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Reconstitution of Natural Killer Cells in HLA-Matched HSCT after Reduced-Intensity Conditioning: Impact on Clinical Outcome

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Recent advances in the development of reduced-intensity conditioning (RIC) have allowed a broader range of patients to access allogeneic hematopoietic stem cell transplantation (HSCT). Reconstitution of an effective immune system post-transplant, including natural killer (NK) cells, is critical for both tumor control and infectious disease control or prevention. The development and functions of NK cells in such settings remain elusive. Here we analyzed NK cell development in HLA-matched HSCT from related or unrelated donors, after RIC that included antithymocyte globulin (N = 45 patients). Our data reveal that NK cells quickly recover after RIC-HSCT, irrespective of donor type. Rapidly re-emerging NK cells, however, remain immature for more than 6 months. Effector functions resemble that of immature NK cells because they poorly produce IFN-γ and TNF-α in response to target cell stimulation, despite a rapid acquisition of degranulation ability and MIP-1β production. Strikingly, rapid reconstitution of cytokine production correlates with a lower relapse incidence ($P = .01$) and a better survival rate ($P < .0001$) at 1 year post-transplant, whereas degranulation capacity was associated with less relapse ($P = .05$). Our study demonstrates rapid quantitative reconstitution of the NK cell compartment despite administration of potent immune suppressive drugs as part of the conditioning regimen and after transplantation. However, there is a prolonged persistence of functional defects, the correction of which positively correlates with clinical outcome.

INTRODUCTION

During the last 3 decades, the use of allogeneic hematopoietic stem cell transplantation (HSCT) as a treatment for hematological malignancies has dramatically increased. It is well established that allogeneic HSCT exerts immune-mediated antitumor effects (the so-called graft-versus-leukemia or graft-versus-tumor effect), although the exact mechanisms that mediate this action remain elusive. These mechanisms require effective donor-derived immune reconstitution in the recipient.

Historically, myeloablative conditioning (MAC) was commonly used to create space in the recipient marrow and for its antitumor effect. However, adverse effects and transplantation-related mortality hampered access to elderly and less fit patients. In attempt to preserve most of the therapeutic effects of allogeneic HSCT and to overcome previously mentioned limitations, reduced-intensity (non-myeloablative) conditioning regimens (RICs) were introduced and tested with success with respect to transplantation-related mortality.

Natural killer (NK) cells are antitumor innate lymphoid cells. Donor-derived NK cells appear rapidly during

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immune reconstitution after allogeneic HSCT following myeloablative conditioning [1,2]. NK cells exert their antileukemic activity both by direct killing of tumor cells and by warning other immune cells via proinflammatory cytokines (such as IFN-γ and TNF-α) or chemokines (such as MIP-1β). NK cells express a large array of activating receptors recognizing stress-induced ligands or adhesion molecules. In contrast, they also express inhibitory receptors that recognize classical and nonclassical HLA-I molecules and are clonally expressed (ie, expression frequencies vary within the pool of NK cells of a given individual) [3-5]. Inhibitory NK receptors include the C-type lectin NKG2A (heterodimerized with CD94) and the killer cell immunoglobulin-like receptors (KIRs). Expression of these receptors varies over the lifetime of an NK cell. Triggering of effector functions is the result of an initial phase of NK cell education, followed by the encounter with target cells. Upon interaction with target cells, activation of NK cells depends on a finely tuned balance between activating and inhibitory signals.

Human NK cells are commonly divided into $CD56^{bright}$ and $CD56^{dim}$ NK cells [1-3,6-9]. NK cells develop from common lymphoid progenitors into $CD56^{bright}$ NK cells expressing high levels of NKG2A. $CD56^{bright}$ NK cells give rise to $CD56^{dim}$ NK cells, partially losing NKG2A expression and acquiring KIRs [1,3-5,10,11]. $CD56^{dim}$ NK cell maturation further continues until terminal differentiation associated with expression of CD57 and NKG2C [1,12-14].

The antitumor activity of allogeneic NK cells has been difficult to show in the past years, until the pioneering work of Ruggeri et al. [15,16] that demonstrated the high potential of alloreactive HLA-haplomismatched NK cells in HSCT. Unfortunately, HSCT with megadoses of immuno-selected $CD34^{+}$ obtained from a related haploidentical donor after administration of a MAC regimen to the recipient causes significant morbidity and is not commonly used outside of rare experienced centers. Today, HLA-matched HSCT remains the most common procedure. HLA matching limits the risk of graft-versus-host disease (GVHD) triggered by alloreactive T cells; whether alloreactive NK cells can exert antitumor effects in this setting remains unclear, because "KIR matching" is the consequence of HLA matching in siblings. Several retrospective studies were conducted to understand the role of NK cells in the graft-versus-leukemia effect of HSCT [4,17-19]. Unfortunately, it is difficult to draw firm conclusions from these studies because of the heterogeneity of cohorts, conditioning, and cell source (matched unrelated [MUD] or related [MRD] donors).

Most published studies that describe NK cell reconstitution after HSCT do not include an accurate evaluation of NK cell phenotype and function. We and others have shown that NK cells develop quickly in recipients after MRD HSCT and express KIRs and NKG2A, with similar profiles compared with donors [1,2,20-23]. However, these studies were based on heterogeneous cohorts of donors/patients or conditioning regimens. Moreover, most studies were performed in recipients who received MAC rather than RIC regimen.

We performed a detailed and sequential analysis of the development and functions of NK cells in a homogeneous cohort of patients treated with MRD or MUD HSCT after a RIC regimen. We analyzed the maturation of NK cells as it conditions effector functions and monitored the capacity of patients' NK cells to develop efficient multiparametric effector functions. Finally, we analyzed the association of all biological parameters regarding NK reconstitution with clinical parameters.

METHODS

Patients and Samples

We analyzed peripheral blood mononuclear cells (PBMCs) of 45 patients who received allogeneic SCT from HLA-matched donors (Table 1). All patients received the same RIC regimen (fludarabine 30 mg/m^2/day, busulfan 3.2 mg/kg/day) and antithymocyte globulin 5 mg/kg for 2 days. The source of stem cells was minimally manipulated granulocyte colony-stimulating factor–mobilized PB (no immune selection of $CD34^{+}$ cells). Post-transplantation GVHD prophylaxis consisted of cyclosporine A up to 100 days and was tapered afterward in the absence of GVHD. Bulk-chimerism was routinely studied as part of clinical procedures and was >80% at day 90 post-HSCT (data not shown).

Before scientific use of samples and data, patients were appropriately informed and asked to consent in writing, in compliance with French and European regulations. This protocol was approved by our institutional review board (no. 12-002). The study was carried out in accordance with the principles of the Declaration of Helsinki.

Samples were obtained at different times postgraft. Whole PB samples were processed by Ficoll density centrifugation and were cryopreserved until use. Healthy control PB from the local blood bank was obtained and processed similarly to patient samples and used as controls in all experiments. Follow-up was measured from the date of transplantation to the date of last information for living patients. Median time to relapse was 3 months (range, 49 to 215 days). Median time to death was 6.3 months (range, 103 to 548 days). Of note, 80% of patients who relapsed died afterward (median survival postrelapse, 67 days).

Cells

NK cell reactivity was evaluated after interaction with the erythroleukemia cell line K562. K562 was maintained in RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin, and 100 U/mL streptomycin (complete medium). The follicular lymphoma cell line RL was cultured in complete medium. Acute myelogenous leukemia blasts and B-cell chronic lymphocytic leukemia (B-CLL) cells were obtained from the local biobank as frozen samples. Only samples containing more than 90% of leukemic cells were used.

Antibodies and Flow Cytometry

The list of antibodies used in the study are presented in Supplementary Table 1. Data were acquired on an LSR Fortessa (BD Biosciences) and analyzed with FlowJo 9.6 (FlowJo, LLC, Ashland, OR). Graphics were

Table 1
Patient Characteristics

	Donor Origin		Total
	MRD	MUD	
N (%)	31 (69)	14 (31)	45
Median age, yr (range)	53 (28-68)	52 (25-69)	53 (25-69)
Diagnosis			
Lymphoid	15 (48)	9 (64)	24 (53)
ALL	3	2	5
CLL	2	2	4
NHL	7	2	9
MM	3	3	6
Myeloid	16 (52)	5 (36)	21 (47)
AML	13	4	17
MDS	3	1	4
GVHD			
Acute	5 (16)	8 (57)	13 (29)
Chronic	13 (42)	5 (36)	18 (40)
Survival*	20 (65)	11 (78)	31 (69)
Relapse	10 (32)	2 (14)	12 (27)
NRM	2 (6)	1 (7)	3 (7)
CR	21 (68)	11 (78)	32 (71)
CMV reactivation	7 (23)	5 (36)	12 (27)
Median follow-up, days (range)	420 (232-553)	411 (222-494)	416 (222-553)

ALL indicates acute lymphoid leukemia; NHL, non-Hodgkin lymphoma; MM, multiple myeloma; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; NRM, nonrelapse mortality; CR, complete remission.
Values are number of incidences with percents in parentheses, unless otherwise indicated.
*Patients alive at the end of the study.