The relationship between circulating natural killer cells after reduced intensity conditioning hematopoietic stem cell transplantation and relapse-free survival and graft-versus-host disease

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ABSTRACT

Background
Natural killer cells are known to have anti-tumor activity in haploidentical hematopoietic stem cell transplantation. We hypothesized that reconstituted circulating natural killer cells may be associated with improved relapse-free survival after HLA-matched hematopoietic stem cell transplantation.

Design and Methods
Serial peripheral blood absolute natural killer cell counts were prospectively measured by flow cytometry of lymphocytes expressing CD56 and CD16 in 167 patients. Cluster analysis was used at engraftment and 60 days post-transplant to distinguish patients with high and low absolute natural killer cell counts. At engraftment 80 patients had high counts (> 22.2/mm^3) and 43 had low counts. At 60 days post-transplant 84 patients had high counts (> 18.2/mm^3) and 38 had low counts. The primary study end-points were death, relapse and acute graft-versus-host disease. The median follow-up was 373 days (range, 67-1767).

Results
Among patients given reduced intensity conditioning, a low absolute natural killer cell count at 60 days post-transplant was independently associated with relapse [adjusted hazard ratio (AHR) = 28.4, 95% confidence interval (CI) 4.3–186.4] and death (AHR = 17.5, 95% CI 4.3–71.3). Furthermore, patients given reduced intensity conditioning who had a high absolute natural killer cell count at 60 days had a significantly better 1-year survival than those with a low count by Kaplan-Meier analysis (83% *vs.* 11%, $p<0.001$). Multivariate analysis confirmed that low 60-day absolute natural killer count in patients given reduced intensity conditioning was independently associated with an increase in relapse or death (AHR = 20.22, 95% CI 4.76–85.40). In contrast, there was no significant association between 60-day absolute natural killer cell counts and clinical outcomes in patients receiving myeloablative conditioning. There was no significant association between absolute natural killer cell count and graft-versus-host disease.

Conclusions
High natural killer cell reconstitution is associated with reduced relapse and death without an increased incidence of graft-versus-host-disease after reduced intensity conditioning allogeneic hematopoietic stem cell transplantation. Measuring reconstituted natural killer cells expressing CD56$^+$/CD16$^+$ post-transplant may have novel prognostic and therapeutic implications.

Key words: natural killer cells, hematopoietic stem cell transplantation, relapse-free survival, graft-versus-host-disease.

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Introduction

Natural killer (NK) cells are cells of the innate immune response capable of lysing 'foreign' targets that do not express protective inhibitory killer cell immunoglobulin-like receptor (KIR) ligands (specific major histocompatibility complex class I allele groups), including infectious organisms, malignant cells, and recipient cells during hematopoietic stem cell transplantation (HSCT).[1-5] The anti-leukemic and anti-rejection activities of NK cells appear to be essential to the complex interplay between graft-versus-tumor effects and graft-versus-host disease (GVHD) after haploidentical HSCT.[2,4,6] However, the role of reconstituted circulating NK cells after HLA-matched HSCT is less well known and was the object of our study.

NK cells originate from $CD34^+$ hematopoietic progenitor cells and have been defined by flow cytometry as $CD56^+/CD16^+$ *typical adult* or *mature* cells, $CD56^+/CD16^-$ *immunoregulatory* cells, and $CD56^-/CD16^+$ *cytotoxic* cells.[2,7] Whether these CD56/CD16 variations or levels of their cell surface expression represent different stages of maturation or, instead, subsets with unique potentials for cytolysis, cytokine production, cellular communication, and reconstitution after HSCT, remains of great clinical and research importance.[2,8,9]

In animal and human *in vivo* studies, NK cells have been associated with decreased rates of relapse and GVHD after HLA-haploidentical HSCT.[1,10,11] Studies performed predominantly in animal *in vivo* and *in vitro* settings have led to the proposal of various mechanisms of these desirable NK attributes, including actions of the NK cells in the donor graft,[12] naturally occuring KIR ligand incompatibility,[13] and actions by certain activating KIR from the donor graft.[14]

We prospectively investigated the potential antitumor effects associated with NK cells and the correlation between post-transplant circulating (*in vivo*) human mature NK ($CD56^+/CD16^+$) reconstitution, and survival, relapse, and GVHD in patients undergoing HLA-matched allogeneic HSCT. Determinants at both engraftment and at 60 days post-transplant were sought as potentially important and clinically relevant time points. We hypothesized that increased circulating levels of NK cells would be associated with improved clinical outcomes. We also analyzed reconstituted NK levels with regard to both the type of transplant conditioning and NK subsets, and correlated these levels with clinical outcomes.

Design and Methods

Patients

We prospectively enrolled 209 consecutive patients, in an Institutional Review Board-approved protocol, undergoing HSCT for predominantly high-risk hematologic malignancies as defined previously.[15] Of these, 197 patients engrafted and flow cytometry was performed on 167 patients at the time of neutrophil engraftment, defined as >500 cells/mm^3 for 3 consecutive days. Most patients received 6/6 HLA-matched stem cells, except for ten patients undergoing cord blood transplantion, and eight patients with a single HLA-mismatch. At 60 days post-transplant, 122 patients were evaluable. Of these, 79 patients had received myeloablative conditioning (43 had received a graft from a related donor and 36 from an unrelated donor) and 43 patients had been given reduced intensity conditioning (RIC, 24 of whom had received a graft from a related donor and 19 from an unrelated donor). After myeloablative conditioning, acute GVHD was defined as grade II-IV within the first 100 days of follow-up and graded using standard diagnostic criteria.[16,17] After RIC, acute GVHD was defined using the emerging definition of patients displaying clinical symptoms, defined as grade II-IV using standard diagnostic criteria, without the 100-day restriction.[18] Chronic GVHD was defined using the National Institutes of Health Consensus Guidelines[19] and graded using standard diagnostic criteria.[20,21]

Conditioning regimens and graft-versus-host disease prophylaxis

Patients received myeloablative conditioning based on previously published regimens.[22] For RIC, patients received either fludarabine (30 mg/kg/day) on days -4, -3, and -2 and 2 Gy of total body irradiation on day 0, or fludarabine (30 mg/kg/day) on days -7 to -2, rabbit antithymocyte globulin (Thymoglobulin®, Genzyme, Cambridge, MA, USA) 1.5 mg/kg/day, or equine antithymocyte globulin 10 mg/kg/day on days -6 to -3, and busulfan 1 mg/kg every 6 hours on days -4 and -3. The fludarabine/total body irradiation regimen was selected for patients who lacked autologous stem cell back-up or had a high risk of graft rejection, including recipients of a transplant from an unrelated donor.

For the fludarabine/total body irradiation regimen, immunosuppressive therapy with oral tacrolimus (0.06 mg/kg twice a day) was started on day -3 and mycophenolate motefil (15 mg/kg twice daily for recipients of grafts from related donors and three times daily for recipients of grafts from unrelated donors) was started 4 to 6 hours after the transplant on day 0. In patients with related donors with no evidence of GVHD, mycophenolate mofetil was discontinued at day 28 without tapering and tacrolimus was tapered from day 60 over 8 weeks. In patients with unrelated donors with no evidence of GVHD, mycophenolate motefil was tapered from day 40 over 8 weeks and tacrolimus was tapered from day 100 over 8 weeks.

For the fludarabine/antithymocyte globulin/busulfan regimen, immunosuppressive therapy with oral tacrolimus (0.06 mg/kg twice a day) was started on day -3. In patients with no GVHD, tacrolimus was tapered from day 60 over 8 weeks (for those with a related donor) or from day 100 over 8 weeks (for those with an unrelated donor).

Immunophenotypic analysis

Peripheral blood samples were collected from donor stem cell products and from the recipients prior to HSCT (baseline) and at various time points after the

transplant. Samples were collected around the time of engraftment, defined as 3 consecutive days with an absolute neutrophil count (ANC) greater than 0.5×10^9/L (500 cells/mm^3). In the case of RIC transplants, the time of engraftment was defined as the day of an increase in the ANC after the nadir. Additional blood samples were collected 60 days post-transplant. Four-color flow cytometric analysis was performed with a FACSCalibur™ flow cytometer, as previously described.[15] Whole blood staining, without a cell washing technique, was used to maximize cell yield. This was followed by red cell lysis with ammonium chloride. For the primary analysis, the peripheral blood absolute NK cell count (ANK) was determined by flow cytometry analysis of lymphocytes staining positive for CD56 and CD16. In sub-analyses, we additionally examined the association of clinical outcomes with alternative phenotypic definitions of NK, such as lymphocytes with single-expressed bright CD56$^+$/CD16$^-$ and CD56$^-$/CD16$^+$ levels, as well as transplant type, such as combinations of related/unrelated donor and myeloablative/RIC. Absolute NK cell number (ANK/mm^3) was determined by multiplying the percentage of CD56$^+$/CD16$^+$ cells by the total number of white blood cells (WBC) per mm^3. This method was repeated for the CD56$^+$/CD16$^-$ and CD56$^-$/CD16$^+$ subsets. Others cells measured by flow cytometry in our analysis included dendritic cells (lin$^-$/HLA-DR$^+$/CD123$^+$ or CD11c$^+$), CD34$^+$ and CD3$^+$ cells, and subsets of T cells (CD4$^+$ and CD8$^+$).

Table 1. Patients' characteristics by study groups.

Baseline characteristics	*Low NK level*	*High NK level*	*p value**
Myeloablative transplant	66%	64%	0.87
Peripheral blood graft source	66%	67%	0.92
Related donor	58%	54%	0.66
Male recipient	47%	56%	0.38
Male donor	47%	60%	0.24
High disease risk	71%	86%	0.06
Recipient age	44±16	48±13	0.13
Log (days to engraftment)	3.1±0.5	3.0±0.3	0.36
Log (NK cell count at engraftment)	2.5±1.6	2.8±1.1	0.25
Log (CD3 cells infused)	1.0±0.6	1.0±0.6	0.63
Log (CD34 cells infused)	1.7±0.6	1.7±0.6	0.80
Log (interleukin-12 level first week post-transplant)	3.0±1.6	3.1±1.9	0.86
Prior clinical events (occurring between transplant and 60 days post-transplant)			
Acute graft-versus-host disease	34%	48%	0.17
Viral infection	32%	37%	0.57
Flow cytometry data at 60 days post-transplant			
Ln(dendritic cell count [1×10^9/L])	1.6±1.1	1.6±1.0	0.82
Ln(CD4 cell count [1×10^9/L])	4.6±0.9	4.5±1.2	0.49
Ln(CD8 cell count [1×10^9/L])	5.2±1.4	4.9±1.5	0.35
Ln(ANC cell count [1×10^9/L])	1.0±0.8	0.9±0.8	0.79

*Comparison of demographics between patients with high and low absolute NK counts at 60 days post-transplant. *Result of χ^2 tests for categorical variables and two-sample t-tests for continuous variables.*

Statistical analysis

Primary study groups were designated based on disjoint cluster analysis of the natural logarithmically transformed NK cell count. Clinical outcomes were assessed using Kaplan-Meier plots and multivariate Cox proportional hazard models. The log-rank test was used to evaluate the statistical significance of differences between the Kaplan-Meier plots. Survival models were constructed from the time of engraftment and from 60 days post-transplant. Cox models were adjusted for baseline characteristics including transplant type (myeloablative or RIC), graft source (peripheral blood or other), recipient age, and type of donor (related or unrelated).

If possible, cells were quantified as the number of cells per given volume. However, for the purposes of reducing variability and to account for non-linear relationships among variables, we used logarithmically transformed levels (preserving the order of values). A positive shift in the location of the values was required in the transformation because of their small or null values. Cox models were generated independently for myeloablative and RIC transplants using backward stepwise variable selection criteria with an inclusion criterion of a type-I error probability of 0.15.

Outcomes were assessed over the full follow-up period of the study. Analyses were conducted at both a planned 1-year post-transplant censorship and at the end of the full follow-up period. For the primary study population, defined as those patients surviving a minimum of 60 days post-transplant, the median follow-up time was 373 days (minimum 67 days, maximum 1767 days). The primary variable of interest to be analyzed for predictive properties with regard to the study end-points was the recipients' engrafted NK cell counts. Using the methods described above, the patients were divided into groups with high and low ANK for analysis.

The primary outcomes chosen for analysis were relapse, death, and acute GVHD. The ANK (CD56$^+$/CD16$^+$) of the engrafted recipient at various time points were the main explanatory variables to be evaluated for their predictive abilities with regard to the study end-points. Numerous peripheral blood cell counts of comparative interest were measured from the cells collected from the donors, as well as the cells collected from the recipients at baseline (prior to HSCT), at engraftment, and 60 days post-transplant. As a sensitivity analysis to account for non-linear associations or an effect related to a specific threshold, we also generated a model for the composite end-point of relapse or death using ANK as a continuous explanatory variable, and CD4, CD8, and ANC as categorical variables (high *vs*. low).

In addition, a composite end-point was defined for an event of relapse or death. Models for relapse alone and acute GVHD were censored if patients were lost from the study population because of death. Univariate comparisons between study groups were conducted with χ^2 tests for categorical variables and Student's t-tests for continuous variables. All analyses were performed with SAS (v.9.1.3, Cary, NC, USA). Alternative definitions of NK cells (CD56$^+$/CD16$^-$ and CD56$^-$/CD16$^+$) and transplant types (myeloablative or RIC) were analyzed as part of pre-planned sub-analyses.

Results

Patients' characteristics

Of the 209 patients enrolled, 197 patients engrafted, and 167 patients were evaluable by flow cytometry performed at the time of sustained neutrophil engraftment of ≥500 cells/mm^3. At the 60-day post-transplant follow-up, the primary study sample included 122 patients with available laboratory measures. ANK levels were designated from the cluster analysis and counts greater than 18.2 cells/mm^3 at 60 days were considered in the high level (n=84) and counts less than this value were considered in the low level (n=38). The characteristics of the study groups (baseline demographics, prior clinical events, and corresponding day 60 post-transplant flow cytometry data) are given in Table 1. There were no statistically significant differences between study groups based on baseline characteristics, clinical events prior to 60 days, or other laboratory parameters measured at 60 days.

Natural killer levels are associated with different outcomes, based on transplant conditioning

There was no statistically significant difference in ANK levels at 60 days according to type of transplant conditioning. However, there was a highly significant association between clinical outcomes and 60-day ANK within RIC recipients: patients with a high 60-day ANK had a lower relapse rate and improved survival compared to patients with a low ANK. The Kaplan-Meier plot for the composite end-point of relapse or death indicated that RIC recipients with a high ANK at 60 days had a 1-year survival rate of 83% compared to 11% among patients with a low ANK (log-rank p value <0.001) (Figure 1). No significant association between 60-day ANK level and outcome was found among patients who received myeloablative conditioning (Figure 2). Given these differences in clinical outcomes according to the reconstituted NK cell level and the conditioning regimen, we studied the differences between the two conditioning groups (myeloablative *vs*. RIC) by baseline demographics, clinical events, and corresponding day 60 flow cytometry data. Compared to the myeloablative group, the RIC group differed by being older (p<0.001), having higher disease risk (p=0.046), a predominance of peripheral blood as the graft source (p<0.001), and were mostly recipients of grafts from male donors (p=0.01). The multivariate Cox model confirmed that, in RIC patients, low day 60 ANK was independently associated with an increase in relapse or death in the first year after the transplant [adjusted hazard ratio (AHR)=20.22, 95% confidence interal (CI) 4.76–85.40], as shown in Table 2. Using log-transformed ANK as a continuous variable also resulted in a statistically significant association (AHR=24.6, 95% CI 5.0–122), associated with a one unit increase in the log-transformed ANK. None of the CD4, CD8 or ANC (high *vs.* low) levels was significant, and using these cell counts as categorical (rather than continuous) variables did not affect the statistical outcome. There were no other statistically significant factors in these models.

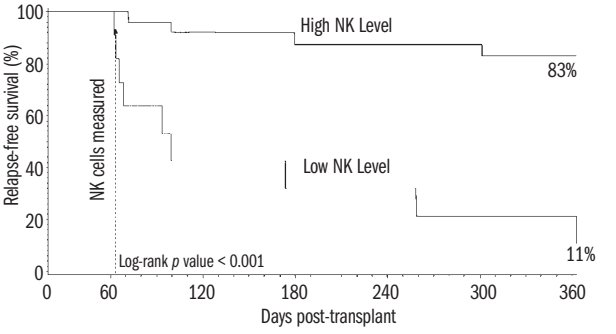

Figure 1. Kaplan-Meier plot of days to relapse or death by natural killer level for patients undergoing reduced intensity conditioning transplantation. Among patients receiving RIC, those with high natural killer reconstitution had composite outcomes of improved survival and less relapse compared to those with low counts.

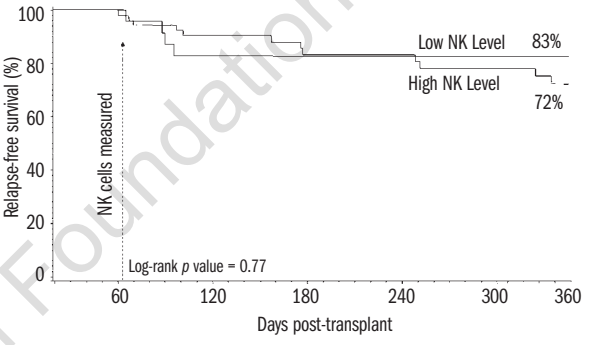

Figure 2. Kaplan-Meier plots of days to relapse or death by natural killer level for patients undergoing myeloablative conditioning transplantation. In patients receiving myeloablative conditioning, high natural killer reconstitution was not associated with improved survival and less relapse.

Lack of association between ANK levels at baseline and at engraftment with clinical outcomes

There was no association between ANK at baseline in patients prior to conditioning and the investigated outcomes of relapse, GVHD, or survival. In addition, there were no significant factors associated with the change between these ANK levels at baseline and engraftment.

Based on cluster analysis, a high ANK level at engraftment was considered to be a count greater than 22.2 cells/mm^3. According to this cut-point, 80 patients had a high ANK and 43 patients had a low ANK at engraftment. ANK level at engraftment was not associated with the investigated outcomes of acute GVHD (p=0.65), death (p=0.49), or relapse (p=0.24) based on Kaplan-Meier plots. Cox models also indicated no significant association between ANK levels at engraftment and clinical outcomes.

Natural killer subsets are associated with different clinical outcomes after reduced intensity hematopoietic stem cell transplantation

A comparison of absolute cell counts of CD56$^+$/CD16$^+$, CD56$^+$/CD16$^-$ and CD56$^-$/CD16$^+$ NK cell subsets at baseline, engraftment and day 60 in

Table 2. Cox model for relapse or death in the first year post-transplant after reduced intensity conditioning hematopoietic stem cell transplantation.

Variable	*Level (Reference group)*	*AHR*	*95% CI*	*p value*
NK level at 60 days	Low (high)	20.22	4.76–85.40	<0.001
Recipient age	Per year	1.04	0.97–1.11	0.28
Donor relation	Not related (related)	0.58	0.08–4.45	0.60
Graft source	Peripheral blood (bone marrow or cord blood)	0.27	0.03 – 2.24	0.22

Multivariate analysis demonstrating low NK level at 60 days post-transplant increases the risk of relapse or death after reduced intensity conditioning.

Table 3. Absolute natural killer cell counts by time point and conditioning regimen.

Cell Population	*Myeloablative (n=115)*[a]	*Reduced intensity (n=53)*[a]	*p value*[b]
	Baseline		
$CD56^+/CD16^+$	40.5 (8.5/24.4/56.3)	44.2 (10.8/33.2/74.4)	0.43
$CD56^+/CD16^-$	109.0 (40.5/75.6/160.4)	96.3 (45.2/75.2/122.1)	0.85
$CD56^-/CD16^+$	25.4 (5.3/11.8/27.0)	29.9 (6.6/15.8/38.3)	0.16
	Engraftment		
$CD56^+/CD16^+$	35.7 (7.0/19.7/41.8)	53.3 (5.4/18.3/39.5)	0.96
$CD56^+/CD16^-$	57.6 (22.1/57.6/111.0)	230.6 (21.6/55.0/111.7)	0.46
$CD56^-/CD16^+$	17.3 (3.8/9.3/23.0)	40.7 (4.0/16.9/38.6)	0.18
	Day 60		
$CD56^+/CD16^+$	59.7 (14.5/37.2/82.0)	53.6 (13.6/32.3/83.3)	0.90
$CD56^+/CD16^-$	94.5 (35.4/65.3/109.1)	115.9 (37.1/74.0/161.7)	0.46
$CD56^-/CD16^+$	30.2 (5.6/17.8/37.3)	28.8 (7.7/20.3/34.2)	0.88

Absolute cell counts of NK subsets at baseline, engraftment and day 60 post-transplant. Reported as cells/mm³: mean (25th percentile/median/75th percentile); [a]sample size at transplantation; [b]based on the non-parametric Wilcoxon's test.

patients divided according to conditioning regimen (myeloablative *vs*. reduced intensity) is shown in Table 3. There were no significant differences between the two groups with respect to the absolute counts of measured NK cell subsets at these time points. The association between 1-year overall outcomes and level of ANK ($CD56^+/CD16^+$), as compared to the alternatively defined NK cell subsets ($CD56^+/CD16^-$, and $CD56^-/CD16^+$) was assessed at 60 days post-transplant for RIC patients (Table 4). Statistically significant associations were found only between $CD56^+/CD16^+$ ANK

Table 4. Clinical outcomes by natural killer subsets after reduced Intensity conditioning hematopoietic stem cell transplantation.

Day 60 level (reference is high level)	*One year adjusted hazard ratio* *Relapse*	*Death*	*Composite end-point of relapse or death*
Low $CD56^+/CD16^+$ level (< 18.2/mm³)	28.4 (4.3–186.4)	17.5 (4.3–71.3)	20.2 (4.8–85.4)
Low $CD56^+/CD16^-$ level (< 17.3/mm³)	1.8 (0.3–9.4)	0.9 (0.3–3.2)	1.1 (0.3–3.9)
Low $CD56^-/CD16^+$ level (< 44.7/mm³)	3.8 (0.8–17.4)	2.2 (0.7–7.2)	2.4 (0.7–8.0)

Multivariate analysis demonstrating the association between clinical outcomes at 60 days post-transplant and levels of $CD56^+/CD16^+$ NK, but not with other commonly measured NK subsets ($CD56^+/CD16^-$ and $CD56^-/CD16^+$).

levels and clinical outcomes. At 1 year, the AHR for relapse was 28.4 (95% CI 4.3–186.4), the AHR for death was 17.5 (95% CI 4.3–71.3), and the composite AHR for death or relapse was 20.2 (95% CI 4.8-85.4).

Circulating natural killer levels are not associated with graft-versus-host disease

For the whole cohort, development of new onset acute GVHD was similar by baseline and engraftment ANK levels. Likewise, new onset acute GVHD was evaluated 60 days after transplantation, and found to be similar by ANK level, occurring in 34% with a low ANK and 48% with a high ANK (p=0.17). Acute GVHD-free survival was evaluated at 100 days post-transplant and found to be similar regardless of ANK level, occurring in 85% with a low ANK and 76% with a high ANK (p=0.62). Kaplan-Meier plots for the composite end-point of relapse or death, in patients stratified by prior onset of acute GVHD, revealed a similar (and also not statistically significant) association with ANK levels. Censored at 1-year post-transplant, the patients with prior acute GVHD had low and high survival proportions of 58% and 79% respectively (p>0.05). For patients without prior acute GVHD these proportions were 56% and 74% (p>0.05). Furthermore, there was no association with chronic GVHD (p=0.35) and ANK levels within 1 year post-transplant.

In RIC patients, there was no evidence for either increased acute GVHD (p=0.82) or chronic GVHD (p=0.54) based on the 60-day ANK level.

Discussion

This prospective analysis of human NK cell reconstitution following predominantly HLA-matched HSCT confirms our hypothesis of potential anti-tumor effects associated with cells having a NK phenotype, particularly after RIC. Our study is the first to demonstrate that a certain magnitude of circulating reconstituted NK cells ($CD56^+/CD16^+$) predicts both relapse and survival after non-T-cell-depleted HSCT. Significantly, we

demonstrate a key role of NK cell reconstitution only after RIC and not after myeloablative transplantation. These demonstrated anti-tumor associations of NK cells complement results from previously well defined studies in animal and human HLA-haploidentical transplants.[1,6,11]

A strong association was found between ANK level and reduced relapse after RIC HSCT, although RIC transplant recipients comprised one-third of our cohort. In contrast, and surprisingly, there does not seem to be a similar effect in myeloablative HSCT recipients even though these patients represented two-thirds of our cohort. This may be associated with many factors innately related to myeloablative conditioning, including ablation of recipient malignant cells and ablation of recipient non-malignant hematopoietic and immune cells, which rely less on NK function for anti-tumor effects.[11] A change in NK level after myeloablative HSCT may, therefore, not be as apparent or clinically significant. In contrast, RIC preserves the host immune environment and the unique interaction of donor and host cells, allowing for differential KIR-associated NK cell lysis of residual tumor cells and differential cell modulation by interleukins/cytokines.[4] Additionally, preliminary studies suggest that post-grafting administration of immunosuppression after RIC HSCT may enhance NK cell-associated graft-versus-leukemia effects.[23] The importance of this NK-associated graft-versus-leukemia activity may explain why a change in NK cell level may be more apparent and clinically significant in this population after RIC HSCT. Our findings are supported by recent work from Clausen *et al*. showing that patients receiving RIC and transplanted with high numbers of donor NK cells had a reduced relapse rate.[24] Our work further supports studies with HLA-matched transplants[25] including the research by Savani *et al*.[26] who found that a high NK cell count at day 30 was associated with improved outcomes in patients with acute leukemia who received T-cell-depleted allografts.

Our prior studies demonstrated an important prognostic role for both interleukin-12 and dendritic cells with regards to clinical outcomes after HSCT.[15,22] We, therefore, incorporated these two important factors in our model since they are known to interact with NK cellular function.[3,27] Perhaps not surprisingly, however, NK cells appear to be independently prognostic in our Cox models suggesting that they may have anti-tumor effects through their innate mechanisms of action, unlike the adaptive actions of dendritic cells.

Surprisingly, although our results indicate strong NK cell-associated graft-versus-leukemia effects, there was a lack of association of GVHD with the level of reconstituted NK cells. Although this phenomenon appeared to be consistent in RIC patients, the sample size was probably not sufficiently powered to conclude definitively that there is no association between GVHD and NK cell level in these patients. Future studies may, therefore, need to include greater numbers of patients when analyzing these end-points. Nevertheless, our results confirm work by Rugerri *et al*., who reported that the graft-versus-leukemia effect can exist without an exacerbation of GVHD.[10] Proposed mechanisms for the predominance of NK cell-associated graft-versus-leukemia as opposed to NK cell-associated graft-versus-host effects include the apparent restriction of NK cell alloreactivity to hematopoietic cells[4] and the exploitation of the innate differences between the donor and recipient (host) MHC class I profiles by NK cells. This latter mechanism of naturally occurring KIR-ligand incompatibility was recently demonstrated to be responsible for the powerful graft-versus-leukemia effect in HSCT patients with acute myeloid leukemia.[13]

In summary, our study demonstrates an association between NK cellular reconstitution and improved relapse-free survival without a significant increase in the incidence of GVHD after RIC HSCT. Significantly, we found that a low ANK level at 60 days post-transplant is associated with a higher risk of death or relapse within the first year after a RIC transplant and that patients receiving RIC with subsequent high NK cell levels at day 60 post-transplant had significantly greater survival at 1 year as compared to patients with low NK cell levels. Our findings are unique in describing that both a magnitude change in reconstituted NK cell level and a measurement at the 60 day post-transplant time point are early predictors of clinical outcomes after RIC HSCT. Further studies are required to investigate factors related to NK cell reconstitution following RIC HSCT for clues to explain the complex interactions between the donor NK cell and host environment,[8,28-30] including their interaction with dendritic cells. Measuring post-transplant NK cell levels may be an asset to investigators, particularly those exploring therapeutic NK cell manipulation, or clinicians desiring to improve strategies of early relapse surveillance, earlier withdrawal of immunosuppression, or maintenance chemotherapy for poor risk patients.

Authorship and Disclosures

ED: data analysis and manuscript writing; MPB: contributed to data analysis and writing of the manuscript; JBL, JS contributed to the statistical analysis; H-UM-K: data analysis and manuscript writing; VR: protocol initiation, obtaining funding, experimental design, laboratory work, data collection and analysis, and writing manuscript. The authors reported no potential conflicts of interest.

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