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BIOLOGY

Expansion of NKG2A⁻LIR1⁻ Natural Killer Cells in HLA-Matched, Killer Cell Immunoglobulin-Like Receptors/HLA-Ligand Mismatched Patients following Hematopoietic Cell Transplantation

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The prognosis after hematopoietic cell transplantation (HCT) for the treatment of leukemia or lymphoma in humans is influenced by donor-derived natural killer (NK) cells, which enhance the graft-versus-leukemia (GVL) effect. Such alloreactive killer cells can be generated in vivo after HCT if the donor expresses killer cell immunoglobulin-like receptors (KIRs), such as KIR2DL1, KIR2DL2/3, or KIR3DL1, for which the recipient lacks HLA class I ligands. We studied effector cells from 22 KIR/HLA-ligand mismatched and 14 KIR/HLA-ligand matched, primarily HLA-matched patient-donor pairs after allogeneic HCT. A novel 8-color flow cytometry panel allowed us to characterize effector-cell populations without "broadly reactive" inhibitory receptors such as CD94/NKG2A or LIR1. The numbers of such NKG2A⁻LIR1⁻ NK cells increased following HCT in patients transplanted by KIR/HLA-ligand mismatched grafts, compared to KIR/HLA-ligand matched grafts, and in patients transplanted from donors of the A/B, compared to A/A, KIR haplotypes. NKG2A⁻LIR1⁻ NK cells expressing only those inhibitory KIRs for which the patient had no HLA class I ligands could be stimulated by HLA class I-deficient cells to express CD107a. Thus, NKG2A⁻LIR1⁻ NK cells may be important GVL effector cells following HCT, even in patients transplanted from HLA-matched donors.

Biol Blood Marrow Transplant 16: 469-481 (2010) © 2010 American Society for Blood and Marrow Transplantation

KEY WORDS: Allogeneic hematopoietic cell transplantation, NK cells, HLA-matched, KIR/HLA-ligand mismatch

INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) is often the only possibility to cure acute myelogenous leukemias, myelodysplastic syndromes, and aggressive leukemias/lymphomas. A key to survival in this situation is the graft-versus-leukemia (GVL) effect, mediated by effectors from the donor that destroy residual leukemia cells in the patient. These include donor-derived natural killer (NK) cells, which have been shown to play an important role in the outcome of allogeneic HCT [1,2]. The function of NK cells is regulated by the balance of signals from clonally distributed inhibitory and activating cell surface receptors [3]. The same receptors can be expressed on resting or activated γδ and αβ T cells [4]. The most important ones are the inhibitory NK receptors, which induce tolerance against self. These include the lectin-like receptor CD94/NKG2A, which is specific for the nonclassical class I molecule HLA-E, and is stabilized by leader peptides from most classical HLA-A, -B, and -C allotypes [5]. The Ig-like receptors, encoded in the leukocyte receptor complex of chromosome 19, can be divided into the killer cell immunoglobulin-like receptors (KIRs) and the leukocyte Ig-like receptors [6]. The leukocyte Ig-like receptors (CD85), previously called immunoglobulin-like transcripts or leukocyte immunoglobulin-like receptors (LIR), are broadly reactive inhibitory receptors against the HLA-A, -B, and -C allotypes. Similar to CD94/NKG2A, they enable NK cells to survey for overall class I expression [7]. In contrast, 3 different KIRs can distinguish between groups

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Financial disclosure: See Acknowledgments on page 480.

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Received September 18, 2008; accepted December 23, 2009

1083-8791/10/164-0002$36.00/0
doi:10.1016/j.bbmt.2009.12.008

of HLA-A, -B, and -C allotypes [8-10]. KIR2DL1 recognize HLA-C allotypes with a lysine at position 80 (C2 ligands such as Cw02, 0307, 0310, 04, 05, or 06). KIR2DL2 and KIR2DL3 recognize HLA-C allotypes with an asparagine at position 80 (C1 ligands, such as Cw01, 03, 07, or 08). KIR3DL1 molecules recognize HLA-B-allotypes with a polymorphic sequence motif at position 77-83 (Bw4 epitope), whereas KIR3DL2 interacts with the HLA-A3 and A11 allotypes.

Besides inhibition, NK cells require activation by virus-infected or leukemic cells to mediate their protective functions [11,12]. Stimulatory receptors on NK cells include lectin-like receptors such as CD94/NKG2C and the KIRDS molecules. The HLA class I specificities and antibody reactivities of activating KIRs overlap with those of the corresponding inhibitory KIRs. Other activating receptors on NK cells are the NKG2D homodimers whose ligands include MHC class-I-related chain A/B (MICA/B) [13]. A third class of activating receptors on NK cells, as well as some T cells, are the natural cytotoxic receptors (NCR), NCR1 (NKp46), NCR2 (NKp44), and NCR3 (NKp30) [9]. Although the ligands for these receptors have not been fully elucidated, they may be important in the recognition of leukemic cells [9,11,12]. Inhibitory NK receptor signals generally overrule signaling through stimulatory receptors [10,14]. Several clinical studies demonstrated improved survival post allogeneic HCT, along with a strong GVL effect against myelogenous leukemias when the patient was lacking 1 of the 3 major KIR/HLA-ligands present in the donor, the Bw4 epitope in HLA-B, or the C1 or C2 ligands in HLA-C [1,15-20]. The likely mechanism for this is that NK cells derived from the graft express a "leukemia-reactive" profile of inhibitory receptors. These inhibitory KIR do not find an HLA class I ligand on the recipient's cells, including residual leukemia cells. Because all KIRs are encoded on chromosome 19, they are segregated independently of the HLA alleles on chromosome 6. Thus, transplants from HLA identical family donors may be KIR-mismatched [21], whereas only transplants from identical twins are HLA- and KIR-matched. We studied regenerating NK and T cells following allogeneic HCT in adult patients, most of which were HLA-matched. According to the expression of KIRs in the donor's blood, the patients were divided into KIR/HLA-ligand matched and mismatched groups. In an effort to better understand the GVL effect we focused our analysis on the regenerating NK cells without expression of broadly reactive inhibitory NK receptors (CD94/NKG2A and LIR1 [CD85j]) and monitored their regeneration in KIR/HLA-ligand matched as well as mismatched patients post-HCT. In addition, we monitored NK receptor expression on other innate effector cell populations that may be relevant for the GVL effect postallogeneic HCT, such as NKT cells, NKT-like cells, and γδ T cells.

MATERIALS AND METHODS

Patients

The study included 36 consecutive adult patients treated by allogeneic HCT in the Division of Allogeneic Stem Cell Transplantation, Department of Hematology/Oncology, Freiburg University Hospital. The details of their transplant history are given in Tables 1, S1, and S2. This study was approved by the University of Freiburg Hospital Ethics Committee; all clinical investigations were conducted according to the Declaration of Helsinki Ethical Principles. The patients provided informed written consent prior to inclusion in this study. All patients received granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood progenitor cells from HLA A-, B-, and C-locus matched donors (32 patients) or HLA A-, B-locus matched, but C-locus mismatched donors (4 patients). The patients were only considered C-locus mismatched if the donor and recipient were assigned to different HLA-C allele groups (C1 or C2). For the purpose of this study we assigned the patients to the groups "KIR/HLA-ligand match" and "KIR/HLA-ligand mismatch," as defined shortly.

Sample Collection and Preparation

Blood samples were drawn from donors and recipients before and after transplantation. The blood samples post-HCT were collected on days 14 (n = 36), 28 (n = 35), 56 (n = 35), 84 (n = 33), 112 (n = 30), 140 (n = 29), 168 (n = 31), 252 (n = 16), and 336 (n = 1). All blood samples were drawn within 1 week of the specified day. In the case of missed collections, samples were obtained at interim time points whenever

Table 1. Characteristics of the KIR-Ligand Mismatched and KIR-Ligand Matched HCT Patients

	KIR-Ligand Mismatch	KIR-Ligand Match
N	22	14
Age	62.5 ± 8	56.5 ± 15
Sex (male / female)	18 / 4	6 / 8
Diagnosis (n)	AML/MDS = 16 B-NHL = 6	AML/MDS = 9 B-NHL = 5
HLA class I mismatch in GVHD direction (n)		
A	0	0
B	0	0
C	3	1
KIR haplotype (n)		
A/B	14	5
A/A	7	4
B/B	1	5
Transplantation (n)		
Related	5 (23%)	9 (64%)
Unrelated	17 (77%)	5 (36%)

AML indicates acute myelogenous leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; GVHD, graft-versus-host disease; KIR, killer cell immunoglobulin-like receptor.

possible. Three relapsed patients were excluded from further analysis when they had to undergo a second transplantation. All blood samples were freshly processed by isolation of peripheral blood mononuclear cells (PBMCs) on Ficoll gradients (LSM 1077, PAA, Pasching, Austria), followed by staining for flow cytometry analysis.

KIR-Polymerase Chain Reaction (PCR) Genotyping

Donor DNA samples were extracted using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). KIR genotyping was performed according to published protocols with minor modifications [20,22] to differentiate inhibitory KIRs from activating KIRs (KIR2DL1/S1, KIR2DL2/S2, KIR2DL3/S3, KIR2DL5, KIR3DL1/S1, KIR3DL2) and determine the KIR haplotypes.

Antibodies and Flow Cytometry

The following mouse antihuman monoclonal antibodies (mAbs) were used: CD3-FITC (UCHT1), TCRγδ-FITC (IMMU 510), NKG2A-PE (Z199), CD85j-PE (HP-F1), TCR Vα24-FITC (C15), NKG2D-PE (ON72), NKp30-PE (Z25), NKp44-PE (Z231), NKp46-PE (BAB281), CD3-ECD (UCHT1), CD244-PE-Cy5 (C1.7.1), CD56-PE-Cy7 [N901(NKH-1)], CD158a,h-APC (EB6.B) (all from Beckman Coulter, Krefeld, Germany), and TCRαβ-FITC (BMA031) (Serotec, Oxford, UK). The mAb TCR Vβ11-PE-Cy7 (C21), CD158e-ECD (Z27), and CD158b,j-APC-Cy7 (GL183) were purchased as custom-conjugates (Beckman Coulter, Custom Design Service). For surface staining, PBMC were incubated with different mAb panels, as shown in Table S3. Following 15 minutes of incubation, 50 μL of OptiLyse B solution (Beckman Coulter) were added for red cell lysis. After 15 minutes 500 μL of double-distilled water (Braun, Melsungen, Germany) were added and the tubes were incubated for a further 20 minutes. The samples were washed (PBS + 1% human serum), fixed in 250 μL 1% paraformaldehyde (IOTest 3 Fixative Solution; Beckman Coulter), and events were acquired on a CyAn™ ADP Flow Cytometer (Beckman Coulter). Voltage of the CyAn FlowCytometer was first adjusted using appropriate isotype controls for each color. Then, compensation beads for each color (antimouse Ig CompBeads, BD) were run on the instrument. Upon analysis of the surface markers automatic compensation was preformed using the FlowJo (Tree Star, Ashland, OR, USA) program.

CD107a and Interferon (IFN)-γ Assay

PBMC were incubated in complete medium (IMDM, supplemented with L-Arginine, L-Asparagine, L-Glutamine, penicillin, streptomycin [all from Gibco, Invitrogen, Karlsruhe, Germany] and heat-inactivated 10% male human serum [PAN-Biotech, Aidenbach, Germany]) for 4 hours at 37°C in the presence of monensin (GolgiStop; BD) the CD107a-PE-Cy5.5 mAb (H4A3) (Beckman Coulter, Custom Design Service) [23-25] and K562 cells (50.000 K562/150.000 PBMC). Then, the cells were washed (phosphate-buffered saline [PBS] + 1% human serum), stained for surface markers (30 minutes), fixed, and permeabilized by IntraPrep Permeabilization Reagent (Beckman Coulter). For intracellular staining we used the IFN-γ-Pacific Blue mAb (4S.B3) (eBioscience, Frankfurt, Germany). Following 30 minutes of incubation, the cells were washed, resuspended in 250 μL IOTest 3 Fixative solution (Beckman Coulter) and analyzed (8 colors) on an LSR II cytometer using automatic compensation and the Diva software (BD Biosciences).

Definition of KIR/HLA-Ligand Match and KIR/HLA-Ligand Mismatch

Donor-recipient pairs were divided into the categories "KIR/HLA-ligand match" and "KIR/HLA-ligand mismatch" according to the receptor (KIR)-ligand (HLA) model [17,18]. 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' PBMC were examined by multicolor flow cytometry for NK cells expressing KIR2DL1/S1 (C2-specific), KIR2DL2/DL3/S2 (C1-specific), or KIR3DL1/S1 (Bw4-specific) (Table S4). A patient was assigned to the group "KIR/HLA-ligand match" if the KIR expressed by the donor's NK cells matched the patient's HLA-C or -Bw4 ligands. Thus, most patients in the KIR-ligand matched group expressed all 3 KIR ligands C1, C2, and Bw4. In contrast, a patient was assigned to the group "KIR/HLA-ligand mismatch" if the donor had NK cells expressing KIR for which the patient had no HLA-C or Bw4 ligand. This would mean that there was a KIR-mismatch in the graft-versus-host (GVH) direction. Although patients #14 and #18 were missing HLA C2 KIR ligands, they were assigned to the KIR/HLA-ligand match group because phenotypic analysis of their donors' effector cells failed to demonstrate KIR2DL1 expression [26].

Statistical Analyses

For a statistical comparison of the study groups 20,000 viable lymphocytes were collected per sample. All data were analyzed using SPSS software (Version 14.0 SPSS, Munich, Germany). The significance of the differences in cell counts between donor and recipient pairs following HCT were tested using the non-parametric Mann-Whitney *U*-test.

RESULTS

A New 6-Color Flow Cytometry Panel Differentiating NK and T Cell Subpopulations

We used special mAb panels to characterize inhibitory receptors on NK and T cells (Table S3, panels 1-4). To identify MHC unrestricted effector cells, we first gated on the NKG2A$^-$ LIR1$^-$ effector cells and, alternatively, on effector cells that expressed either NKG2A or LIR1. Then, we identified the NK (CD3$^-$ CD56$^+$) (panel 1), NKT-like (TCR $\alpha\beta^+$ CD56$^+$) (panel 2), $\gamma\delta$ T cell (TCR $\gamma\delta^+$) (panel 3), and NKT (Vα24$^+$ Vβ11$^+$) (panel 4) cell subsets within this population. Because both NKG2A/CD94 and LIR1 (CD85j) represent inhibitory receptors with broad reactivity against different HLA class I alleles, we designated NKG2A$^-$ LIR1$^-$ NK cells as "potentially alloreactive." The NKG2A$^-$ LIR1$^-$ NK cell subset was further gated on cells which were single-, double-, or triple-positive for KIR2DL1/S1 (CD158a,h), KIR2DL2/L3/S2, (CD158b,j), and KIR3DL1/S1 (CD158e). In addition, we used 4- or 5-color flow cytometry to investigate the expression of activating receptors NKG2D, CD244 (2B4), NKp30, NKp44, and NKp46 on each of these effector cell subsets (Table S3, panels 5-8).

NKG2A$^-$ LIR1$^-$ NK Cells in the Course of HCT

Initially, we compared patients before HCT and their healthy donors for the presence of MHC unrestricted killer cells without broadly reactive NK-inhibitory receptors, designated here the NKG2A$^-$ LIR1$^-$ phenotype (Figure 1A). Only NK cells of the NKG2A$^-$ LIR1$^-$ phenotype, but not NKG2A$^-$ LIR1$^-$ NKT-like T cells or $\gamma\delta$ T cells, were decreased in the patients before HCT, when compared to the healthy donors ($P < .001$). In contrast, donors and patients had similar levels of NK cells expressing 1 or both of the broadly reactive NK inhibitory receptors NKG2A and LIR1 (Figure 1B). NKG2A$^-$ LIR1$^-$ NK cells were already detectable in the donors and became a prominent NK cell subpopulation in the late time course following HCT in some of the patients (Figure 2). There was a relatively sharp rise of NKG2A$^-$ LIR1$^-$ NK cells between days 28 and 84 post-HCT. Although NKG2A$^-$ LIR1$^-$ NK cells were, overall, a relatively small population, compared to NK cells expressing NKG2A and/or LIR1, this population continued to rise until approximately day 112 post-HCT, when the levels of the other NK cells were decreasing (Figure 3). As determined by donor KIR genotyping, increased levels of NKG2A$^-$ LIR1$^-$ NK cells post-HCT were more prominent in patients of the A/B than A/A haplotypes, but there was no apparent difference in the levels of NKG2A$^+$ and/or LIR1$^+$ NK cells (Figure 3). Interestingly, patients remaining in complete remission (CR) had significantly more NKG2A$^-$ LIR1$^-$ NK cells, but not NKG2A$^+$ and/or LIR1$^+$ NK cells, compared to relapsed patients as observed at 3 independent time points following HCT (Figure 4).

Increased NKG2A$^-$ LIR1$^-$ NK Cells in KIR/HLA-Ligand Mismatched Patients following HCT

We monitored NKG2A$^-$ LIR1$^-$ NK cells from day 14, up to day 168 post-HCT (Figure 5). Assignment of the patients to the "KIR/HLA-ligand match" and "mismatch" groups was performed according to the "KIR/HLA-ligand model" (see the Methods section) [18,26]. We noticed significantly increased levels of NKG2A$^-$ LIR1$^-$ NK cells in the blood of the KIR/HLA-ligand mismatched patients compared to the matched patients (Figure 5). This difference was mainly caused by an increase in the amount of NKG2A$^-$ LIR1$^-$ CD56-dim NK cells (Figure S1a). Nevertheless, the KIR/HLA-ligand mismatched patients also had a tendency toward higher levels of NKG2A$^-$ LIR1$^-$ CD56-bright NK cells after HCT than the matched patients (Figure S1b). These statistically significant differences were present at several independent time points following HCT, suggesting that these were biologically relevant.

Next, we analyzed the NKG2A$^-$ LIR1$^-$ NK cells in more detail with regard to expression of the relevant KIR, as well as functional capacity using 8-color flow cytometry. NKG2A$^-$ LIR1$^-$ NK cells expressing KIR for which the patient had no HLA class I ligand (designated KIR-mismatched NK cells) were detectable at relatively low levels before HCT. Following HCT these KIR mismatched NK cells increased in most patients to higher levels than observed in the particular donors with the 2 representative patients shown in Figure 6. Patient #21 had 2 KIR/HLA-ligand mismatches in the presence of an HLA-C group mismatch (donor C1/C2 and patient C2/C2), whereas patient #28 was HLA identical to his donor but still had a KIR3DL1-Bw4 mismatch (Tables 2 and S1). Because the donors for patients #21 and #28 did not carry KIR3DS1, all NK cells expressing KIR3DL1/S1 in these patients following HCT expressed the inhibitory form KIR3DL1. However, patient #21 carried the KIR2DL2/3 and KIR2DS2/3 genes; therefore, both forms of this receptor could be expressed by the single KIR2DL2/L3/S2$^+$ cells in this patient. We examined the functional activity of these potentially alloreactive NK cells by measuring their surface CD107a expression and IFN-γ secretion following stimulation by K562 cells [23-25]. NKG2A$^-$ LIR1$^-$ NK cells expressing the mismatched KIRs, as well as other inhibitory KIRs (marked by an arrow in Figure 6A) could clearly be stimulated by K562 cells, revealing their alloreactive potential against target cells that did not express

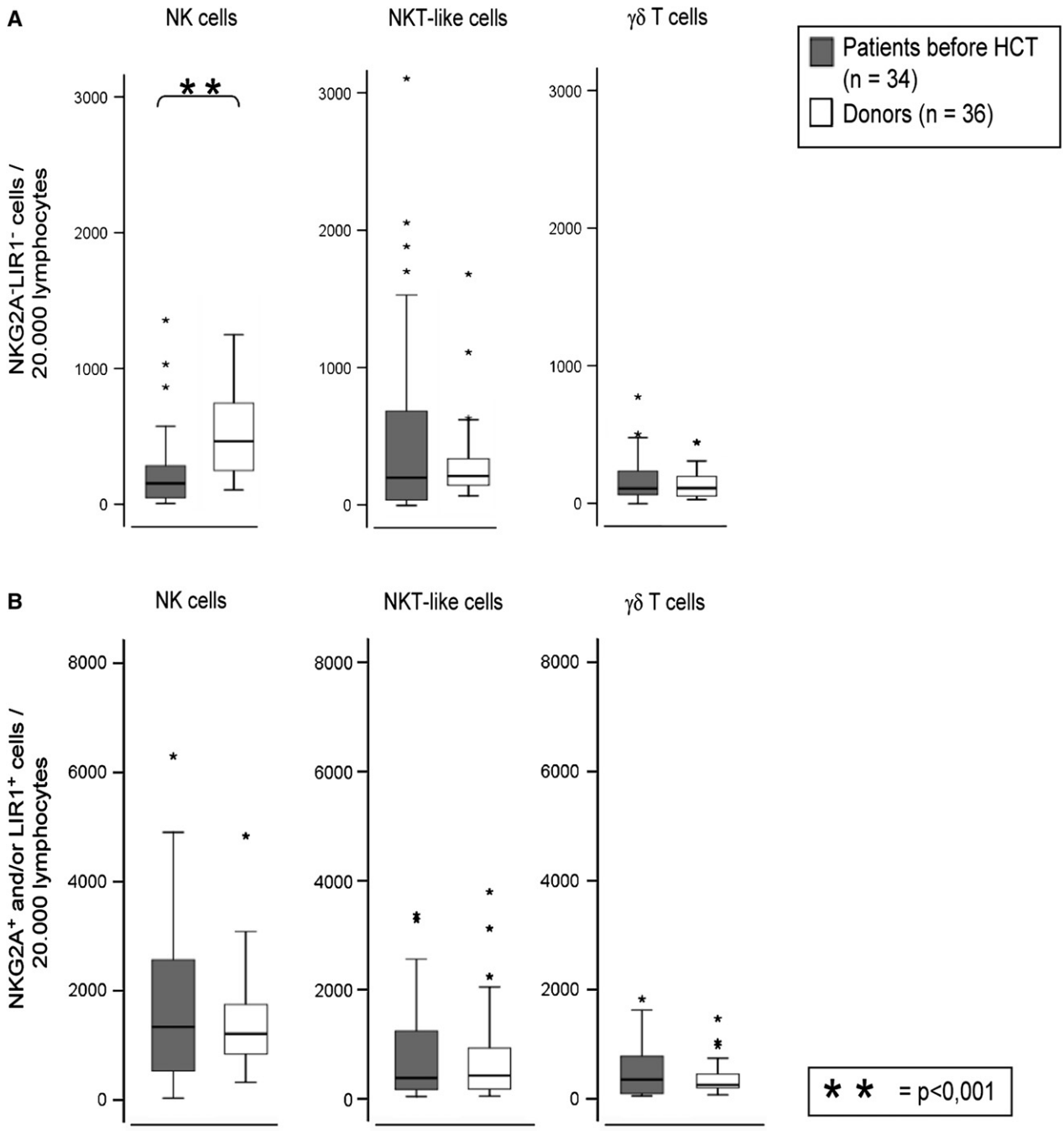

Figure 1. Characterization of effector cells positive or negative for NKG2A/LIR1 inhibitory receptors in patients before HCT and their donors. The numbers of NK cells, NKT-like cells (TCR $\alpha\beta^+$, CD56$^+$) and $\gamma\delta$ T cells were gated on (A) all NKG2A$^-$ LIR1$^-$, as well as on (B) NKG2A$^+$ and/or LIR1$^+$ effector cells. "NKG2A$^+$ and/or LIR1$^+$ effector cells" include the NKG2A$^+$ LIR1$^-$, the LIR1$^-$ NKG2A$^+$, and the LIR1$^+$ NKG2A$^+$ cell populations. Cell numbers were determined by flow cytometry from a total of 20,000 cells in patients before HCT and their donors. Highly statistical significant differences (Mann-Whitney *U*-Test) ($P < .001$) are indicated by **.

a corresponding inhibitory HLA class I ligand (Figure 6B). In addition, we gated on NKG2A$^-$ LIR1$^-$ NK cells that expressed only the mismatched KIR but none of the KIR/HLA-ligand matched inhibitory or activating receptors because such NK cells should find no inhibitory HLA class I ligands on the patient's leukemia cells. Remarkably, even these single mismatched KIR$^+$ NK cells appeared to be functional effector cells showing a 2- to 4-fold increase in CD107a expression while there was some, albeit lower, induction of INF-γ secretion.

Activating Receptors on NK Cells Post-HCT

Besides analysis of the diverse KIR, NKG2A, and LIR1, we studied expression of the activating receptors NKG2D, 2B4, NKp30, NKp44, and NKp46 on

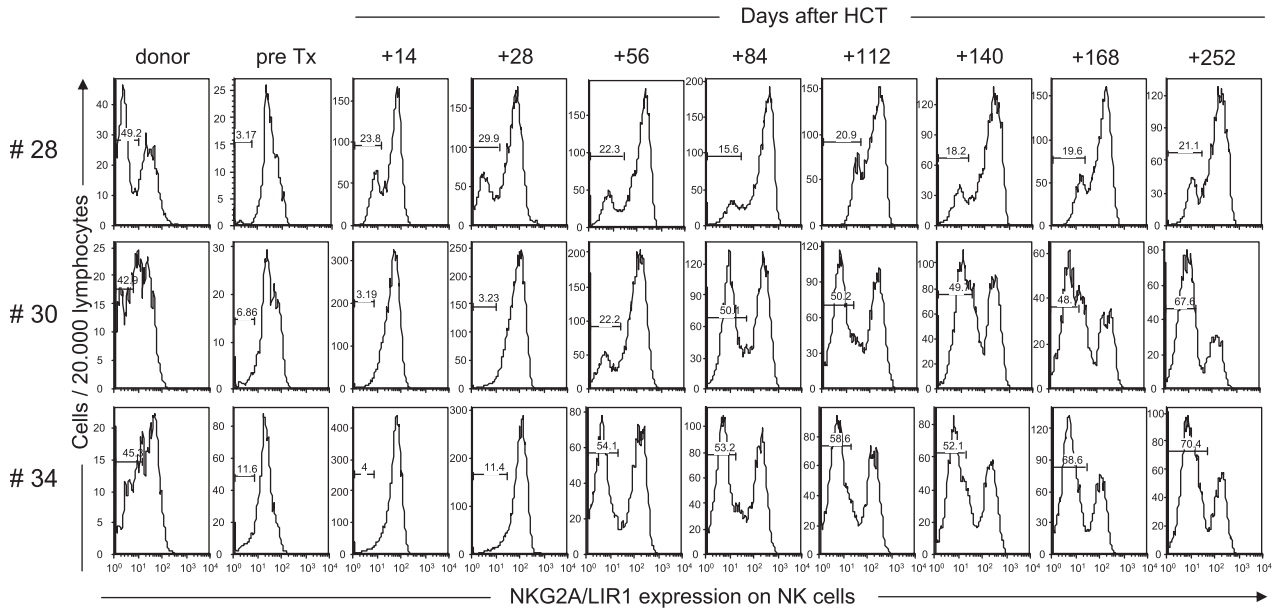

Figure 2. NKG2A/LIR1 expression on NK cells of representative KIR/HLA-ligand mismatched donor/patient pairs. The NK cells of particular donors and representative patients #28, #30, and #34 were analyzed for NKG2A/LIR1 expression by gating on $CD3^-$ $CD56^+$ lymphocytes before HCT (pre Tx) and at different time points following HCT.

patients' NK cells using separate mAb panels (Figure 7, Table S3, panels 5-8). The mean-fluorescence index (MFI) was more informative than percentages of positive cells because most NK cells expressed NKG2D, 2B4, and NKp46, whereas NKp44 was only expressed by the CD56-bright NK cells (Figure 7, and data not shown). During the observation period (168 days) there was a remarkable increase in the expression levels of NKG2D in both KIR/HLA-ligand matched as well as mismatched patients, although it appears that the KIR/HLA-ligand mismatched patients might have transiently higher expression levels of NKG2D than KIR/HLA-ligand matched patients. Similarly, the expression levels of NKp30, NKp44, and NKp46 increased following HCT, and this phenomenon was observed in both the KIR/HLA-ligand mismatched and matched patients. These expression levels were measured on all NK cells because gating on the $NKG2A^-$ $LIR1^-$ NK cell subpopulation would have involved additional customized mAb that were unavailable at the time of our study.

NK Receptor Expression on T Cells Post-HCT

Besides typical NK cells we also measured the blood percentages of the other effector cell subsets (NKT-like, γδ T cells, αβ T cells) and their differential expression of KIR (Table S3). The percentages of NKT cells and γδ T cells in our patients were rather low compared to NK cells (data not shown). KIR expression on these subsets increased posttransplant in only a few of the patients. Typical NKT cells ($V\alpha24^+$ $V\beta11^+$) were extremely rare in the patients' and donors' blood. Therefore, the amount of blood obtained and the samples stained did not allow detailed analysis of the NKT cells. However, when staining more lymphocytes we found that most $V\alpha24^+$ $V\beta11^+$ NKT cells expressed KIRs, whereas T cells that were single positive for Vα24 or Vβ11 did not (data not shown). Naturally, conventional αβ T cells of donor origin increased in these patients post-HCT, but KIR expression on these αβ T cells was only noticed in exceptional patients.

DISCUSSION

In normal donors 20% to 50% of NK cells belong to the $NKG2A^-$ $LIR1^-$ subset, whereas approximately 40% to 50% of NK cells express NKG2A and 15% to 50% LIR1, representing nonoverlapping NK cell subsets (our unpublished results). To our knowledge, $KG2A^-$ $LIR1^-$ NK cells have not been specifically examined to date [19,27]. This subset was increased in healthy donors compared to the patients before HCT, in patients that remained in remission compared to relapsed patients, and in KIR-ligand mismatched patients compared to KIR/HLA-ligand matched patients. Interestingly, there was no difference between patients in remission and relapsed patients in the percentages of $NKG2A^+$ NK cells. The most straightforward interpretation of our observations is that $NKG2A^-$ $LIR1^-$ NK cells play a protective role in the GVL effect. The tendency toward significance of different $NKG2A^-$ $LIR1^-$ NK cell numbers in KIR/HLA-ligand mismatched and matched patients was already present by days 56 and 84 post-HCT (Figure 5). Because at these time points most

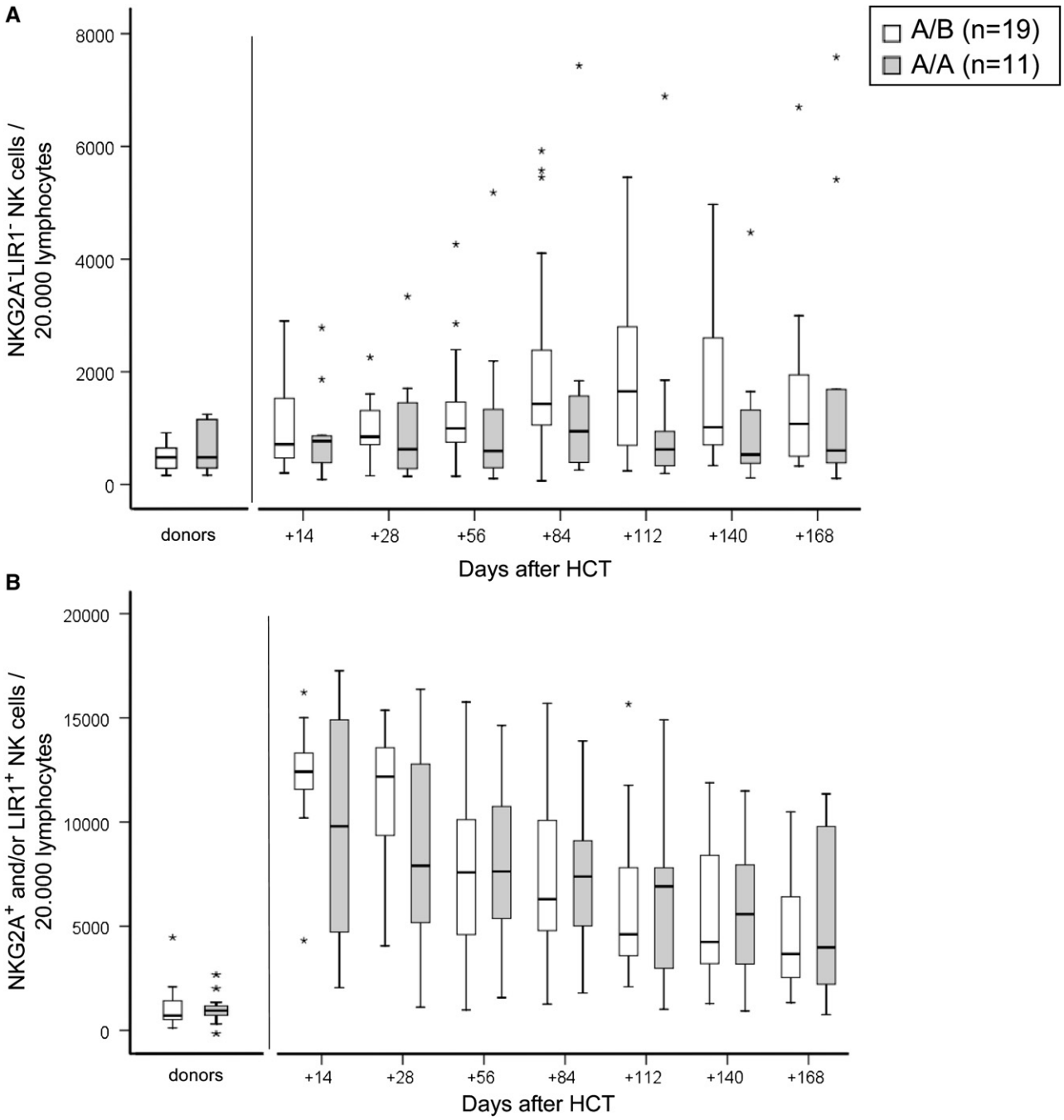

Figure 3. NK cell levels following HCT analyzed by the donor KIR haplotype. The numbers of (A) NKG2A⁻ LIR1⁻ NK cells and (B) NKG2A⁺ and/or LIR1⁺ NK cells, determined from a total of 20,000 effector cells by flow cytometry at different time points following HCT, were analyzed in the donors and their recipients according to the A/B and A/A KIR haplotypes. Patients of the B/B haplotype were not included in this analysis because 5 of 6 B/B patients were KIR-ligand matched.

patients were still in clinical remission, this suggests that decreasing percentages of NKG2A⁻ LIR1⁻ NK cells preceded leukemic relapses and not vice versa. Thus, higher numbers of antileukemic NK cells may be important for maintaining remission in the time period after day 112. Extrapolating from these data we propose that the adoptive transfer of donor NK cells [28] at some point during the time period between 1 and 3 months after HCT may protect against leukemia relapse, and that prophylactic infusions of enriched NKG2A⁻ LIR1⁻ NK cells may be particularly protective. Furthermore, we observed that expansion of NKG2A⁻ LIR1⁻ NK cells was more evident in patients with the A/B haplotype than in patients with the A/A haplotype. However, there was no apparent difference in the post-HCT rise of the NKG2A⁺ and/or LIR1⁺ NK cells associated with the different KIR haplotypes (Figure 3). Patients with A/B KIR haplotypes carry

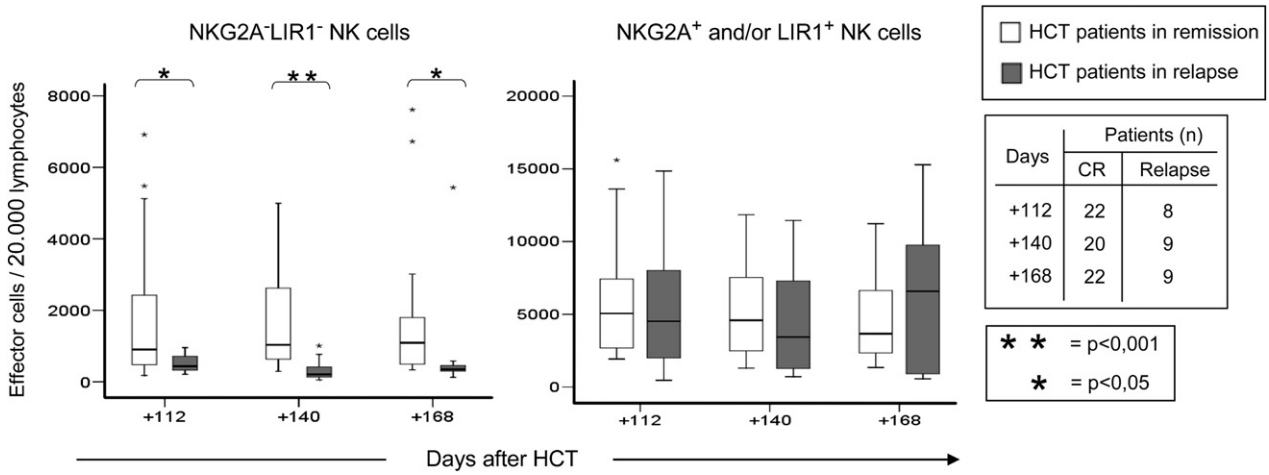

Figure 4. Effector cells positive or negative for NKG2A/LIR1 inhibitory receptors in the posttransplant course. The numbers of NKG2A$^-$ LIR1$^-$ NK cells as well as NKG2A$^+$ and/or LIR1$^+$ NK cells in patients in CR and in relapse were measured by flow cytometry on days 112, 140, and 168 following HCT. "NKG2A$^+$ and/or LIR1 $^+$ NK cells" include the NKG2A$^+$ LIR1$^-$, the LIR1$^-$ NKG2A$^+$, and the LIR1$^+$ NKG2A$^+$ subpopulations. The number of patients (n) in both groups at each of these time points is shown on the right. Statistically significant differences (Mann-Whitney *U*-test) ($P < .05$) are indicated by * and highly significant differences ($P < .001$) are indicated by **.

a greater diversity of HLA allele-specific inhibitory and activating receptors than A/A or B/B patients. The presence of the A-haplotype with allele-specific inhibitory KIRs may be required for education of NKG2A$^-$ LIR1$^-$ NK cells because these cells express less inhibitory receptors than NKG2A$^+$ and/or LIR1$^+$ NK cells. In addition, the presence of activating KIR-genes in the B-haplotypes may enhance NK cell proliferation and the GVL effect [29,30].

Most of the NKG2A$^-$ LIR1$^-$ NK cells that were increased in the KIR/HLA-ligand mismatched patients were of the CD56-dim phenotype (Figure S1). CD56-dim NK cells are the more cytotoxic NK cell subset, naturally expressing lower levels of NKG2A than CD56-bright NK cells that produce cytokines [31]. The NKG2A$^-$ LIR1$^-$ NK cells could be considered developmentally immature, but this probably only applies to NKG2A$^-$ LIR1$^-$ KIR$^-$ NK cells that produce less IFN-γ and lack cytotoxic potential [32]. However, the vast majority of the NKG2A$^-$ LIR1$^-$ NK cells are mature NK cells because they express various KIRs and are functional effector cells against HLA deficient target cells (Figure 6). The engrafted NKG2A$^-$ LIR1$^-$ NK cells expressing KIRs for which the patient had no HLA class I ligand also increased post-HCT and remained at these levels for at least 6 to 9 months, suggesting that their GVL effect is maintained for a long period of time [33,34]. The potential for alloreactivity by single KIR$^+$ NKG2A$^-$ LIR1$^-$ NK cells was documented for several patients using the CD107a assay following stimulation by K562 cells [23] (Figure 6). These experiments confirmed that, regardless of potential expression of an activating KIR, these were functional alloreactive NK cells. In a recent related study [35], single KIR$^+$ NK clones from transplanted pediatric patients were tested for lysis of autologous leukemia cells using conventional 51-Cr release cytotoxicity assays. Our CD107a flow cytometry assay

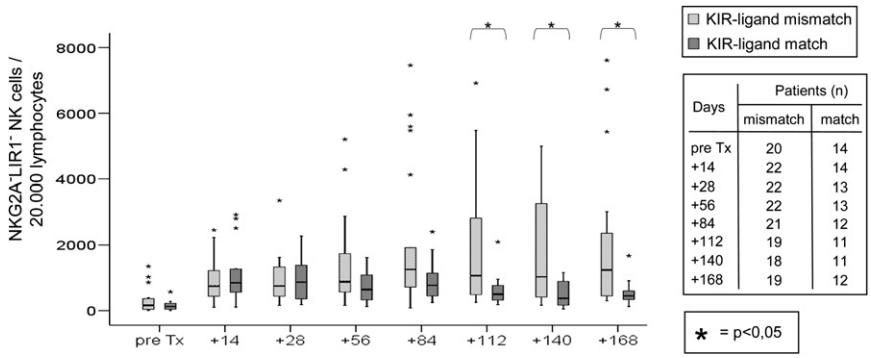

Figure 5. Comparison of NKG2A$^-$ LIR1$^-$ NK cell subpopulations in KIR/HLA-ligand mismatched and KIR/HLA-ligand matched patients. NKG2A$^-$ LIR1$^-$ NK cells were measured in KIR/HLA-ligand mismatched and KIR/HLA-ligand matched transplanted patients before HCT (pre Tx) and at different time points following HCT until day 168. The numbers of all NKG2A$^-$ LIR1$^-$ NK cells from a total of 20,000 lymphocytes for each patient are compared. For each time point, the numbers of patients (n) analyzed in the KIR/HLA-ligand mismatched and matched groups are given. Statistically significant differences between the groups are indicated by * (Mann-Whitney *U*-test).

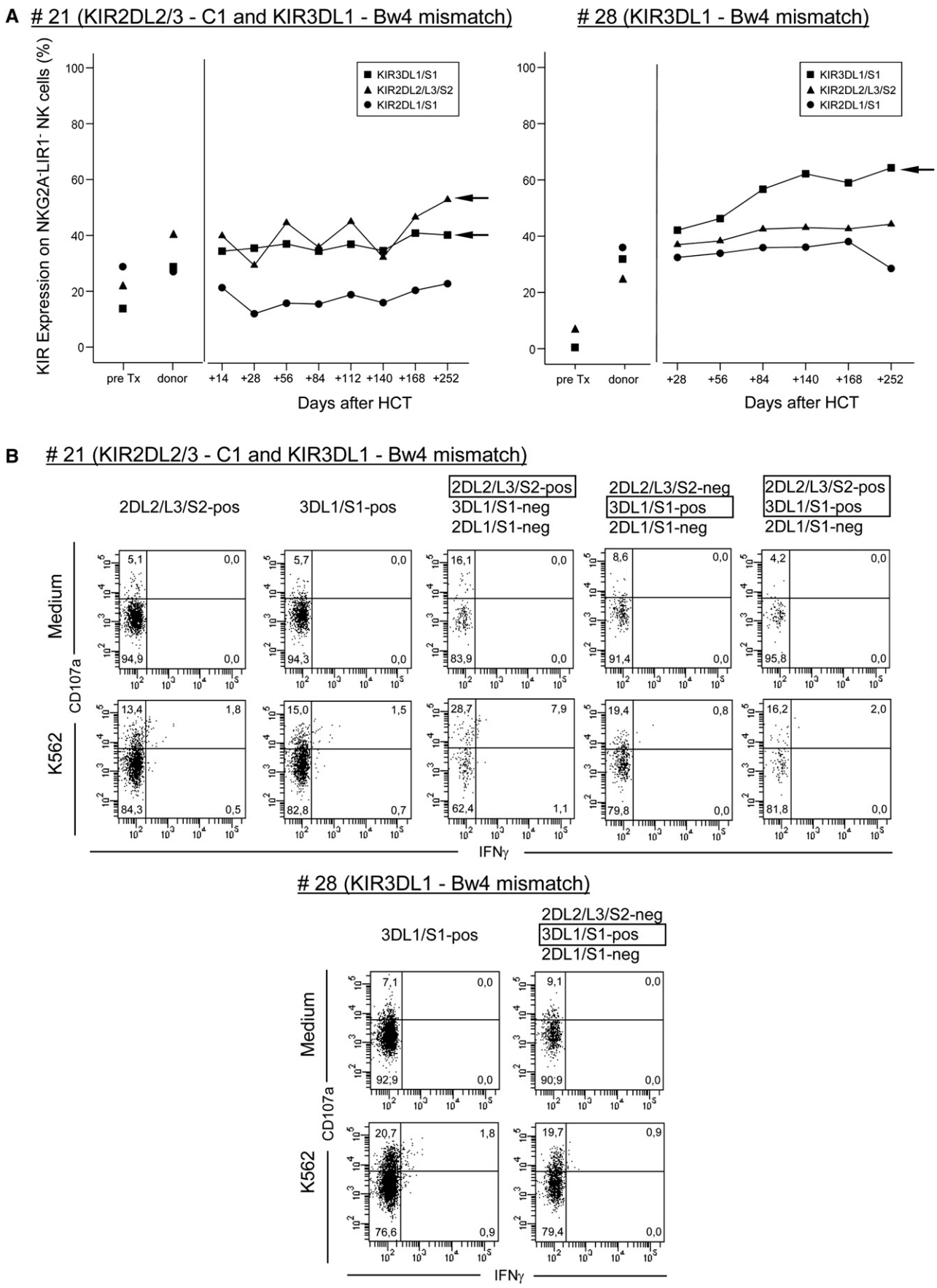
A
21 (KIR2DL2/3 - C1 and KIR3DL1 - Bw4 mismatch)
28 (KIR3DL1 - Bw4 mismatch)
KIR Expression on NKG2A-LIR1- NK cells (%)
KIR3DL1/S1
KIR2DL2/L3/S2
KIR2DL1/S1
pre Tx
donor
+14 +28 +56 +84 +112 +140 +168 +252
+28 +56 +84 +140 +168 +252
Days after HCT
B
21 (KIR2DL2/3 - C1 and KIR3DL1 - Bw4 mismatch)
2DL2/L3/S2-pos
3DL1/S1-pos
2DL2/L3/S2-pos 3DL1/S1-neg 2DL1/S1-neg
2DL2/L3/S2-neg 3DL1/S1-pos 2DL1/S1-neg
2DL2/L3/S2-pos 3DL1/S1-pos 2DL1/S1-neg
Medium
K562
CD107a
IFNγ
5,1 0,0 94,9 0,0
5,7 0,0 94,3 0,0
16,1 0,0 83,9 0,0
8,6 0,0 91,4 0,0
4,2 0,0 95,8 0,0
13,4 1,8 84,3 0,5
15,0 1,5 82,8 0,7
28,7 7,9 62,4 1,1
19,4 0,8 79,8 0,0
16,2 2,0 81,8 0,0
28 (KIR3DL1 - Bw4 mismatch)
3DL1/S1-pos
2DL2/L3/S2-neg 3DL1/S1-pos 2DL1/S1-neg
7,1 0,0 92,9 0,0
9,1 0,0 90,9 0,0
20,7 1,8 76,6 0,9
19,7 0,9 79,4 0,0

Table 2. Clinical Details of KIR-Ligand Mismatched Transplanted Patients #21 and #28 Who Were Examined in Detail in Figure 6

	# 21	# 28
Age/sex	57/f	52/m
Diagnosis	MDS	MDS
Disease status at HCT	untreated	untreated
Donor type	unrelated	unrelated
KIR ligand (donor)	C1,C2	C1,C2
KIR ligand (patient)	C2	C1,C2
KIR haplotype (donor)	A/B	A/A
Inhibitory KIR (donor)	2DL1,2DL2/3,3DL1	2DL1,2DL3,3DL1
Activating KIR (donor)	2DS2/3	No
KIR-ligand mismatch	KIR2DL2/3 - HLA-C1 KIR3DL1 - HLA-Bw4	KIR3DL1 - HLA-Bw4
GVHD (acute)	Grade 1 (skin)	Grade 4 (skin, gut)
GVHD (chronic)	mild day +192 (skin)	severe day +179 (gut) until day +195
Donor chimerism	Yes	Yes
Outcome	CR (+32 months) Alive	CR (+30 months) Alive

MDS indicates myelodysplastic syndrome; f, female; m, male; CR, complete remission; KIR, killer cell immunoglobulin-like receptor; HCT, hematopoietic cell transplant; GVHD, graft-versus-host disease.

offers the advantage of analyzing very rare NK cell subpopulations that have not been activated by IL-2 in vitro directly upon isolation from the patients' blood. Expression of CD107a on the surface of NK cells correlates with degranulation following stimulation and represents a sensitive marker of NK cell activity [23,36]. Testing autologous leukemia cells for "the lack of inhibition" of NK cell lysis by a missing HLA class I ligand offers, in our opinion, no definitive advantage to K562 cells. Nevertheless, K562 cells differ from autologous leukemia cells in the spectrum of ligands for NK activating receptors, most of which are yet uncharacterized [11,12]. Autologous leukemia cells were only available in a few patients, and are frequently contaminated by normal bone marrow cells. These facts and the condition of the leukemia cells following thawing prevented meaningful functional experiments in our system. This may be why other studies used fresh allogeneic leukemia cells as well as the HLA class I A, B, and C negative Epstein-Barr virus transformed B cell line .221 as target cells [34].

The GVL effect by NK cells implies that leukemia cells express ligands for activating NK receptors. Because ligands for these receptors on leukemia cells remain poorly characterized [9,11,12], it is still impossible to predict which patients' leukemia cells will be GVL targets. Although the expression of activating receptors on leukemia cells cannot be influenced, it is possible to select donors expressing an inhibitory KIR without a corresponding HLA class I ligand on the recipient's leukemia cells [37]. This can occur in both the HLA-mismatched or HLA-matched setting of HCT. Nevertheless, most studies on the protective role of potentially alloreactive NK cells were conducted in the haploidentical setting of HCT [15,17,33-35,38-40] or investigated both HLA-matched and mismatched patients [19,20]. Only a few groups have focused their analysis on HLA-matched patients or HLA-identical siblings [18,21,30,41,42]. However, there is clearly a lower incidence of treatment related complications following HLA matched allogeneic HCT [37], whereas a mismatch at a single or multiple loci is associated with higher treatment-related mortality and graft-versus-host disease, regardless of other factors such as patient age, disease status, or use of T cell depletion [43]. For fully HLA-matched patients, haplotype matching seems to further decrease the likelihood of severe graft-versus-host disease [44]. Thus, practically all patients in our institution were HLA-matched because our donors were HLA-identical siblings or unrelated donors, primarily selected based on a match in HLA A, B and -DRB1.

One may question if functional alloreactive NK cells exist post-HCT in the situation of an HLA-match. We found at least 9 donors in our relatively small study that expressed KIR for which they themselves lacked an HLA class I ligand ("aberrant" KIR). Most aberrant KIRs were expressed by NK cells that coexpressed additional inhibitory receptors. Although

Figure 6. Subpopulations of NKG2A$^-$ LIR1$^-$ NK cells expressing individual KIR and their alloreactive potential. (A) The numbers of NKG2A$^-$ LIR1$^-$ NK cells, expressing different KIR, from KIR/HLA-ligand mismatched patients #21 and #28 before (pre Tx) and at several time points following HCT, compared to their donors. NK cell subsets expressing the KIR for which the patients had no HLA class I ligand (marked by an arrow). (B) CD107a expression and intracellular IFN-γ secretion following stimulation by medium or K562 cells were measured by flow cytometry in subpopulations of the NKG2A$^-$ LIR1$^-$ NK cells from patients #21 (days 331 post-HCT) and #28 (day 357) (subsets indicated above the dot plots), expressing KIR for which the patients had no HLA class I ligands (outlined by a frame). The percentages of positive cells are indicated in the quadrants. KIR2DL2/L3/S2$^+$ or KIR3DL1/S1$^+$ gated dot plots included NKG2A$^-$ LIR1$^-$ NK cells expressing other inhibitory KIR. Analysis of dot plots gated on multiple KIR was restricted to NK cells expressing the mismatched KIR as their only inhibitory receptors (for patient #21 KIR2DL2/L3/S2$^+$ and/or KIR3DL1/S1$^+$ and for #28 KIR3DL1/S1$^+$) (for the KIR genotype see Tables 2/S1).

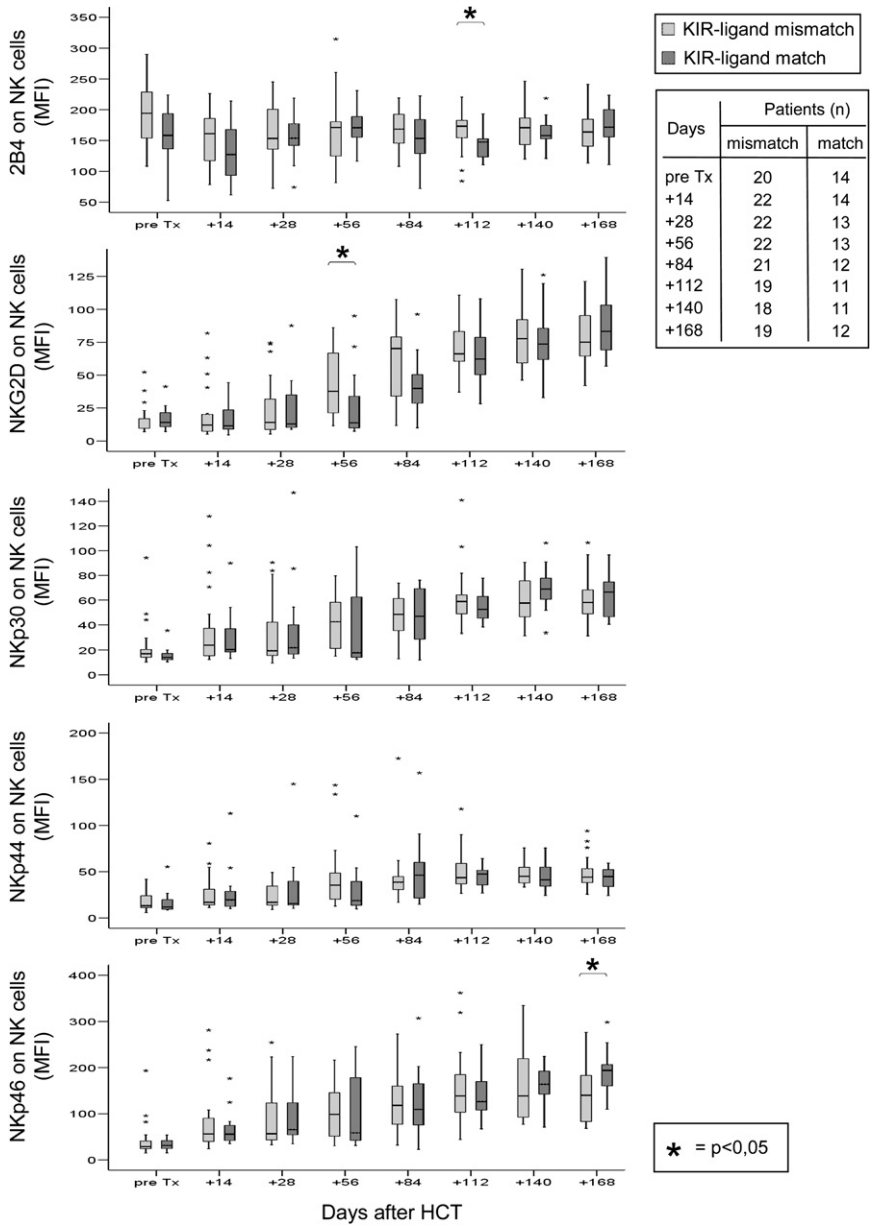

Figure 7. Activating receptors on NK cells following KIR/HLA-ligand matched and mismatched HCT. The mean fluorescence index (MFI) was determined by flow cytometry for the activating receptors 2B4, NKG2D, NKp30, NKp44, and NKp46 expressed by NK cells from KIR/HLA-ligand mismatched and KIR/HLA-ligand matched transplanted patients before HCT (pre Tx) and at different time points following HCT. The numbers of patients (n) in the KIR/HLA-ligand mismatched and matched groups for each time point are indicated on the right. Statistically significant differences between the KIR/HLA-ligand mismatched and matched patients are indicated (*).

this would prevent most autoreactivity against normal cells, such NK cells may still mediate a significant GVL effect because leukemia cells may express ligands for activating NK receptors so that the total activating signals may prevail over the inhibitory signals [9,11]. In addition, leukemia cells may lose single HLA class I alleles that would make them sensitive for GVL effects by rare NK cell subsets [45]. We show that such NK cells expressing aberrant KIR increase post-HCT (eg, KIR3DL1$^+$ cells in patients #21 and #28; Figure 6). Although NK cells lacking self-HLA class I receptors are normally considered to be developmentally and functionally immature [24,46], the present data and a study published recently [42] suggest that NK cells expressing the HLA-mismatched KIRs, and no other inhibitory receptors, may actually be functional following HCT (Figure 6B). Nevertheless, we cannot exclude that they are somewhat functionally impaired, as has been suggested by another study in a haploidentical setting [34]. Such single KIR$^+$ NK cells with alloreactive potential may be present in all HLA-matched HCT-patients who are not positive for all 3 KIR/HLA ligands (C1, C2, and Bw4). An intriguing hypothesis is that, even in the healthy donors,

such NK cells might have a "leukemia-protective" effect. Further experiments are needed to investigate how NK education following stem cell transplantation might differ from the physiological development of NK cells.

Expression levels of the activating receptors NKG2D, NKp30, NKp44, and NKp46 on NK cells increased in both patient groups during the observation period following HCT (until day 168) (Figure 7). Most remarkable was the rise in NKG2D-expression on NK cells that could be observed in KIR/HLA-ligand mismatched patients around day +56 and in the KIR/HLA-ligand matched patients around day +112. Increased expression levels of NK activating receptors at later time points following HCT may represent a mechanism in maintaining remission following HLA mismatched, as well as matched transplants.

In addition to NK cells, we also investigated the expression of NK receptors on other effector cell populations such as NKT cells, NKT-like cells, as well as other αβ and γδ T cells. It appears that NKG2A$^-$ LIR1$^-$ NKT-like cells and NKG2A$^-$ LIR1$^-$ γδ T cells expressing KIRs transiently increased following HCT compared to the levels in the patient pre-HCT or those seen in the donor. The vast majority of cytotoxic γδ T cells expressed CD94/NKG2A [4,47] and the NKG2A$^-$ LIR1$^-$ phenotype on T cells is rather infrequent. Expression of broadly reactive inhibitory receptors is restricted to T cells with a potential for autoreactivity. These receptors downmodulate the activating signals from the TCR and protect from the autoimmune capacity of these effector cells. In other words, T cells expressing broadly reactive inhibitory receptors need additional signals from the TCR to play a role in GVH and GVL responses, and the antigenic specificities of these cells depend on the T cell repertoire of the particular donor. Our data showing relatively few KIR-expressing T cells after HCT suggest that such MHC unrestricted and potentially autoreactive T cells are less important for the GVL effect than NK cells.

In summary, our data suggest that NKG2A$^-$ LIR1$^-$ NK cells are important effector cells in GVL. NK cells, expressing only 1 inhibitory KIR for which the patient has no ligand, may mediate GVL effects in haploidentical, KIR/HLA-ligand mismatched, as well as in HLA-matched, KIR/HLA-ligand mismatched HCT patients. The function of such NK cells following HCT needs to be compared to similar NK cells present in normal donors, as well as to regular NKG2A$^+$ NK cells.

ACKNOWLEDGMENTS

The authors thank the staff of the bone marrow transplantation clinics, laboratories, and Station Löhr for their assistance and support, Irmgard Matt for organizing the clinical and HLA typing data, and Roland Mertelsmann and Martin Werner for support and encouragement.

Financial disclosure: This work was supported by grant DJCLS R 05/01 from the Deutsche José Carreras Leukämie Stiftung to J.F. and P.F. and SFB 620 Z2 to P.F. Markus Kaymer is employed by Beckman Coulter, Krefeld, Germany, a company acting in health care. The other authors declare no competing financial interests.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbmt.2009.12.008.

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