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NK cell maturation to CD56^{dim} subset associated with high levels of NCRs overrides the inhibitory effect of NKG2A and recovers impaired NK cell cytolytic potential after allogeneic hematopoietic stem cell transplantation

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ABSTRACT

NK cell cytotoxicity against residual leukemic cells is crucial for immune system reconstitution after hematopoietic stem cell transplantation (HSCT). Since immune recovery after transplant still remains a major concern, we studied the counterbalance of NK cell receptors after HSCT and its importance in NK cell functional recovery. We investigated NK cell reconstitution in 27 acute leukemia patients at different time points following HLA-matched allogeneic HSCT compared to those of donors. NK cells were evaluated for their cytotoxicity in a standard ^{51}Cr-release assay against target cells and also analyzed for their receptors expression using flow cytometry. Early after transplant, we found higher percentage of CD56^{bright} NK cells, increased levels of NKG2A and NCRs as well as decreased levels of KIRs expression on NK cells associated with an impaired cytotoxicity of these cells. All the abnormalities were normalized by one year after HSCT when CD56^{bright} NK cells gradually differentiated into CD56^{dim} subset. Collectively, we confirmed a gradual increase of CD56^{dim} NK cells expressing NCRs with the significant decrease in NKG2A expression on NK cells. This finding was also associated with the recovery of NK cell cytotoxicity that suggests an important role for the kinetics of NK cell receptors during cell maturation in HSCT outcome.

1. Introduction

For more than three decades, hematopoietic stem cell transplantation (HSCT) has been considered as a standard treatment for certain malignant and non-malignant hematopoietic disorders [1,2]. Immune reconstitution after HSCT could be affected by different factors including type of graft, underlying diseases, conditioning regimen, GvHD, age, cytokines, post-transplant immune suppressive or immune modulating medications and infections [3].

Several lines of evidence indicate that the NK cells of innate immunity reconstitute quicker than the T cells of adaptive immunity after transplant [3–5]. This faster recovery supports the early reconstitution of innate immune response to tumor cells via direct anti-tumor effect of NK cells that can prevent relapse after allogeneic HSCT [6,7]. A number of studies suggests an anti-tumor effect of NK cells particularly in AML patients who have undergone haplo-identical HSCT with T cell depletion [8]. Graft-versus-leukemia (GvL) is often crucial to the eradication of residual malignant cells that have survived the conditioning regimen prior to HSCT. A growing body of evidence shows an important role for NK cells in GvL [9,10]. Hence, a better understanding of the mechanisms involved in NK cell cytotoxicity against tumor cells has generated an increasing interest in the reconstitution of these cells after HSCT. Human NK cells are classically presented as CD3$^-$CD56$^+$ cells and according to their levels of CD56 expression, these cells are subdivided into two major subsets of CD56^{dim} and CD56^{bright} [11].

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CD56^{dim} NK cells are fully mature, comprising approximately 90% of NK cells in peripheral blood. These cells predominantly mediate cytotoxicity responses. In contrast, CD56^{bright} cells comprise approximately 5–15% of total NK cells which are mostly immature with a limited role in cytolytic responses [12]. In addition to the mentioned maturation status, a delicate balance between activatory and inhibitory signals transduced by different types of NK cell receptors supports functional potential of these cells [13,14].

Among NK cell receptors, NKG2D and NCRs (NKp30, NKp44 and NKp46) appear to be the most important receptors that support cytolytic responses to tumor target cells, while FcγRIIIA (CD16) mediates ADCC against target cells opsonized with IgG [12,15]. The Inhibitory KIRs and NKG2A/CD94 are the most important inhibitory receptors and prevent autoreactivity through the recognition of HLA class-I ligands expressed on the surface of healthy cells [16,17]. Consistently, the presence of immature state of NK cells (CD56^{bright} subset) may affect their activities. Several lines of evidence indicate that early reconstitution of NK cell receptors after transplant may play an important role in the improvement of the immune responses leading to a successful transplant with better outcomes in patients [8,10,18]. Considering the important role of T cells in NK cell maturation [19,20], we decided to study the patterns of NK cell reconstitution in correlation with their cytolytic potentials in patients undergoing non-T cell depleted HLA-matched HSCT.

2. Patients and methods

2.1. Study cohort

Our study group included 27 leukemic patients and their relevant donors with one year follow-up investigation. The patients received peripheral blood stem cells from the donors with HLA-matched allogeneic HSCT. Donor selection was performed using molecular typing for HLA-A, -B, -Cw, -DRB1, -DQ and -DP by the Australian Red Cross Blood Service. All donors and patients allele were matched for HLA-A, -B, -Cw, -DRB1, -DQ. Samples were collected from the donors and patients before the commencement of the conditioning regimen and also from post-HSCT patients at 1st, 2nd, 3rd, 6th, 9th, 12th months. Characteristics of the study cohort are given in Table 1. Conditioning regimen was TBI-based or Fludarabine/Melphalan/Campath. Dose of TBI was either 12cGy for standard risk patients or 13.2 cGy for high risk patients. Dose of Fludarabine was 25 mg/m^2 from day −7 to −3, dose of melphalan was 140 mg/m^2 on day −2 and dose of campath was 10 mg per day from −8 to −4. GvHD prophylaxis was CSA/Methotrexate. Dose of CSA was 3 mg/kg/day from day −1 and adjusted to keep trough levels to 100–300 μg/L. Dose of Methotrexate was 8 mg/m^2 at day +2, +4, +8 and +12. Written consent was obtained from all patients and donors. This study has been reviewed and approved by the Ethics Committee at the Alfred Hospital in Melbourne of Australia (Project No 30/07) [10,21].

Table 1
Characteristics of the study cohort.

Characteristics	*n* (%) or median (range)
Patients	27
Median age at transplantation, years (range)	47 (23–64)
Male	17 (63)
Underlying diagnosis	
AML	16 (60)
ALL	11 (40)
Donors	27
Median age, years (range)	44 (22–57)
Male	20 (74)
Transplantation	
Matched at the allele level for HLA-A, -B, -Cw, -DRB1, -DQ	27 (100)
Peripheral blood stem cells	27 (100)
Conditioning regimen	
TBI-based	19 (70)
Fludarabine/Melphalan/Campath	8 (30)
GvHD prophylaxis	
CSA/Methotrexate	27 (100)

CSA, cyclosporine A; GvHD, graft-versus-host disease; TBI, total body irradiation.

2.2. Cell isolation and culture

MNCs were isolated by Ficoll-Paque density gradient centrifugation. NK cells were separated from MNCs by MACS-NK cell isolation kit (Miltenyi Biotec Inc., Germany). The purity of the enriched NK cells was evaluated by flow cytometry using anti-human mAbs against CD56 and CD3 markers. NK cells were cultured for two weeks in the presence of 30 Gy irradiated autologous MNCs (1:5) and IL-2 (100 IU/mL; R&D Systems Inc., MN, USA) in RPMI 1640 containing 10% heat-inactivated autologous plasma, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Gibco BRL, Grand Island, NY, USA). Erythroleukemia cell line K562 culture was performed in complete medium containing RPMI 1640 (Gibco BRL, USA) supplemented with 10% heat-inactivated FCS (JPH Bioscience, USA), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Gibco BRL, USA).

2.3. Fluorescence-activated cell sorter (FACS)

Surface phenotype of cells was determined with the following mouse anti-human mAbs: CD3 (SK7)-PerCP; CD3 (UCHT1)-FITC; CD56 (B159)-PE and APC, KIR2DL1 (HP-3E4)-FITC, KIR2DL2 (CH-L)-FITC, NKp30 (Z25)-PE; NKp46 (BAB281)-PE, NKp44 (Z231)-PE and NKG2D (ON72)-APC all from BD (Sanjose, CA, USA), KIR3DL1 (DX9)-APC from R&D systems (Minneapolis, MN, USA) and NKG2A (Z199)-PE from Immunotech (Marseille, France). Corresponding isotype matched control mAbs were also included in all analyses. All samples were assessed by a 4-color FACSCalibur.

NK and T cell counts in blood were calculated as follows:

The percentage of ($CD3^-CD56^+$) NK or ($CD3^+CD56^-$) T cells in the lymphocyte gate × the total number of lymphocytes (determined by automated cell counter).

2.4. Cytotoxicity assay

The cytotoxicity of NK cells from donors and leukemic patients at different time points after HSCT was assessed against K562 and MNCs derived from pre-HSCT leukemic patients using a standard 4-h ^{51}Cr-release assay, as previously described [20].

The restoration of cytolytic potential of NK cells from leukemic patients was assessed in the presence of anti-NKG2A (clone Z199, 20 μg/mL), anti-CD94 (clone HP-3B1, 20 μg/mL) and anti-HLA class-I (clone W6/32, 10 μg/mL) mAbs. Corresponding isotype matched control mAbs (IgG_{2b}, $IgG_{2a,k}$ and IgG_1) were also included in all analyses to assess non-specific binding.

2.5. Statistical analysis

Data were analyzed using one-way ANOVA and Dunnett's Multiple Comparison Test as well as unpaired *t* test with Welch's correction. *p* values <0.05 were considered as statistically significant and tests are two-sided.