 cells divided by the percentage of CD107a-positive cells following incubation in medium alone.

Statistical analyses

Flow cytometric analyses determined the total numbers of live cells in the thawed patients' samples (mean ± s.d.) as follows: pre-HCT 127 080 (±33 959), donor 83 460 (±40 740), day +30 HCT 86 753 (±54 425), day +100 HCT 129 898 (±31 275), and day +300 HCT 135 615 (±36 960). The plots and analyses in Figures 2, 3, 4b and 5b were created using MATLAB (MathWorks Inc., Natick, MA, United States). As the individual cell counts range over several orders of magnitude, the analyses were performed on the logarithmic scale. In order not to lose the information about absent cellular subpopulations by the log-transformations, zero counts were replaced by an estimate of the detection limit for each individual marker. The limit was calculated from the two smallest counts unequal to zero, denoted as Min_1 and Min_2. The detection limit was estimated as the maximum of $Min_1/2$ and $Min_2 - 2 \times (Min_2 - Min_1)$. For testing expression differences of NKG2D and NKp46 in the course of HCT, a two-sided, two-sample *t*-test with pooled variances was applied. The normality assumption was checked by investigating the residuals within each group. Coexpression of CD107a and NKp46 or NKG2D on NK cells following stimulation by K562 cells or medium were analyzed by considering the log-ratios of cell counts. As the raw ratios exhibited outliers, only those ratios (SI = (number of positive cells in the quadrants stimulated by K562)/(number of positive cells in the quadrants cultured in medium)) were considered where for both treatments

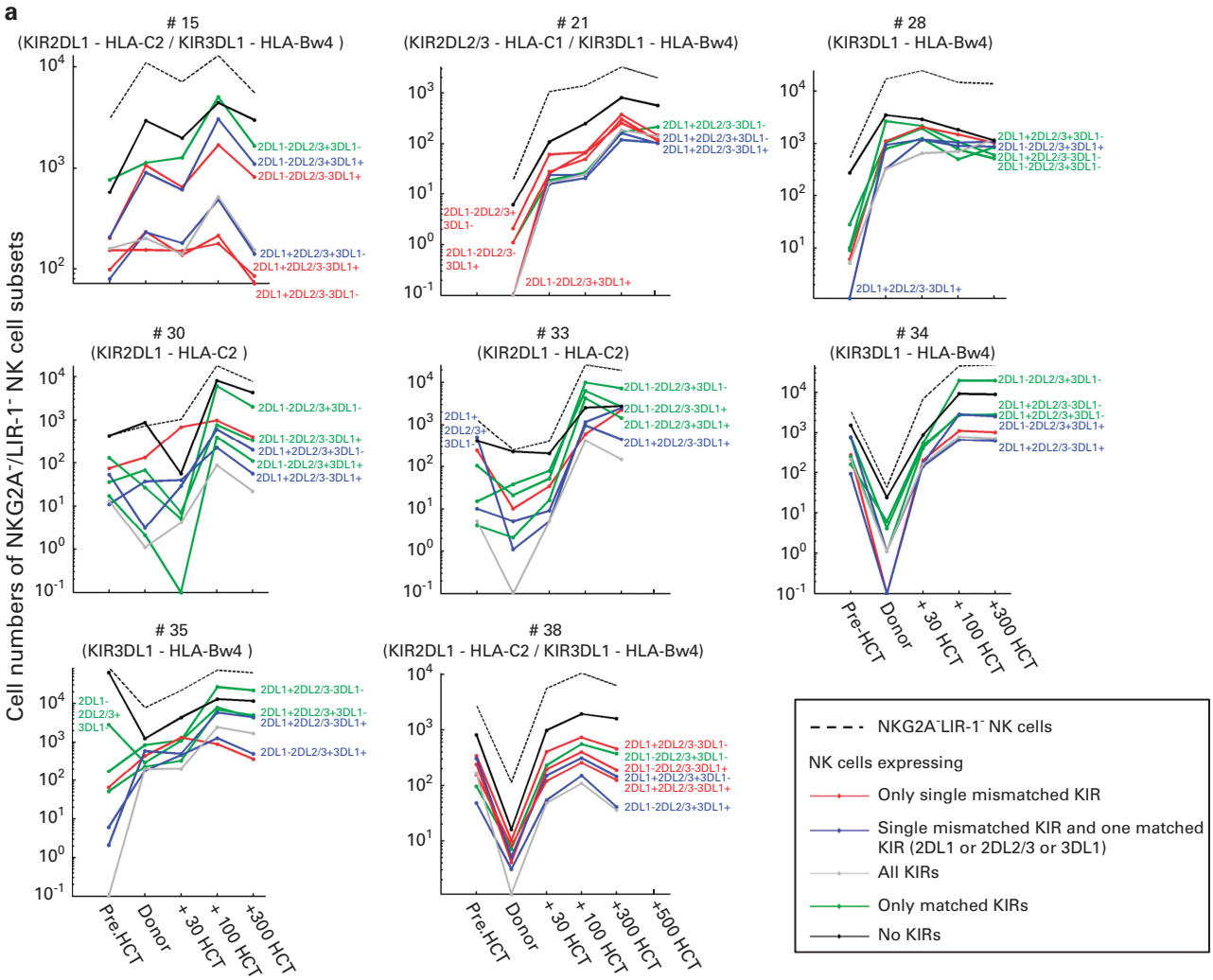

Figure 2. NK cell subpopulations in the course of HCT. The counts of NKG2A$^-$/LIR-1$^-$ (**a**), as well as NKG2A$^+$ and/or LIR-1$^+$ NK cells (**b**), expressing different patterns of KIRs are shown for each of the eight patients before HCT (pre-HCT), on post-transplantation days +30, +100 and +300, as well as for their particular donor. The particular mismatched KIR(s) in the donor and the missing HLA ligand in the patient are shown above each plot. The colors assigned to each type of KIR-expressing subpopulation are defined in the inset. The black dotted line indicates the time course of all NKG2A$^-$/LIR-1$^-$ (**a**), as well as NKG2A$^+$ and/or LIR-1$^+$ (**b**), expressing NK cells. The single KIR$^+$ NK cells expressing the particular mismatched KIR(s) are shown as red lines in each plot.

more than five cells were observed and either the K562 treatment or the negative control yielded >100 cells. The two-sided, nonparametric Wilcoxon rank-sum test was applied to test for ratios unequal to one. As we investigated several distinct hypotheses and the analysis did not provide an unambiguous definition of which tests and hypotheses has to be considered as a multiple testing setting, we report unadjusted *P*-values throughout. *P*-values $\leqslant 0.05$ were considered to be significant and labeled with '*', *P*-values $\leqslant 0.001$ were considered to be highly significant and marked by '**'. All reported *P*-values from *t*-tests were compared with the *t*-test for heterogeneous variances to ensure that the classifications '*' and '**' does not depend on this assumption.

RESULTS

Identification of NK cell subpopulations matched or mismatched for different inhibitory receptors

We utilized our nine-color flow cytometry mAbs panel for analysis of the NK cell subpopulations in the donor and patient before and after HCT. First, we assessed the presence of CD3$^-$/CD56$^+$ effector cells (Figures 1a–c). Subsequently, we subtyped the NK cells into effector cells that did or did not express inhibitory receptors for a broad specificity for diverse HLA class I molecules by gating on NKG2A$^-$/LIR-1$^-$ or NKG2A$^+$ and/or LIR-1$^+$ NK cells (Figure 1d). Depending on the presence or absence of the corresponding HLA-ligand in the particular patient (Table 1), these two NK cell subpopulations in each donor–patient pair were further typed Figures 1e and f for (a) 'KIR-mismatched NK cells' (NK cells expressing only one or two mismatched KIRs but no matched KIRs), (b) NK cells expressing only one mismatched KIR and one matched KIR (2DL1 or 2DL2/3 or 3DL1), (c) NK cells expressing all KIRs, (d) 'KIR-matched NK cells' (NK cells expressing one or two matched KIRs but no mismatched KIR) and (e) NK cells devoid of all KIRs. Within these five subpopulations, we further quantified CD107a$^+$ cells (Figure 1g), as well as NK cells expressing the activating receptors NKp46 (Figure 1h) or NKG2D (Figure 1i). The donor/patient KIR/HLA class I ligand mismatch was determined by donor KIR genotyping, patient HLA typing and donor/patient KIR phenotyping (Table 1).

NK cell subpopulations expressing diverse KIRs in the course of HCT

NK cell subsets expressing the NKG2A$^-$/LIR-1$^-$ or the NKG2A+ and/or LIR-1+ phenotype markedly increased in numbers in the course of HCT in comparison with the corresponding NK cell

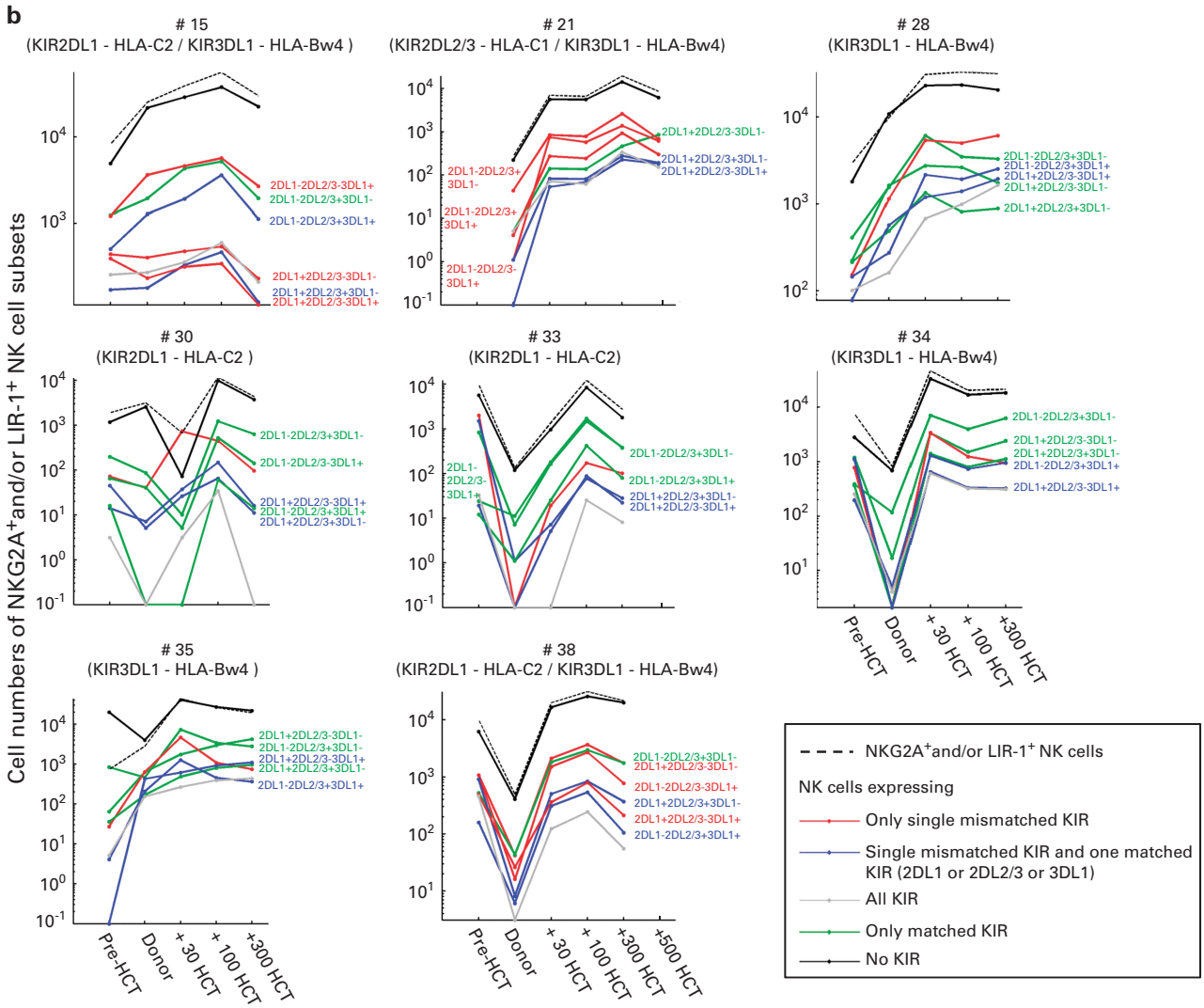

Figure 2. Continued.

subsets prior to HCT from the patients and the donors (black dashed lines in Figures 2a and b). Seven out of the eight patients (except #28) also displayed a marked rise in the numbers of NKG2A$^-$/LIR-1$^-$ NK cells (logarithmic scale) between 30 and 100 days after HCT. Between day +100 and day +300 after HCT, the cell numbers of these NK cell subsets remained at very high levels in all patients, although they were slightly decreasing in five of the eight patients (#15, 30, 21, 33 and 38). In parallel with monitoring the total numbers of NKG2A$^-$/LIR-1$^-$ and NKG2A$^+$ and/or LIR-1$^+$ NK cells (black dashed lines in Figures 2a and b), we analyzed specific NK cell subpopulations, namely, NK cells with different patterns of mismatched and/or matched KIRs, as well as NK cells devoid of all KIRs (Figure 2). The numbers of these NK cell subpopulations followed the general course of NK cells in the post-transplant period in all patients. In seven out of the eight patients, the course of the single KIR-mismatched NK cells (red lines in Figure 2) paralleled the course of the other NK cell subsets, whereas in Patient #30, the single KIR-mismatched KIR2DL1$^+$ NK cells expanded more by day +30 after HCT than the other NK cell subsets. This expansion in Patient #30 involved both the NKG2A$^-$/LIR-1$^-$ and the NKG2A$^+$ and/or LIR-1$^+$ KIR2DL1$^+$ subsets of NK cells. In Patient #33, the single KIR-mismatched KIR2DL1$^+$ NKG2A$^-$/LIR-1$^-$ NK cells continued to increase between days +100 and +300 after HCT, while the remaining NK cell subpopulations showed a tendency to decline slightly. In Patient #21, the very high numbers of all NK subsets persisted up to day +500 after HCT.

Cytotoxicity of NK cells subpopulations and their expression of NKG2D, NKp46 in the course of HCT

The expression levels of CD107a and of the activating NK receptors NKG2D (CD314) and NKp46 (CD335) were analyzed in NKG2A$^-$/LIR-1$^-$ and NKG2A$^+$ and/or LIR-1$^+$ NK cell subsets present in the donors' and patients' PBMCs (Figure 3). After a 4-h incubation with medium alone or stimulation by K562 cells, the CD107a expression was measured and the SIs were calculated (see Subjects and methods section). In general, the NKG2A$^-$/LIR-1$^-$ NK cells showed significantly lower SIs than the sample-matched NKG2A$^+$ and/or LIR-1$^+$ NK cells (merged data of the red, blue, green, gray and black lines in Figure 3a vs b, all time points after HCT, $P^{(1)} \leqslant 0.001$; the superscript numbers in brackets enumerating specific *P*-values are also displayed in the figure). In a direct comparison of individual time points, this difference was significant on day +30 ($P^{(2)} \leqslant 0.05$,) and day +300, ($P^{(3)} \leqslant 0.001$). Only the NKG2A$^-$/LIR-1$^-$ NK population expressing all KIRs (gray lines) showed higher K562-SIs on day +100 post HCT, (although not statistically significant) than the corresponding NKG2A$^+$ and/or LIR-1$^+$ NK populations (Figure 3a vs b). NK cells

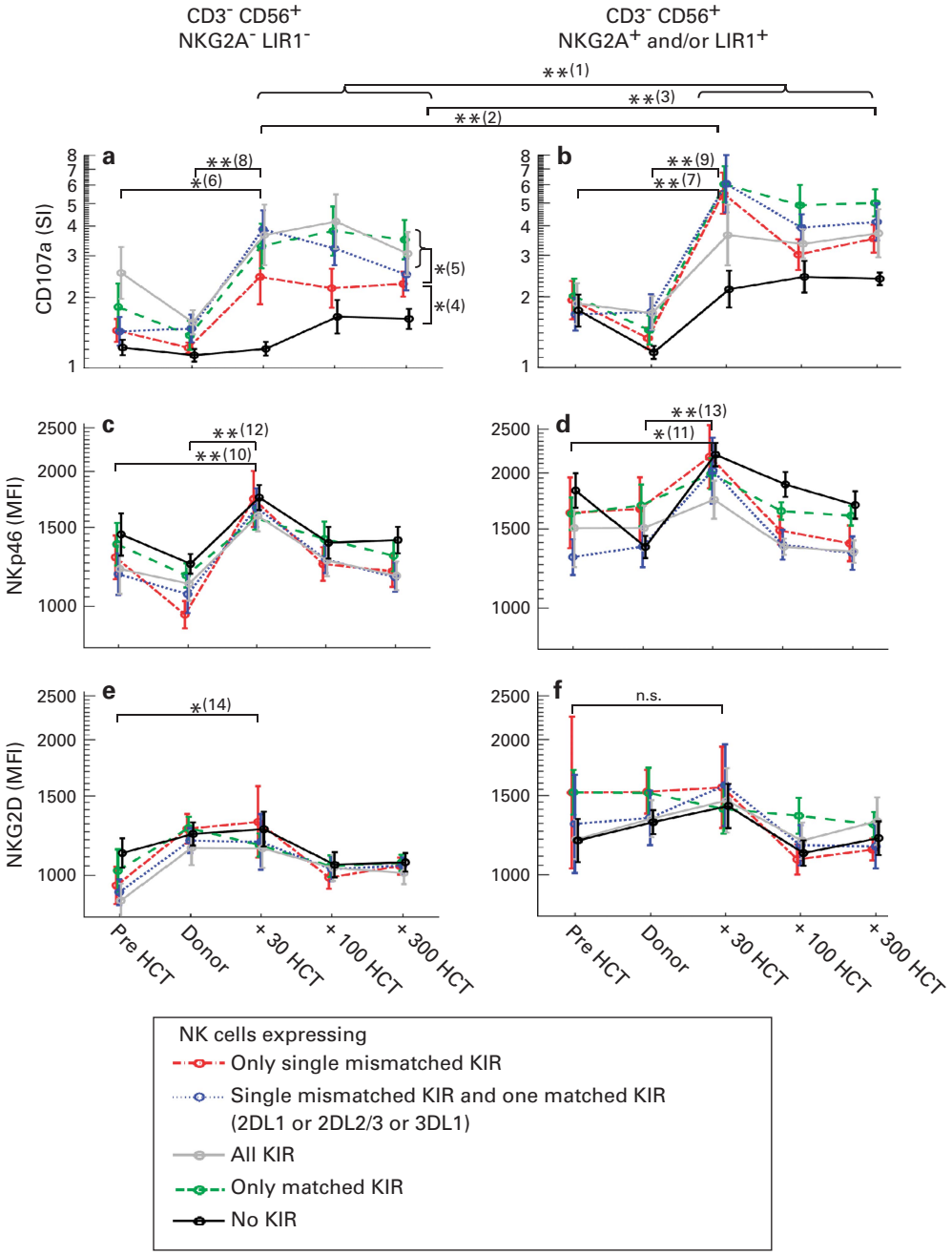

Figure 3. Cytotoxic capacity, NKp46 and NKG2D expression of NKG2A$^-$/LIR-1$^-$ and NKG2A$^+$ and/or LIR-1$^+$ expressing NK cells during the HCT course. The K562 SIs (**a**, **b**) calculated as the percentage of CD107a-positive cells after K562 stimulation divided by the percentage of CD107a-positive cells after a parallel incubation in medium only (for details, see Subjects and methods section) and the relative expression levels (MFIs) of NKp46 (**c**, **d**) and NKG2D (**e**, **f**) in unstimulated NKG2A$^-$/LIR-1$^-$ (**a**, **c**, **e**) and NKG2A$^+$ and/or LIR-1$^+$ (**b**, **d**, **f**) NK cell subsets were analyzed for all patients and donors at the same time points (indicated on the abscissas) as described in Figure 2. Each error bar above and below the mean value represents one s.d. Different NK subpopulations expressing distinct combinations of mismatched and/or matched KIR (s) are defined in the inset. The color-code labeling is the same as in Figure 2. The numbers in brackets enumerate *P*-values mentioned in the text; Statistically significant differences ($P \leqslant 0.05$) are indicated by asterisk (*) and highly significant differences ($P \leqslant 0.001$) are indicated by double asterisks (**). NS, not significant.

devoid of all KIRs (black lines) had the lowest cytotoxic activity in both groups, the NKG2A$^-$/LIR-1$^-$ and NKG2A$^+$ and/or LIR-1$^+$ NK cells (Figures 3a and b, both $P \leqslant 0.001$) compared with all other NK cell subpopulations. The NKG2A$^-$/LIR-1$^-$ NK cells lacking all KIRs (black line in Figure 3a) showed a weak increase in NK activation between days +30 and +100 following HCT, although their overall cytotoxicity was significantly lower than that of the corresponding NKG2A$^+$ and/or LIR-1$^+$ NK cell population (black line in Figure 3b, $P \leqslant 0.001$). Interestingly, NKG2A$^-$/LIR-1$^-$ NK cells expressing only the inhibitory KIR(s) for which the patient had no HLA class I ligand (the KIR-mismatched NK cells) had a statistically significant 'intermediate' level of cytotoxic potential (red line in Figure 3a), which was higher than that of the NK cells lacking all KIRs (black line, $P^{(4)} \leqslant 0.05$), but lower than the cytotoxic potential of other KIR-expressing NK subpopulations (blue, green and gray lines, $P^{(5)} \leqslant 0.05$). Moreover, these KIR-mismatched NKG2A$^-$/LIR-1$^-$ NK

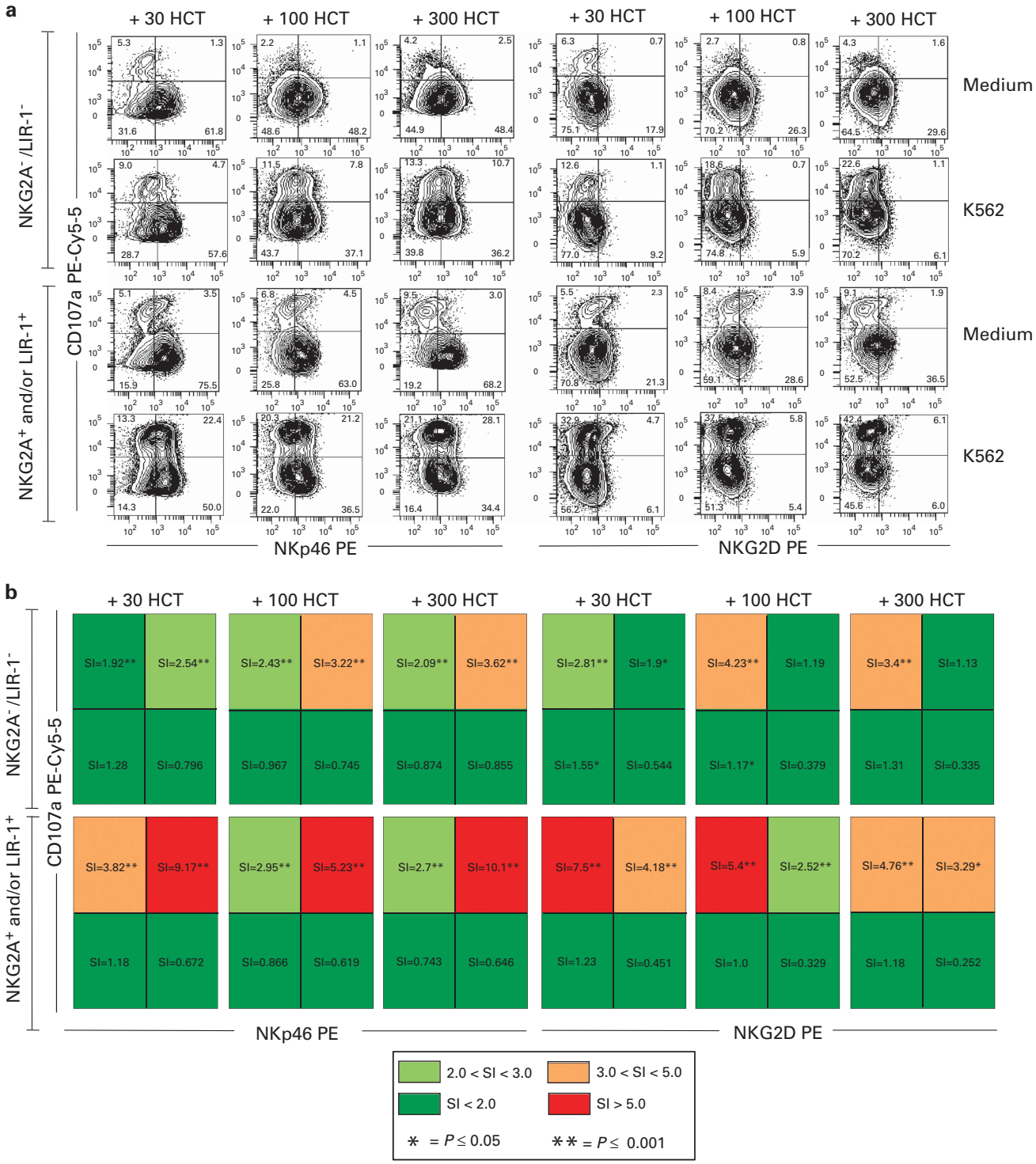

Figure 4. Expression profiles of CD107a, NKp46 and NKG2D in NKG2A$^-$/LIR-1$^-$ and NKG2A$^+$ and/or LIR-1$^+$ NK cells after stimulation with K562. (**a**) NKG2A$^-$/LIR-1$^-$ and NKG2A$^+$ and/or LIR-1$^+$ NK cells from a representative patient (Patient #34). PBMCs were obtained and cryopreserved at three different time points (that is, days +30, +100 and +300) and were analyzed for CD107a (*y* axis), as well as NKp46 (*x* axis, left) or NKG2D (*x* axis, right) expression after short-term cultures (4 h) in the absence (medium) or presence of K562 cells. The percentages of positive cells are indicated in the quadrants. (**b**) Statistical analyses of the CD107a, NKp46 and NKG2D expression profiles of all eight patients on the given days after HCT match the corresponding plots in (**a**). The SI is defined as the 'median percentage of cells after the incubation with K562' divided by the 'median percentage of cells after the incubation in medium alone'. SIs were categorized into four levels marked with different colors (dark green SI $<$ 2.0, light green 2.0 $<$ SI $<$ 3.0, orange 3.0 $<$ SI $<$ 5.0, red SI $>$ 5.0). Statistically significant differences ($P \leqslant 0.05$) are indicated by asterisk (*) and highly significant differences ($P \leqslant 0.001$) are indicated by double asterisks (**).

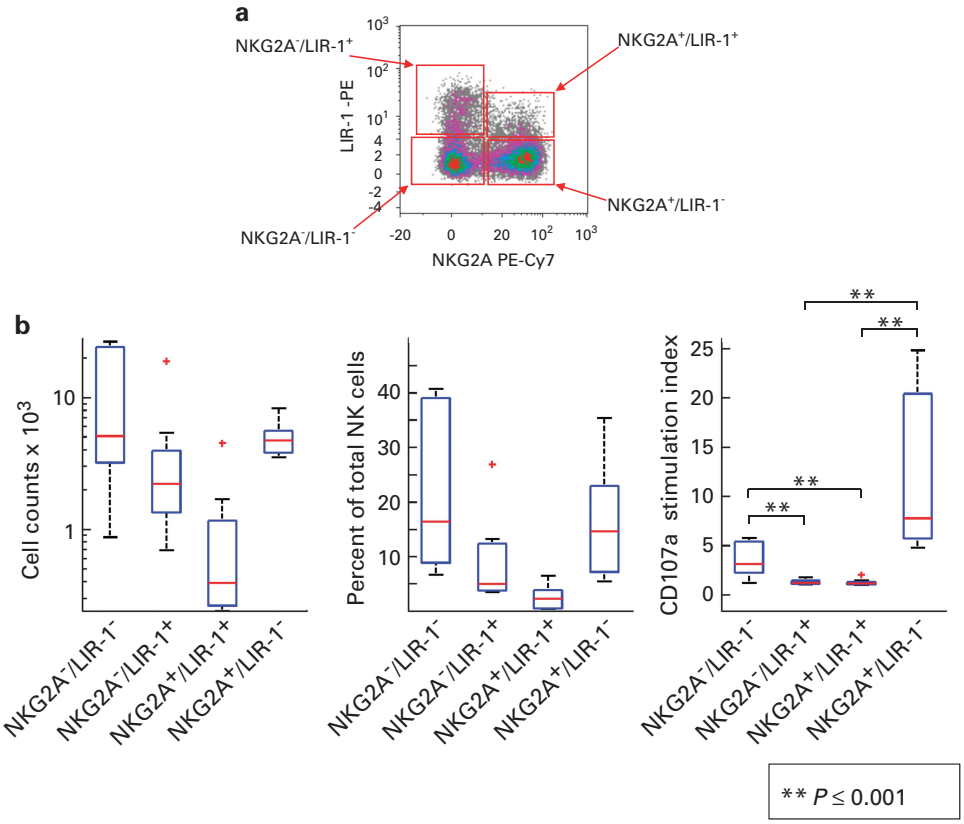

Figure 5. Distribution and function of NK cells with and without the expression of NKG2A and/or LIR-1 following HCT. Cryopreserved PBMC from all 8 patients around day 500 of HCT were thawed and analyzed by flow cytometry. (**a**) The four quadrants show live NK cells ($CD3^-/CD56^+$) from a representative patient gated on the $NKG2A^+/LIR\text{-}1^-$, $NKG2A^+/LIR\text{-}1^+$, $NKG2A^-/LIR\text{-}1^+$ and $NKG2A^-/LIR\text{-}1^-$ phenotypes. (**b**) The data from all eight patients were analyzed for numbers and the percentages of total NK cells in each quadrant and the cytotoxicity against K562 cells by using the CD107a degranulation assay. Statistical significance was determined using the two-sided *t*-test with pooled variances.

cells had a significantly lower cytotoxic potential than the corresponding $NKG2A^+$ and/or $LIR\text{-}1^+$ ones (red lines in Figure 3a vs b, $P \leqslant 0.05$). The cytotoxic capacity (reflected by the level of CD107a expression) of patients' NK cells was markedly increased around day +30 after HCT in comparison with that in the corresponding pre-HCT patients ($P^{(6)} \leqslant 0.05$ for $NKG2A^-/LIR\text{-}1^-$ NK cells and $P^{(7)} \leqslant 0.001$ for $NKG2A^+$ and/or $LIR\text{-}1^+$ NK cells) and in the corresponding donors ($P^{(8)} \leqslant 0.001$ for $NKG2A^-/LIR\text{-}1^-$ NK cells and $P^{(9)} \leqslant 0.001$ for $NKG2A^+$ and/or $LIR\text{-}1^+$ NK cells). This activation was detected in all different NK cell subpopulations of all patients and persisted for up to 1 year following HCT.

The NKp46 expression on unstimulated NK cell subpopulations (Figures 3c and d) was significantly higher on day +30 after HCT as compared with that in the same patients before HCT ($P^{(10)} \leqslant 0.001$ for $NKG2A^-/LIR\text{-}1^-$ NK cells and $P^{(11)} \leqslant 0.05$ for $NKG2A^+$ and/or $LIR\text{-}1^+$ NK cells), as well as with the NKp46 expression in the donors ($P^{(12)} \leqslant 0.001$ for $NKG2A^-/LIR\text{-}1^-$ NK cells and $P^{(13)} \leqslant 0.001$ for $NKG2A^+$ and/or $LIR\text{-}1^+$ NK cells). In comparison with their $NKG2A^-/LIR\text{-}1^-$ counterparts, the $NKG2A^+$ and/or $LIR\text{-}1^+$ NK cells expressed significantly higher amounts of NKp46 when analyzed on days +30, +100 or +300 (Figure 3c vs d, $P \leqslant 0.05$). In contrast to NKp46, the levels of NKG2D on patients' NK cells were augmented to a lower degree in the post-transplant period (Figures 3e and f). which was only significant for the $NKG2A^-/LIR\text{-}1^-$ NK cells on day +30 of HCT ($P^{(14)} \leqslant 0.05$), whereas this was not significant for the $NKG2A^+$ and/or $LIR\text{-}1^+$ NK cells. However, unlike the CD107a-associated K562 SIs, there was no relevant difference (factor 1.022) in NKG2D expression between the $NKG2A^-/LIR\text{-}1^-$ and $NKG2A^+$ and/or $LIR\text{-}1^+$ NK cell subsets. It is also noteworthy that the NK cells lacking all KIRs (black lines in Figures 3a–f) expressed similarly elevated levels of NKG2D and NKp46 as the KIR-expressing NK cell subpopulations expressing self-specific inhibitory KIRs (blue, gray and green lines) and the single KIR-mismatched NK cells (red lines).

As the expression levels of NK-activating receptors might be causally related to the activation of NK cell cytotoxicity, we investigated the correlation between mean fluorescence intensities (MFIs) of the NKp46 or NKG2D stainings and CD107a SIs. We observed significant correlations between the CD107a SIs and the NKp46 MFIs (but not with the NKG2D MFIs) in $NKG2A^-/LIR\text{-}1^-$ cells on day +30 following HCT in 'NK cells with a single mismatched KIR and one matched KIR' ($P \leqslant 0.05$), 'NK cells expressing all KIRs' ($P \leqslant 0.05$) and in 'KIR-mismatched NK cells' ($P = 0.058$) (data not shown). This correlation between the CD107a expression and the NKp46 MFI was only observed on day 30 following HCT but not at the other time points examined (days +100 and +300).

The NKp46 and NKG2D expression in the in vitro-stimulated NK cell subsets

In this set of experiments, we assessed the influence of K562 stimulation on the levels of surface-expressed NKp46 or NKG2D molecules. The patients' PBMCs, obtained on days +30, +100 and +300 after HCT, were incubated *in vitro* in the presence or absence (control) of K562 cells and analyzed by multicolor flow cytometry. Figure 4a shows representative results illustrating the surface expression of CD107a and NKp46 or NKG2D in Patient #34's PBMCs, while Figure 4b shows the corresponding statistical

Table 2. Distribution and function of NK cells with and without expression of NKG2A and/or LIR-1 (CD85j) following HCT

Days post-HCT	*Patient #*	*Living cell counts*	*NK cell counts (%)*[a]	*Counts (% NK)* [b]*NKG2A$^-$ LIR-1$^+$/CD107a SI*	*Counts (% NK)*[b]*NKG2A$^+$ LIR-1$^+$/CD107a SI*	*Counts (% NK)*[b]*NKG2A$^-$ LIR-1$^-$/CD107a SI*	*Counts (% NK)*[b]*NKG2A$^+$ LIR-1$^-$/CD107a SI*
30	15	55 061	19 419 (35.3[a])	7804 (40.2[b])/2.8	1244 (7.9[b])/4.9	2493 (12.8[b])/4.8	7783 (40.1[b])/29.6
	21	9918	4017 (40.5)	197 (4.9)/2.6	297 (4.1)/1.9	490 (12.2)/5.8	2903 (72.2)/22.0
	28	81 929	22 591 (27.6)	2485 (11.0)/1.2	1412 (15.3)/1.4	7886 (34.9)/6.5	10 417 (46.1)/33.7
	30	48 049	22 161 (46.1)	1162 (5.2)/1.3	880 (7.8)/2.8	6816 (30.8)/2.8	12 516 (56.5)/18.0
	33	16 455	1757 (10.7)	360 (20.5)/0.5	121 (5.4)/0.9	269 (15.3)/0.8	932 (53.5)/5.4
	34	59 325	20 563 (34.7)	379 (1.8)/1.1	155 (5.5)/1.8	4408 (21.4)/13.1	15 510 (80.2)/41.6
	35	102 853	68 675 (66.8)	399 (0.6)/1.0	124 (0.4)/2.0	9595 (28.5)/8.0	47 989 (73.5)/41.7
	38	82 761	19 008 (23.0)	97 (0.5)/0.6	81 (1.0)/2.2	4568 (24.0)/2.1	14 202 (76.0)/5.8
100	15	100 302	62 528 (62.3)	32 466 (50.7)/2.9	3433 (6.2)/10.3	12 136 (19.4)/3.7	13 120 (21.4)/9.5
	21	85 032	29 144 (34.3)	427 (1.9)/1.1	365 (2.0)/1.2	4980 (17.1)/8.1	21 695 (78.5)/25.0
	28	122 417	62 023 (50.5)	701 (5.8)/1.3	781 (10.6)/3.7	16 736 (27.0)/1.5	35 886 (61.1)/5.8
	30	81 561	37 896 (46.5)	462 (0.6)/1.2	220 (0.5)/1.2	19 807 (52.3)/7.3	13 519 (46.6)/13.5
	33	81 598	30 495 (37.4)	374 (1.1)/2.3	173 (0.7)/2.2	19 825 (65.0)/19.7	9570 (32.9)/21.1
	34	87 263	34 050 (39.0)	600 (0.7)/1.2	386 (0.5)/1.1	21 453 (63.0)/18.2	10 980 (33.5)/36.2
	35	99 063	59 084 (59.6)	1345 (0.3)/1.3	565 (0.2)/1.1	38 307 (64.9)/18.4	16 840 (33.1)/36.1
	38	97 538	32 909 (33.7)	378 (0.2)/0.6	146 (0.2)/0.9	6998 (21.3)/0.9	21 973 (76.4)/2.3
500	15	59 817	20 301 (33.9)	5351 (26.4)/1.2	1684 (8.3)/1.4	4968 (24.5)/3.7	8237 (40.6)/7.0
	21	75 708	7131 (9.4)	1615 (22.7)/1.1	504 (7.1)/1.2	1472 (20.6)/1.2	3500 (49.1)/5.5
	28	66 262	11 830 (17.9)	1074 (9.1)/1.1	541 (5.4)/1.2	4876 (41.2)/2.4	4998 (42.3)/6.0
	30	103 526	35 034 (33.8)	18 792 (53.6)/1.8	4469 (12.8)/2.0	5169 (14.8)/5.1	6173 (17.6)/21.3
	33	103 409	33 421 (32.3)	2473 (7.4)/1.4	265 (0.8)/1.0	25 968 (77.7)/5.8	4816 (14.4)/24.8
	34	76 850	28 565 (37.1)	1965 (6.9)/1.2	275 (1.0)/1.0	22 388 (78.4)/5.8	3982 (13.9)/19.6
	35	85 062	32 546 (38.3)	2443 (7.5)/1.5	258 (0.6)/1.0	26 513 (81.5)/2.6	3549 (10.9)/4.8
	38	24 732	6557 (26.5)	687 (10.5)/1.0	239 (3.6)/1.1	864 (13.2)/2.1	4625 (70.5)/8.5

Abbreviations: HCT = hematopoietic cell transplantation; NK = natural killer; SI = stimulation index. Flow cytometry data (Figure 5) with the total lymphocyte counts, NK cells (CD3$^-$/CD56$^+$) counts and relative proportions of the given NK cell subsets and their CD107a SI with K562 target cells on days 30, 100 and 500 after HCT. [a]Percentage of living cells. [b]Percentage of NK cells.

analyses in all eight patients. Remarkably, K562 stimulation *in vitro* induced a highly significant CD107a single-marker and CD107a/NKp46 double expression on NKG2A$^-$/LIR-1$^-$ and NKG2A$^+$ and/or LIR-1$^+$ NK cells in PBMCs obtained on days +30, +100 or +300 after HCT (Figures 4a and b, $P \leqslant 0.001$). The SIs (Figure 4b) were higher in the CD107a/NKp46 double-expressing cells than in the CD107a single-marker expressors.

This effect of K562 stimulation was stronger in the NKG2A$^+$ and/or LIR-1$^+$ NK cell subset (SI > 5.0) cells than in the NKG2A$^-$/LIR-1$^-$ NK cell subset (SI < 5.0), although unstimulated NKG2A$^+$ and/or LIR-1$^+$ NK cells already expressed higher levels of NKp46 than unstimulated NKG2A$^-$/LIR-1$^-$ NK cells (Figures 3c and d, $P \leqslant 0.05$). The NKG2A$^-$/LIR-1$^-$ NK cells showed lower relative K562-mediated induction of CD107a single-marker expression (SI < 2.0) and CD107a/NKp46 double expression (2.0 < SI < 3.0) on day +30 as compared with the measurements on day +100 (3.0 < SI < 5.0) and day +300 (3.0 < SI < 5.0). Unlike NKp46, a significant induction of CD107a/NKG2D double expression *in vitro* was noted in the NKG2A$^+$ and/or LIR-1$^+$ NK cell subset on day +30 (3.0 < SI < 5.0, $P \leqslant 0.001$), day +100 (2.0 < SI < 3.0, $P \leqslant 0.001$) and day +300 (3.0 < SI < 5.0, $P \leqslant 0.05$) but not in the NKG2A$^-$/LIR-1$^-$ NK cell subset (SI < 2.0). Thus the NKG2A$^-$/LIR-1$^-$ NK cell subset remained mostly NKG2D negative after K562 stimulation *in vitro*.

Interestingly, there was a marked decrease of NKG2D expression on NK cells following K562 stimulation, compared with stimulation by medium alone that could be observed on days 30, 100 and 300 following HCT (Figure 4). This down-modulation of NKG2D on the cell surface may function to prevent NK cell overactivation possibly caused by multiple interactions with K562 cells. This could be a self-limiting factor preventing NK cell overactivation. Alternatively, K562 cells attached to NK cells might block the NKG2D epitope that binds the anti-NKG2D mAb.

Distribution and cytotoxic activity of NKG2A$^+$/LIR-1$^-$, NKG2A$^+$/LIR-1$^+$, NKG2A$^-$/LIR-1$^+$ and NKG2A$^-$/LIR-1$^-$ NK cells following HCT

In the last set of experiments, we investigated cell numbers and cytotoxicity of NK cell subpopulations differentially expressing NKG2A and/or LIR-1 (CD85j). These NK subsets clustered into four quadrants in the patients (Figure 5a) and in healthy donors (data not shown). Approximately 40–50% of NK cells in healthy donors express NKG2A, 15–50% express LIR-1 and 20–50% belong to the NKG2A$^-$/LIR-1$^-$ subset (our unpublished results). Most patients had only a few LIR-1$^+$ NK cells (NKG2A$^+$/LIR-1$^+$, NKG2A$^-$/LIR-1$^+$ subsets), while individual patients had elevated numbers of LIR-1$^+$ NK cells that were mostly NKG2A$^-$ (Table 2, Figure 5b). Remarkably increased numbers of LIR-1$^+$ NK cells were observed in patient #15 around days 30, 100 and 500 following HCT and in Patient #30 around day 500 of HCT (Table 2). The LIR-1$^+$ NK cell subsets in all patients (NKG2A$^+$/LIR-1$^+$, NKG2A$^-$/LIR-1$^+$) displayed significantly lower cytotoxic capacities (CD107a degranulation SIs; $P \leqslant 0.001$) than the LIR-1$^-$ subsets ((NKG2A$^+$/LIR-1$^-$, NKG2A$^-$/LIR-1$^-$).

DISCUSSION

In a hematopoietic cell transplant recipient lacking HLA class I ligand for the inhibitory KIRs expressed by the transplant donor, the licensed donor's NK cells may become alloreactive.[1,2,19] The three most important inhibitory receptor–ligand interactions are KIR2DL1 (CD158a) with HLA C2 proteins (C group 2 with lysine at position 80 such as *C*0401*), KIR2DL3 (CD158b2) with HLA C1 proteins (C group 1 with asparagine at position 80 such as *C*0702*) and KIR3DL1 (CD158e) with most products of *HLA-B* alleles carrying the Bw4 epitope (such as *B*2702*), which is also present on products of some *HLA-A* alleles (such as *A*2402*).[27] Similar to KIR2DL3, KIR2DL2 also interacts with HLA-C1 proteins, but

depending on the particular *KIR2DL2* allele, it cross-reacts more than KIR2DL3 with several C2 allotypes and some HLA-B allotypes that share polymorphisms with HLA-C.[27–29] Matching of the *HLA -A*, *-B* and *-C* loci between HCT donors and recipients does not prevent NK cell alloreactivity because human *HLA class I* genes (chromosome 6p21.3) segregate independently from the *KIR* genes (human chromosome 19q13.4).

As initially shown in haploidentical HCT,[1–3,30,31] the incidence of relapse in HLA-matched HCT for AML and myelodysplastic syndrome was lower in patients who lacked one or two HLA ligands for donor-inhibitory KIRs.[32–36] NK alloreactivity following HCT may be stronger against residual leukemia cells than against normal tissues as, in addition to a 'lack of inhibition' in the absence of KIR ligands, leukemia cells might activate NK cells through overexpressed ligands for stimulating KIRs, NKG2D or the natural cytotoxic receptors, such as NKp46.[5] It is conceivable that NK cells of donor origin that develop in the hematopoietic allograft recipient and lack the relevant inhibitory receptors, such as NKG2A, KIRs and potentially LIR-1, could contribute to this post-transplant GvL effect. However, according to the 'at least one rule',[37,38] licensed NK cells must express at least one inhibitory receptor for self-MHC; in humans, NKG2A, LIR-1 or a self-specific KIR that calibrate NK cell effector capacities.[7] Indeed, NK cells that lack inhibitory KIRs for self-MHC class I molecules have been found hyporeactive in animal models,[37–39] as well as in healthy humans.[15,16,40] More recent studies in humans revealed that such 'unlicensed' NK cells, that is, NK cells without self-specific inhibitory NK receptors (NKG2A or KIRs), occur less frequently in the NK repertoire.[15,16] Whether or not unlicensed NK cells can expand after HCT and contribute to the GvL effect remains controversial.[19] Although some studies have found that unlicensed posttransplant NK cells were hyporeactive,[22,23,41] Hsu *et al.*[34] demonstrated that unlicensed posttransplant NK cells might be cytotoxic and thus that 'tolerance' could be broken.[33] This was also observed in the autologous setting of immune stimulation by interleukin-2 and immunotherapy leading to NK cell activation through antibody-dependent cell-mediated cytotoxicity.[34,42,43] Our present experiments addressed this controversy by studying NK cell activities over an approximately 1-year period following HCT. We monitored the levels and function of licensed and unlicensed NK cells in eight mostly HLA-matched but KIR-mismatched patient–donor pairs at multiple time points.

We found that the numerical expansions of different NK cell subpopulations followed a parallel course in all eight patients studied (Figures 2a and b, note the logarithmic scale of the *y* axis). The increased numbers of NK cells persisted throughout the entire observation period of at least 300 days after HCT. In the four patients with the KIR2DL1–HLA C2 mismatch (#15, 30, 33, 38) and the six patients with the KIR3DL1–Bw4 mismatch (#15, 21, 28, 34, 35 and 38), the single KIR-mismatched NK cells (red lines in Figure 2a, single KIR2DL1$^+$ and KIR3DL1$^+$) seemed to follow the expansion course of other NK cell populations. In the absence of an HLA ligand for these receptors, one might have expected that these 'unlicensed' cells would not be selected and expanded like the other NK cell subsets, but this was clearly not the case. In the single patient with a KIR2DL2/3–HLA C1 mismatch (Patient #21), the single KIR2DL2/3$^+$ population followed a similar parallel course, although one could argue that this population was licensed by the cross-reacting HLA-C2 ligand.[16,29] However, Patient #21 carried an additional KIR3DL1–Bw4 mismatch and all four KIR-mismatched NK cell subpopulations (KIR2DL3$^+$/KIRDL1$^-$, KIR2DL3$^-$/KIRDL1$^+$, KIR2DL3$^+$/KIRDL1$^-$, KIR2DL3$^+$/KIRDL1$^+$) ran a parallel course. Our data do not indicate that patients' unlicensed or licensed NK cells had any proliferative advantage *in vivo* or that the NK cell repertoire was influenced by licensing during the first year following HCT.

We analyzed the cytotoxic potential of the diverse NK cell subpopulations (Figures 3a and b). The unlicensed NK cells (NKG2A$^-$/LIR-1$^-$ NK cells, expressing only mismatched KIRs (represented by the red line in Figure 3a), had an 'intermediate level' of cytotoxic potential, relative to the NK cell subpopulations with licensed KIRs (blue, green and gray lines in Figure 3a) and the 'immature NK cells' (devoid of all KIRs and NKG2A and LIR-1, black line in Figure 3a). This can be viewed as a 'negative control' consistent with the data reported by Cooley *et al.*,[44] suggesting that this subpopulation represents functionally immature cells with a low cytotoxic capacity and poor interferon-γ production. However, this view has been challenged by observations in mice suggesting that unlicensed NK cells, which are NK cells devoid of inhibitory receptors for self-MHC, may have an important role in some viral infections.[20,45] Our data demonstrate that even this 'uneducated' NK cell subpopulation became activated in the course of HCT, which is obvious when comparing the SIs on day +100 with those from earlier time points in the same patients and the corresponding donors. Moreover, the NKG2A$^+$ and/or LIR-1$^+$ NK cells 'devoid of all KIR' (Figure 3b) showed higher levels of cytotoxic potential during the course of HCT than the corresponding KIR$^-$ NKG2A$^-$ LIR-1$^-$ NK cells, suggesting that expression of these broadly reactive NK inhibitory receptors and their interactions with HLA class I molecules during NK cell development may enhance the cytotoxic potential of these cells against HLA class I$^-$ target cells, such as K562. We extend previous results showing that NKG2A$^+$ NK cell subsets display higher levels of cytotoxicity than NKG2A$^-$ subsets[22,23] and our study is, to our best knowledge, the first one that considered LIR-1 expression in the analysis of NK education following HCT.

Our data clearly show that unlicensed donor's NK cells are activated in the post-HCT period and that their cytotoxic potential against K562 cells (and potentially also against the recipient's leukemia cells) is augmented following HCT compared with pre-HCT. The most likely explanation for this 'break in tolerance' is that the NK cells are activated in the cytokine-rich environment of the reconstituting hematopoietic system.[46] Thus the unlicensed ('tolerant') NK cells are not inert, they are just weaker in their MHC unrestricted killing capacity than are the 'licensed' NK cells pre-HCT and somewhat more reactive following HCT. This validates earlier findings showing that unlicensed NK cells can be activated after allogeneic HCT[43] or in neuroblastoma patients treated by autologous HCT[47] or anti-GD2 antibody-dependent cell-mediated cytotoxicity.[42,48] However, this challenges the conclusions of others that unlicensed NK cells remain hyporesponsive following HCT.[22,23,44] What could be the possible explanations for these inconsistent conclusions? By gating on the NKG2A$^-$/LIR-1$^-$ NK subset, we excluded LIR-1$^+$ NK cells that are otherwise included in NKG2A$^-$, KIR$^-$ NK cells and may account for a significant proportion of NK cells in some patients (Table 2). As LIR-1$^+$ NK cells display much lower levels of cytotoxic capacity than LIR-1$^-$ NK cells (Table 2, Figure 5), this must have lowered the CD107a SIs of single KIR$^+$ NKG2A$^-$ NK subsets in previous studies.[22,23,44] In healthy donors, LIR-1 expression on fresh NK cells and by most NK cell clones is also associated with lower levels of cellular cytotoxicity[49,50] (our unpublished results). As LIR-1 expression on CD8$^+$ T cells was associated with CMV reactivation in lung transplant patients,[6,9] it is expected that CMV reactivation can induce LIR-1 expression on T and NK cells following HCT. Our relatively small study allows clear conclusions as we focused primarily on analyzing the cytotoxic capacity of unlicensed vs licensed NK cells than other larger retrospective studies[22,23] utilizing heterogeneous pools of patients with different conditioning (TBI, antithymocyte globulin) and GvHD prophylaxis regimens.

It was apparent that the increased expression of activating receptors NKG2D and NKp46[51] peaked around day 30 after HCT in all NK cell subsets, regardless of their KIR status (Figures 3c–f). Interestingly, similar amounts of NKG2D and NKp46 were present

in NK cell subpopulations with different cytotoxic potentials (Figures 3a and b). The NKp46, but not NKG2D, expression was significantly higher in the studied NK cell subsets around day 30 after HCT compared with before HCT or to the corresponding donors' NK cells (Figures 3c–f). This higher NKp46 expression positively correlated with the K562 SI, but only in the NKG2A$^-$/LIR-1$^-$ NK cell subset. Thus it is conceivable that the NK cell subpopulations could be activated via as yet poorly characterized NKp46-ligands around day +30, resulting in increased numbers of NK cells (Figure 2) and cytotoxicity (Figures 3a and b). The CD107a/NKp46 and, to a lesser extent, CD107a/NKG2D double expression appeared to be better induced by K562 cells *in vitro* on NKG2A$^+$ and/or LIR$^+$ NK cells than on NKG2A$^-$/LIR$^-$ NK cells (Figures 4a and b). We propose that additional *in vivo* education by these broadly reactive inhibitory NK cell receptors might result in 'hyperlicensing' of the NKG2A$^+$ NK cells as suggested by their higher cytotoxic potential and NKp46 and NKG2D expression of this subset as compared with NKG2A$^-$/LIR$^-$ NK cells (Figures 3a–f).

In summary, we propose that there is a 'partial break in tolerance' at relatively early time points (that is, around day +30) following HCT. This break is incomplete, because single KIR-mismatched NKG2A$^-$/LIR-1$^-$ NK cells that are hyporeactive in the donor clearly show an augmentation of their cytotoxic capacity following HCT. This stimulation of cytotoxic capacity is significantly higher than that of unlicensed KIR$^-$ NKG2A$^-$/LIR-1$^-$ NK cells but lower than detected in 'licensed' NK cells with self-reactive KIRs. This may explain somewhat incongruent data indicating that NK cells expressing inhibitory KIR for non-self-ligands remain 'self-tolerant' in HLA-matched sibling stem-cell transplantations and that tolerance to self in such NK cells may be broken. The observed 'partial break in tolerance' after HCT, which factually means that the very low cytotoxic capacity of unlicensed NK cells against target cells that do not express self-HLA class I is augmented following HCT, could be potentially influenced by endogenous danger signals, including inflammatory cytokines,[18,46,52,53] and may impact immune responsiveness and transplantation outcome. Clearly, further studies of the mechanisms regulating NK cell reactivity are necessary to elucidate how the activity of NK cells is regulated under various physiological and pathophysiological conditions.

CONFLICT OF INTEREST

NW is an employee of Beckman Coulter. The remaining authors declare no conflict of interest.

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