Expansion of CD56-Negative, CD16-Positive, KIR-Expressing Natural Killer Cells after T Cell-Depleted Haploidentical Hematopoietic Stem Cell Transplantation

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Introduction
Natural killer (NK) cells are an important constituent of the innate immune system, exert cytotoxicity against infected and transformed cells, and produce cytokines and chemokines that regulate adaptive immune responses [1–3]. In humans, NK cell function is regulated by clonally distributed inhibitory receptors termed killer cell immunoglobulin-like receptors (KIR). KIR recognize epitopes (KIR ligands) shared by human leukocyte antigen (HLA) class I antigens. Functional NK cells express at least one receptor for self HLA class I. Those which express, as their only inhibitory receptor for self, a KIR for the HLA class I group which is absent on allogeneic targets sense the missing expression of the self class I KIR ligand and mediate alloreactions [4–10].

Donor-versus-recipient NK cell alloreactions may occur in HLA haplotype mismatched (haploidentical) hematopoietic stem cell transplantation (HSCT) with KIR ligand incompatibility in the graft-versus-host direction. NK cell alloreactivity impacts beneficially on outcome after haploidentical transplants [11, 12], as it reduces the risk of leukemia relapse without causing graft-versus-
host disease, and thereby markedly improves leukemia-free survival in humans and in murine models [13–16].

In our studies on the NK cell repertoire reconstitution pattern in haploidential HSCT recipients, we detected expansion of an unusual KIR-expressing CD16+/CD56− NK cell subset. Here, we describe its phenotype, posttransplant recovery kinetics, function and alloreactive potential.

Materials and Methods

Patients

Fifty haploidential HSCT recipients were included in this study. Approval was obtained from the Umbria Region Ethics Committee and from the Perugia University institutional review board. Informed consent was provided in accordance with the Declaration of Helsinki. Diagnoses were acute myeloid leukemia in 27 patients, acute lymphoblastic leukemia in 12, Hodgkin’s lymphoma in 7, non-Hodgkin’s lymphoma in 2, chronic myeloid leukemia in 1 and multiple myeloma in 1. Patients received CD34-selected peripheral blood hematopoietic stem cell grafts after conditioning with total body irradiation, thiotepa, fludarabine and either antithymocyte globulin or the monoclonal anti-CD3 antibody OKT3. No pharmacological immunosuppression was given after transplantation.

Flow Cytometry and Immunophenotyping

To study reconstituting NK cell subsets after transplantation we performed immunofluorescence analyses on NK cells purified by the RosetteSep human NK cell enrichment cocktail method (StemCell Technologies, Vancouver, B.C., Canada) which consistently allowed purification of a population >99% negative for CD3+ T lymphocytes (representative plot in fig. 1a), CD19+ B lymphocytes, CD66b+ granulocytes and CD36+ monocytes.

To characterize purified NK cells, APC-conjugated anti-CD56 (IgG1; Miltenyi, Bergisch-Gladbach, Germany) was used in combination with either: unconjugated anti-CD16 (IgM; BD Bioscience, San Jose, Calif., USA) developed with fluorescein isothiocyanate (FITC)-conjugated IgM (Southern Biotech, Birmingham, Ala., USA) and phycoerythrin (PE)-conjugated anti-NKp46 (IgG1; Beckman Coulter, Fullerton, Calif., USA); PE-conjugated anti-NKp30 (IgG1; Beckman Coulter); PE-conjugated anti-CD244 (2B4, IgG1; eBioscience, San Diego, Calif., USA); PE-conjugated anti-CD161 (NKR-P1A, IgG2a; Miltenyi); (PE)-conjugated anti-KIR2DL2/3/S2, anti-KIR2DL1/S1 and anti-KIR3DL1/S1 (all IgG1 from Beckman Coulter) as well as unconjugated anti-NKG2A (IgG2b, kindly donated by A. Moretta, University of Genoa, Italy) developed with FITC-conjugated goat anti-mouse IgG2b antibodies (Southern Biotech); unconjugated anti-NKG2P80 or unconjugated anti-NKG2D (IgG1, kindly donated by A. Moretta, University of Genoa, Italy) developed with FITC-conjugated goat anti-mouse IgG1 antibodies (Southern Biotech).

Enumeration of KIR+/NKG2A−/CD3−/CD56− and KIR+/NKG2A−/CD3−/CD56+ NK cells was determined from peripheral blood mononuclear cells by four-color immunofluorescence using the antibodies described above in conjunction with PE-Cy7-conjugated anti-CD3 (IgG1; eBioscience).

HLA Missing Self-Response and ADCC

To assess missing self-HLA response in CD56+ and CD56− NK cells, purified NK cells were incubated with the HLA-deficient erythroleukemia cell line K562. The capacity for ADCC was measured by co-incubating purified NK cells with the EBV-transformed B cell line W00 (which expresses all relevant KIR ligands) in the presence of saturating concentrations of the chimeric IgG anti-CD20 antibody (Rituximab; Roche, Basel, Switzerland). The NK cell response in both assays was measured by assessing IFN-γ secretion (by intracellular cytokine staining) and degranulation (by assessing surface CD107a) [17]. As negative controls we co-incubated NK cells with autologous phytohemagglutinin (PHA) lymphoblasts (degranulation and IFN-γ production <1% in both CD56+ and CD56− subsets); positive controls were effector cells with PMA/ionomycin (degranulation and IFN-γ production >30% in both subsets).

NK Cell Cloning and Cytotoxicity Assay

NK cells were separated into CD56+ and CD56− subsets by immunomagnetic sorting using PE-conjugated anti-CD56 antibodies and anti-PE antibody-conjugated microbeads (Miltenyi). Purity of subfractions, as assessed by flow cytometry, was >95% for CD56+ and >99% for CD56− cells. CD56+ and CD56− NK cells were plated in 96-well round-bottom plates at concentrations of 20, 10 and 5 cells/well, activated with PHA (Biochrom KG, Berlin, Germany) and cultured with IL-2 (Chiron BV, Amsterdam, The Netherlands) on irradiated feeder cells for 3 weeks. Specific lysis by cloned NK cells was tested by standard 51Cr release assay using an effector-target ratio of 5:1 against K562 cells, KIR ligand-mismatched allogeneic PHA lymphoblasts, autologous PHA lymphoblasts and leukemic blasts in selected patients. In tests against KIR ligand-mismatched target cells, mean lysis of alloreactive clones only (specific lysis >5%) within the donor’s repertoire is reported.

Statistical Analyses

Characteristics of CD56+ and CD56− NK cells were compared by the Mann-Whitney U test (unpaired comparisons) and Wilcoxon’s rank test (paired comparisons), where appropriate. Two-sided p values of <0.05 were considered statistically significant.

Results

Identification of NK Subsets Reconstitution in Recipients of Haploidential Transplantation

Flow-cytometric analyses of reconstituting NK cells in recipients of T cell-depleted haploidential HSCT revealed an unusual subset of CD3− lymphocytes expressing KIR but not CD56 molecules (fig. 1b). Upon further investigation, these CD56− cells expressed NK cell-associated and NK cell-specific surface antigens such as CD16, 2B4 (CD244), NKR-P1A (CD161) as well as the natural cytotoxicity receptors NKp30 and NKp46 (fig. 1c–e show representative flow-cytometric plots). Compared to their CD56-expressing counterparts, CD56− NK cells expressed CD16, KIR and CD161 at similar frequencies,
whereas the expression of NKG2A, NKp46, NKp30 and 2B4 was significantly lower in CD56– compared to CD56+ NK cells (table 1).

As expression of multiple KIR identifies a subset of very mature NK cells, we analyzed both overall KIR expression and co-expression of multiple KIR. While overall expression of KIR was similar between the two subsets, expression of more than one KIR was higher in CD56+ NK cells than in CD56– NK cells, although this difference reached statistical significance only for one combination of KIR: KIR2DL1/S1+/KIR2DL2,3/S2+ (19 ± 18% vs. 6 ± 6.6%, p = 0.015), KIR2DL2,3/S2+/KIR3DL1/S1+ (11 ± 11% vs. 3.2 ± 3%, p = 0.06) and KIR2DL1/S1+/KIR3DL1/S1+ (5.2 ± 4.3% vs. 1.6 ± 1.7%, p = 0.10).

In an attempt to identify a marker that detects the largest possible number of NK cells in transplant recipients, we evaluated several candidate antigens with a broad ex-

Fig. 1. Immunophenotype of CD56+ and CD56– NK cells after T cell-depleted haploidentical HSCT. a Detection of a CD56–/CD3– population after NK cell purification. b–e Expression of KIR (b), CD16 (c), NKp46 (d) and 2B4 (CD244; e) on CD56– and CD56+ NK cells in patients after T cell-depleted haploidentical HSCT. All analyses carried out on RosetteSep-purified NK cells.
pression on NK cells. On total NK cells, NKp46 was detected in a mean of 83% (±24%), CD244 (2B4) in 99% (±1%), NKG2D in 91% (±6%), and NKp80 in 94% (±4%). Therefore, some of these markers might be more sensitive in detecting NK cells than CD56. For example, CD244 (2B4) was consistently expressed on almost all NK cells at all time points. In contrast, a significant NK cell population lacking the CD56 antigen could be detected up to day 150 after transplant (fig. 2a).

Post-Transplant Reconstitution of KIR+/NKG2A– NK Cells

As KIR+ NK cells contain the effectors of graft-versus-leukemia reactions in haploidentical HSCT, we investigated the kinetics of KIR+/NKG2A– NK cells which emerged after haploidentical HSCT in the CD56+ and the CD56– subsets. At the earliest time point (day 30), CD56–/KIR+/NKG2A– NK cells outnumbered CD56+/KIR+– NKG2A– NK cells (33.5 ± 10^6 vs. 11 ± 10^6 cells/l, p = 0.10). At all later time points the KIR+/NKG2A– NK cell count was higher in the CD56+ subset than in the CD56– subset, although the difference did not reach the level of statistical significance (fig. 2b). The KIR+/NKG2A– NK cell count peaked in both the CD56– (85 ± 10^6 cells/l) and CD56+ NK cell subsets (136 ± 10^6 cells/l, p = 0.49) on day 120 and declined thereafter.

Functional Profile of CD56– NK Cells

To investigate CD56– NK cells functionally, we tested degranulation and cytokine production in response to stimulation with appropriate target cells. Figure 3a exemplifies degranulation as assessed by surface expression of CD107a after incubation with the HLA-deficient erythroleukemia cell line K562 in CD56– and CD56 dim NK cells. In agreement with previously published data [18], activation of NK cells by co-incubation with K562 in transplant recipients was lower than that reported for healthy donor NK cells [19]. Degranulation in CD56 dim NK cells (6 ± 7%) was comparable to that of CD56– NK cells (5 ± 6%, p = 0.26; fig. 3b). In contrast, IFN-γ production was significantly lower (3 ± 3%) in CD56– NK cells compared to CD56 dim NK cells (4 ± 5%, p = 0.007; fig. 3b) after exposure to K562 cells. However, both the degranulation and cytokine production assays suggested that CD56– respond to missing self-stimulation.

In ADCC assays using a B cell line as target cell in conjunction with the anti-CD20 antibody rituximab, 10 ± 5% of CD56 dim NK cells degranulated compared to 8 ± 5% of CD56– NK cells (p = 0.02). A similar trend was seen

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**Table 1. Immunophenotype of NK cells which reconstitute after T cell-depleted haploidentical transplantation**

<table>
<thead>
<tr>
<th>Antigen(s)</th>
<th>CD56+ NK cells</th>
<th>CD56– NK cells</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16</td>
<td>83 ± 9</td>
<td>82 ± 12</td>
<td>0.97</td>
</tr>
<tr>
<td>KIR</td>
<td>42 ± 21</td>
<td>46 ± 23</td>
<td>0.44</td>
</tr>
<tr>
<td>NKG2A</td>
<td>62 ± 24</td>
<td>33 ± 19</td>
<td>0.0001</td>
</tr>
<tr>
<td>KIR+/NKG2A–</td>
<td>38 ± 24</td>
<td>32 ± 25</td>
<td>0.02</td>
</tr>
<tr>
<td>NKp46</td>
<td>82 ± 19</td>
<td>55 ± 26</td>
<td>0.0001</td>
</tr>
<tr>
<td>NKp30</td>
<td>42 ± 21</td>
<td>35 ± 20</td>
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</tr>
<tr>
<td>CD161</td>
<td>68 ± 27</td>
<td>58 ± 27</td>
<td>0.11</td>
</tr>
<tr>
<td>2B4</td>
<td>99 ± 1</td>
<td>89 ± 12</td>
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</tbody>
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**Fig. 2.** Post-transplant reconstitution of CD56+ and CD56– NK cells. a Absolute numbers of CD56+ and CD56– NK cells (mean ± SD) in the first 5 months after haploidentical HSCT. b Post-transplant reconstitution of KIR+/NKG2A– NK cells in CD56+ and CD56– subsets (mean ± SD, n = 25).
for IFN-γ production which was detected in 12 ± 10% of CD56dim NK cells compared to 8 ± 6% of CD56− NK cells (p = 0.06; fig. 3e). No significant degranulation or cytokine production was measured for the CD56bright NK cell subset in either assay [20].

**Alloreactivity of Clonally Expanded CD56+ and CD56− NK Cells**

Cytotoxicity assays were performed with clonally expanded NK cells against K562 and KIR ligand-mismatched allogeneic targets. Importantly, CD56− NK cells acquired CD56 expression during 15–20 days of stimulation with IL-2, as has previously been shown for CD56− NK cells in HIV patients [21]. The proliferative potential of CD56− versus CD56+ NK cells appeared to be similar as indicated by overlapping cloning efficiencies (mean 1 cell in 300, p = 0.60) and number of cells obtained for each clone (range 2 × 10^3 to 4 × 10^5) in the two populations.

Clones derived from CD56+ and CD56− NK cells exerted similar cytotoxicity against K562: 50 ± 10% vs. 44 ± 17%, p = 0.37 (fig. 4a). Alloreactivity was further tested against allogeneic targets lacking C1 or C2 expression. Lysis of PHA-lymphoblasts lacking HLA-C1 was comparable (mean lysis 23 ± 4% in 6/96 CD56+ NK clones; mean lysis 21 ± 2% in 7/165 CD56− NK clones, p = 0.10; fig. 4d). Similarly, no significant differences were seen between CD56+ and CD56− NK cell clones in their capacity to lyse leukemia blasts lacking HLA-C2 (mean lysis 20 ± 1% in 3/107 CD56− NK clones; and mean lysis 22 ± 1% in 3/93 CD56+ NK clones, p = 0.65; fig. 4e).
Discussion

In healthy donors, the majority (approx. 90%) of NK cells are CD56dim, whereas approximately 10% of NK cells are CD56bright [1]. NK cells lacking CD56 expression are rare in healthy donors, were originally described in HIV-infected individuals [21, 22] and have more recently been found at elevated levels in other chronic infectious diseases, such as hepatitis C [23, 24] and Chagas disease [25]. However, in these settings, NK cells lacking CD56 expression are functionally defective as they display decreased levels of activating receptors and fail to kill target cells.

The present study demonstrates that in recipients of T cell-depleted haploidentical HSCT, a significant number of reconstituting NK cells do not express CD56. We observed a remarkable post-transplant expansion of these unusual CD56– NK cells. They express the natural cytotoxicity receptors NKp46 and NKp30 and activating coreceptors such as CD244, lyse HLA-deficient target cells, express KIRs and show cytolytic activity against allogeneic targets and leukemic cells which do not express cognate class I ligands. KIR expression occurs as a late event during NK cell development and NK cells expressing more than one KIR are among the most mature [26, 27]. We found that NK cells expressing more than one KIR were preferentially CD56+. However, our data clearly show that CD56– NK cells are functional. When analyzed at the clonal level, the unusual CD3–/CD56– subset contained similar frequencies of alloreactive NK cells as the conventional CD3–/CD56+ subset.

Fig. 4. Cytolytic activities of clonally expanded CD56– versus CD56+ NK cells. Mean specific lysis of NK clones derived from CD56+ and CD56– NK cells against K562 target cells (a) and against allogeneic PHA lymphoblasts and leukemic target cells lacking expression of the HLA C1 and C2 specificities (b-e) (n = 15).
An expansion of CD56− NK cells may be common to different forms of HSCT, as CD56−/CD16+ NK cells has previously been described after cord blood transplantation [28], and after HLA-matched bone marrow/peripheral blood stem cell transplantation [29]. Little is known, however, about the function of CD56− NK cells arising after HSCT. Our data suggest that CD56− NK cells may contribute to graft-versus-leukemia effects after haploidentical and possibly other types of HSCT [30].

Finally, the present data have a bearing on recent attempts to assess the frequencies of potentially alloreactive NK cells by flow cytometry [31]. Our data indicate that gating on conventional CD3−/CD56+ cells may considerably underestimate the alloreactive NK cell frequency and that a gating strategy using a more broadly expressed receptor such as CD244 in combination with CD3 may be more appropriate.

In conclusion, we show that in recipients of T cell-depleted haploidentical HSCT, a significant number of NK cells lack expression of CD56. CD56− NK cells appear to be functional and capable of graft-versus-leukemia effects.

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