Haploidentical allogeneic hematopoietic cell transplantation in adults using CD3/CD19 depletion and reduced intensity conditioning: a phase II study

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The online version of this article has a Suplementary Appendix.

ABSTRACT

Background

We report a prospective multicenter phase II study of haploidentical hematopoietic stem cell transplantation using CD3/CD19-depleted grafts after reduced intensity conditioning with fludarabine, thiotepa, melphalan and OKT-3.

Design and Methods

Sixty-one adults with a median age of 46 years (range 19-65 years) have been enrolled. Diagnoses were acute myeloid leukemia (n=38), acute lymphoblastic leukemia (n=8), non-Hodgkin's lymphoma (n=6), myeloma (n=4), chronic myeloid leukemia (n=3), chronic lymphatic leukemia (n=1) and myelodysplastic syndrome (n=1). Patients were considered high risk because of refractory disease (n=18), cytogenetics (n=6), complete remission (≥2) (n=9), chemosensitive relapse in partial remission (n=4) or relapse after prior hematopoietic stem cell transplantation (n=15 allogeneic, n=8 autologous, n=1 both). At haploidentical hematopoietic stem cell transplantation, 30 patients were in complete remission and 31 in partial remission. Grafts contained a median of 7.0×10^6 (range 3.2-22) $CD34^+$ cells/kg, 4.2×10^4 (range 0.6-44) $CD3^+$ T cells/kg and 2.7×10^7 (range 0.00-37.3) $CD56^+$ cells/kg.

Results

Engraftment was rapid with a median of 12 days to granulocytes more than 0.5×10^9/L (range 9-50 days) and 11 days to platelets more than 20×10^9 (range 7-38 days). Incidence of grade II-IV acute graft-*versus*-host-disease and chronic graft-*versus*-host-disease was 46% and 18%, respectively. Non-relapse mortality on Day 100 was 23% and 42% at two years. Cumulative incidence of relapse/progression at two years was 31%. Kaplan-Meier estimated 1-year and 2-year overall survival with median follow up of 869 days (range 181-1932) is 41% and 28%, respectively.

Conclusions

This regimen allows successful haploidentical hematopoietic stem cell transplantation with reduced intensity conditioning in high-risk patients lacking a suitable donor. *(clinicaltrials.gov identifier:NCT00202917).*

Key words: survivin, Aurora-B kinase, myelodysplastic syndromes.

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Introduction

The availability of a suitable HLA-matched donor is one of the major limitations to the widespread application of allogeneic hematopoietic stem cell transplantation (HSCT). A matched related donor can only be found for 30% of patients, and a matched unrelated donor for only up to 70%.[1] The search for a donor can be even more difficult for patients from ethnic minorities or if the aggressive course of the disease requires fast identification of a suitable donor. Since virtually every patient has a suitable haploidentical related donor within the family, a successful strategy for haploidentical allogeneic hematopoietic stem cell transplantation (haplo-HSCT) would eliminate the problem of the lack of donors.

However, initial trials of haplo-HSCT were complicated by a high incidence of graft-*versus*-host-disease (GVHD), engraftment failure, and infectious complications resulting in unacceptably high treatment-related morbidity and mortality (TRM).[2] Graft rejection and GVHD is primarily mediated by host and donor T cells. Therefore, attempts to overcome the HLA-barrier were focused on strategies for effective host and graft T-cell depletion. The Perugia group pioneered an approach for graft T-cell depletion by positive selection of $CD34^+$ stem cells combined with transplantation of a megadose of these cells ($>10\times10^6$ $CD34^+$ cells/kg body weight) in order to overcome the HLA barrier in the haploidentical setting.[3,4] The strategy allowed successful haplo-HSCT with a low rate of GVHD and a promising event free survival for patients transplanted in complete remission (CR).[5] However, this strategy for haplo-HSCT relies on intensive myeloablative conditioning regimens and CD34-selection for T-cell depletion that may lead to high toxicity and slow immune reconstitution. The reported incidence of non-relapse mortality (NRM) is up to 57% and is mainly related to toxicities and infections.[6] In our own experience of haplo-HSCT using such an approach, we observed high toxicity from the conditioning regimen, high NRM, slow engraftment (namely of platelets) and delayed immune reconstitution.[7] A delayed engraftment was observed particularly with CD34 doses of less than 8×10^6/kg body weight.[8] This means elderly, heavily pretreated and comorbid patients can not benefit from such an approach. New strategies of graft manipulation using immunomagnetic cell depletion aim to improve engraftment, making haplo-HSCT feasible even after reduced intensity conditioning (RIC) and without megadoses of $CD34^+$ cells. Based on the promising experiences gained at St. Judes Children's Research Hospital, Memphis, in the pediatric population,[9,10] a new regimen was developed using graft CD3/CD19 depletion with microbeads coated with anti-CD3 and anti-CD19 on a CliniMACS device. This approach allows the transplantation of an "untouched" graft product in contrast to CD34 selected stem cells that are coated with CD34-specific microbeads, potentially altering the characteristics of the stem cells transplanted. CD3/CD19 depleted grafts not only contain $CD34^+$ stem cells, but also CD34-negative ($CD34^-$) progenitors, natural killer (NK), dendritic and other graft-facilitating cells,[11-16] and enable haplo-HSCT after RIC using fludarabine, thiothepa and melphalan. T- and B-cell depletion was employed to reduce the risk of GVHD and to prevent EBV-lymphoproliferative disease (LPD). In addition to chemotherapy, the anti-CD3 mAb OKT-3 was used to deplete remaining host T cells, avoiding graft rejection. In contrast to the frequently used polyclonal anti-thymocyte globulin (ATG), OKT-3 spars incoming engraftment-facilitating cells such as NK cells.

Using CD3/CD19 depleted haploidentical grafts and RIC with fludarabine, thiotepa and melphalan, we observed sustained and, compared with CD34 selection, accelerated engraftment without a megadose of $CD34^+$ stem cells[7,17] even in an elderly and heavily pre-treated patient population. We report the results of a prospective multicenter phase II study evaluating this approach.

Design and Methods

Study protocol and centers

Patients were enrolled between 2003 and 2009 in a multicenter phase II study of haploidentical hematopoietic cell transplantation with CD3/CD19 depleted grafts after a reduced intensity conditioning regimen for adult patients with therapy refractory hematologic diseases (*ClinicalTrials.gov N. NCT00202917*). The participating centers were all in Germany; these were the Medical Centers of the Universities of Tuebingen, Dresden, Essen, Halle, Muenster and Wuerzburg, and the Deutsche Klinik für Diagnostik Wiesbaden. The study protocol was approved by the local institutional review boards and the German federal agency the "Paul Ehrlich Institute" that is responsible for approval of trials in HSCT. All patients gave their written informed consent. The primary objective was the evaluation of TRM on Day+100 after haplo-HSCT. Secondary objectives were engraftment, toxicity, infections, immune reconstitution, GVHD, rate of relapse and event free survival (EFS).

Eligibility criteria

Inclusion criteria were patients with high-risk hematologic diseases curable with allogeneic HSCT. These were acute myeloblastic leukemia (AML), myelodysplastic syndrome (MDS), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), paroxysmal nocturnal hematuria (PNH), multiple myeloma (MM), non-Hodgkin's lymphoma (NHL) or Hodgkin's disease (HD). Inclusion criteria included relapsed disease refractory to conventional chemotherapy or relapse after preceding autologous or allogeneic HSCT. Further inclusion criteria were age 18-65 years, Karnofsky score over 60%, and no HLA-identical donor with up to one antigen or two allelic mismatches.

Exclusion criteria were: less than three months after preceding HSCT, CNS involvement, more than 30% (amended to 10% after 18 patients enrolled) blasts in bone marrow in ALL, AML or CML, non-response to chemotherapy, prior myocardial infarction, uncontrolled fungal infections, liver function abnormalities with bilirubin more than 2 mg/dL and transaminases more than two times the upper limit of normal, chronic active viral hepatitis, ejection fraction less than 40%, patients with over grade II hypertension by CTC criteria, creatinine clearance less than 50 mL/min, respiratory failure necessitating supplemental oxygen or diffusing capacity for carbon monoxide less than 30%, allergy against murine antibodies, HIV-infection, pregnancy or breast feeding, no adequate contraceptive method.

Flow cytometry

Analysis of the initial leukapheresis product and CD3/CD19 depleted grafts was performed using flow cytometry. Cells were analyzed for CD3, CD19, CD56 and CD34 with fluorochrome-labeled antibodies (all Becton Dickinson, Heidelberg, Germany). Flow cytometric analysis was performed using a flow cytometer

(FACScalibur, Becton Dickinson, Heidelberg, Germany). CD34 determination followed the guidelines of the International Society for Hematotherapy and Graft Engineering.[18]

Donors, HLA-typing, matching, stem cell mobilization and collection

Family members were assessed for HLA-compatibility by high-resolution molecular typing methods. Peripheral blood mononuclear cells (PBMCs) were mobilized with human G-CSF (Lenograstim, Granocyte®, Chugai Pharma, Germany) at a dose of 2 x 5 μg/kg/day for five days, and stem cells were collected on Days 5 and 6 using a Cobe-Spectra (CaridianBCT Inc., Lakewood, CO, USA) or Baxter CS3000 PLUS (Baxter, Germany) cell separator. A total of 10-15 liters of blood was processed at a flow of 50-80 mL/min on one or two consecutive days. We sought to obtain at least 6×10^6 $CD34^+$ cells/kg recipient body weight.

CD3/CD19 depletion, conditioning regimen and transplantation

CD3/CD19 depletion was performed by negative selection using the automated CliniMACS device as described (Miltenyi Biotec, Bergisch-Gladbach, Germany).[9,19] In brief, PBMCs were processed either immediately or mixed with an equal volume of autologous plasma and stored overnight at 4°C. PBMC were washed once with CliniMACS PBS buffer (phosphate-buffered saline supplemented with 1 mM EDTA and 0.4% of human albumin) and incubated with anti-CD3 and anti-CD19 antibodies directly conjugated to magnetic microbeads (Miltenyi, Bergisch-Gladbach, Germany). The amount of antibody used was calculated according to the manufacturer's instructions. One vial was used for every $4x10^{10}$ MNC with a maximum of 15×10^9 $CD3^+$ T cells and a maximum of 5×10^9 $CD19^+$ cells. Cells were then incubated under continuous agitation at room temperature for 30 min, washed once with CliniMACS PBS buffer, resuspended in 100-300 mL buffer, and then processed with the fully automated Clinimacs device (Miltenyi) equipped with LS tubing set (162.01) separation columns using the program Depletion 2.1 for CD3/CD19 depletion, according to the manufacturer's instructions.

Reduced intensity conditioning regimen consisted of fludarabine (150 mg/m^2, n=57 or 200 mg/m^2, n=4), thiotepa (10 mg/kg), melphalan (120 mg/m^2) and OKT-3 (5 mg/day, Days -5 to +14). Patients received fresh or cryopreserved peripheral blood stem cells (PBSC) processed with CD3/CD19 depletion as described above on Day 0. The patients received no G-CSF support post transplant. Mycophenolate mofetil (MMF, 15 mg/kg bid) was used as postgrafting immunosuppression only if the T-cell content in the graft exceeded 5×10^4 $CD3^+$ cells/kg.

Monitoring of patients for engraftment, chimerism, immune reconstitution, disease and donor lymphocyte infusions

Hematopoietic donor cell chimerism in mononuclear cells (MNC) was monitored in all patients using microsatellite markers as described.[20] Additionally, in some patients, T- and NK-cell chimerism was evaluated by flow cytometry.[21] Engraftment and immune reconstitution was assessed by peripheral blood counts and flow cytometry. The reconstitution of $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ and $CD56^+$ cells was monitored at 2-4 week intervals in the early post transplant period and then every three months. Engraftment was defined as the first day on which the absolute neutrophil count (ANC) was consistently over 0.5×10^9/L. Platelet engraftment was defined as the first day with platelets consistently more than 20×10^9/L. Response was assessed according to the criteria of the International Working Group.[22] GVHD was assessed and graded as described.[23] Toxicities were scored according to the National Cancer Institute Common Toxicity Criteria (3.0). Patients received additional donor lymphocyte infusions (DLI) in case of relapse or progression of underlying disease. PBMCs were freshly obtained from the donor and adjusted to a defined $CD3^+$ T-lymphocyte content (range 1×10^4 to 3×10^8 $CD3^+$ cells/kg) and given at various time points after transplantation.

Statistical analysis

Sample size calculation for this exploratory phase II study was based on an expected TRM/NRM of 25% until Day +100. With a sample size of 60 patients, a two-sided 95% confidence interval (CI) will extend 11% from the observed proportion. Therefore, a sample size of 60 was considered to be sufficient to evaluate TRM/NRM until Day +100 as primary objective of this phase II study. The sample size was calculated using nQuery 4.0 (Statistical Solutions Ltd, Cork, Ireland). SAS version 9.1.3 (SAS Institute, Cary, NC, USA) biostatistical software was used for statistical analysis. Actuarial curves for overall survival (OS) and EFS were estimated according to the Kaplan-Meier method. The log rank test was used to compare Kaplan-Meier estimates (OS and EFS) between different groups of patients. OS was measured as the number of days from transplantation to death from any cause. Patients who were still alive at follow up were censored at the last follow-up date. Time to NRM included only deceased patients who died without preceding relapse. Cumulative incidence curves for NRM and relapse were adjusted for competing risks. EFS was calculated as the number of days from HSCT until relapse/progression/death. Patients were censored at the last follow-up date without evidence of disease progression/relapse or date of death. Risk factors for survival, relapse/progression and NRM were evaluated using univariate comparisons and Cox's regression model. Cox's regression model for NRM was adjusted for competing risk relapse/progression using the free Riskcox software.[24] The following factors were included in the regression models: gender, age (<55 *vs.* ≥ 55 years), prior HSCT, achievement of CR prior to haplo-HSCT (yes *vs.* no), grade of acute GVHD (aGVHD) (<3 *vs.* ≥ 3), chronic GVHD (cGVHD), diagnosis (AML *vs.* other). A landmark analysis was used to assess the impact of cGVHD on survival.[25] The first occurrence of cGVHD in our cohort at eight months was used as landmark. All patients who were alive and in CR at eight months after haplo-HSCT were included in this analysis.

Results

Patients and donors

Sixty-one patients were enrolled in the study. Patients' characteristics are shown in Table 1. Patients were heavily pre-treated with a median of 4 (range 1-9) lines of prior chemotherapy. Median EBMT-HSCT risk score was 6 (range 5-7).[26,27] All donor-recipient pairs had at least a two-loci mismatch and 38 patients had in addition a KIR mismatch in GVH direction using the KIR-ligand model.[11] If a choice of multiple donors was available, the donor with a KIR mismatch was chosen.

CD3/CD19 depletion

T- and B-cell depletion was 4.1 log. The grafts contained 1.48% $CD34^+$ cells due to the high content of non-$CD34^+$ cells such as NK cells, monocytes, granulocytes and antigen presenting cells. Average recovery of $CD34^+$ cells was 59%. *Online Supplementary Table S1* gives details of graft composition and CD3/CD19 depletion in 26 patients treated within the study in Tuebingen.

Graft content

All patients received HSCT with CD3/CD19 depleted haploidentical grafts. The CD3/CD19 depleted grafts contained a median of 7.0×10^6 (range $3.2\text{-}22\times10^6$) $CD34^+$ cells/kg, 4.2×10^4 (range $0.6\text{-}44\times10^4$) $CD3^+$T-cells/kg and 2.7×10^7 (range $0.00\text{-}37.3\times10^7$) $CD56^+$ cells/kg. Twenty-seven patients received MMF as their graft CD3-content exceeded 5×10^4 $CD3^+$ cells/kg.

Engraftment

All but 5 patients engrafted with full donor chimerism by Day 7-126 after haplo-HSCT. Two of these patients died due to NRM within 14 days after HSCT before engraftment could occur. Median time to engraftment was 12 (range 9-50) days to more than granulocytes 0.5×10^9/L and 11 (range 7-38) days to platelets more than 20×10^9/L (*Online Supplementary Figure S1*). Data on transfusion support was available for 51 patients. Fifty of 51 patients (98%) required platelet support (median 7, range 0-53). Forty-eight of 51 (94%) patients received red blood cell transfusions (median 8, range 0-46). Four cases of secondary graft rejection were observed; 2 of these were rescued by a consecutive haplo-HSCT with CD3/CD19 depleted cells from an alternative haploidentical donor.

Immune reconstitution

Detailed studies on immune reconstitution were performed and results have been published.[28] In the present study, immune reconstitution was analyzed in 24 patients. In brief, on Day 20, a median of 248 $CD16^+56^+CD3^-$ NK-cells/μL (range 1-886) were observed (*Online Supplementary Figure S2*). T cells regenerated with a median of 191 $CD3^+$ cells/μL (range 38-799) on Day 100 (*Online Supplementary Figure S2*). On Day 100 a median of 66 $CD8^+$ cells/μL (range 8-170) *versus* 70 $CD4^+$ cells/μL (range 12-301), and on Day 400 a median of 157 $CD8^+$ cells/μL (range 19-980) *versus* 181 $CD4^+$ cells/μL (range 32-379) were observed. The subset of naive T cells showed slower regeneration compared to memory T cells with a median of 28 $CD4^+45RA^+$ (range 0-152) *versus* 79 $CD4^+45R0^+$ cells/μL (range 14-310) and 166 (range 21-2396) *versus* 237 (range 46-252) on Days 100 and 400, respectively. The T-cell repertoire was skewed with oligoclonal T-cell expansion to Day 100 and normalization after Day 200. B-cell reconstitution reached a median of 32 (range 0-407) $CD19^+20^+$ cells/μL on Day 100. Six of these 24 patients received donor lymphocyte infusions (DLI) for relapse or mixed chimerism resulting in acceleration of immune recovery in T and NK cells.

Toxicity, infections, GVHD and NRM

The regimen was well tolerated; maximum acute toxicity was grade 2-3 mucositis, nausea and loss of appetite. Initially, we observed severe neurotoxicity in 4 patients administered 200 mg/m^2 fludarabine. Consequently, fludarabine dose was reduced to 150 mg/m^2. NRM in the first 100 days was 14 of 61 (23%) and 25 of 61 (41%) after two years. Table 2 shows the causes of NRM and time to death after haplo-HSCT. Cumulative incidence (CI) of NRM adjusted for relapse as competing risk was 23% on Day 100 (95% CI: 0.147-0.359) and 42% at two years (95% CI: 0.291-0.538) (Figure 1A). Causes of infectious deaths are shown in Table 2. *Online Supplementary Table S2* provides details of immune reconstitution and infectious deaths of patients treated in Tuebingen. Incidence of grade II-IV aGVHD was 46% and 18% of chronic cGVHD (n=11, limited n=7, extensive n=4). The occurrence of acute and chronic GVDH in relation to T-cell dose in the graft is shown in the *Online Supplementary Figure S3*. In patients receiving a graft with $CD3^+$ T cells more than 7.5×10^4/kg, the proportion of GVHD is increased as shown in the *Online Supplementary Figure S4* (aGVHD P=0.018; cGVHD P=0.062).

Table 1. Patients' characteristics.

Characteristics	N=61	
Patients	Female=23 Male=38	
Donors	Siblings=23 Mothers= 7 Children= 24 Cousins=7	
Diagnosis	AML=38 ALL=8 NHL=6 MM=4 CML=3 MDS= 1 CLL=1	
Stage at HSCT	CR=30 PR=31	
Median age, years (range)	46 (19-65)	
Engraftment, days to median (range)	>500/μL ANC: >20x10^9/L PLT:	12 (range, 9-50) 11 (range, 7-38)
Acute GVHD ≥2	28 (46%) II=17 III=7 IV=4	
Chronic GVHD	11 (18%) limited=7 extensive=4	
NRM	26 (43%) infections=18 IPS=1 cardiac failure=2 tetraparesis of unkown origin=1 GVHD=4	
Relapse	19 (31%)	

Disease response, event free and overall survival

Forty-two patients achieved CR after HSCT, 19 relapsed. Overall survival is 16 of 61 patients (26%) with a median follow up of 869 days (range 181-1932). The Kaplan-Meier estimate of EFS and OS is 34% and 41% at one year and 25% and 28% at two years, respectively (Figure 1B). Kaplan-Meier estimated 2-year OS was 29% in AML, 0% in ALL and 50% in NHL patients (Figure 2A). For patients with AML in CR at time of haplo-HSCT, 2-year estimated OS was 32% (Figure 2B). All but one patient transplanted for advanced ALL died either due to relapse (n=4) or NRM (n=3).

Cumulative incidence of relapse/progression adjusted for competing risk NRM at two years was 31% (95% CI: 0.197-0.433). Patients with limited cGVHD had a better 2-year survival with 67% *versus* 24% without any cGVHD (P=0.0864), as evaluated by a landmark analysis at eight months after haplo-HSCT (Figure 2C). Graft CD3 content

Table 2. Causes and timing of non-relapse mortality

Category	Cause/Grade	Days from haplo-HSCT to death
Infections (n=16)	Meningitis	595
	Cerebral toxoplamosis	178
	CMV pneumonia	100
	CMV pneumonia	186
	CMV pneumonia	299
	Pneumonia	83
	Pneumonia	86
	Viral pneumonia	18
	Viral pneumonia	375
	Viral pneumonia	47
	Bacterial pneumonia	63
	Fungal infection	99
	Fungal infection	187
	Sepsis, MOF	260
	Sepsis, MOF	87
	Sepsis, MOF	2
GVHD (n=4)	Acute GVHD IV	67
	Acute GVHD IV	99
	Acute GVHD IV	105
	Chronic GVHD liver	268
Other (n=6)	Progressive multifocal leukencephalopathy	58
	Progressive multifocal leukencephalopathy	144
	Idiopathic pneumonia syndrome	68
	Cardiac failure	11
	Cardiac failure	108
	Tetraparesis of unknown origin	74

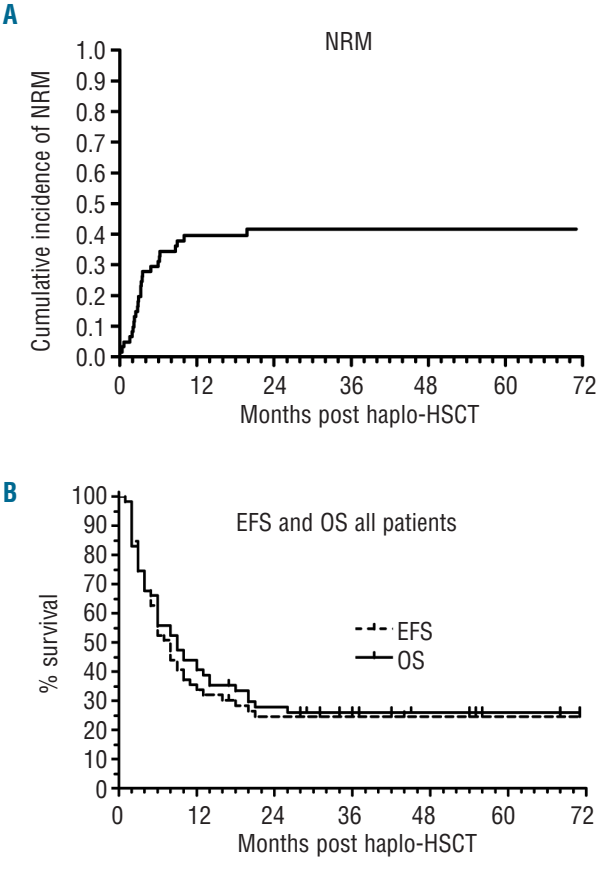

Figure 1. (A) Cumulative incidence of NRM adjusted for relapse as competing risk. (B) Kaplan-Meier estimate of disease free and overall survival. Overall survival (OS): number of days from transplantation to death from any cause. Patients who were still alive at follow up were censored at the last follow-up date. Event free survival (EFS): number of days from haplo-HSCT until relapse/progression/death. Patients without evidence of relapse/progression were censored at last follow-up date or date of death.

(>75,000 $CD3^+$ cells/kg *vs.* <75,000 $CD3^+$ cells/kg) impacted OS as follows: Kaplan-Meier estimate for 1-year OS was 35% with the higher *versus* 45% with the lower T-cell content and 21% *versus* 33% for 2-year OS (P=0.27) (Figure 2D).

KIR mismatches

Using the KIR-ligand model,[3,29] 38 patients were transplanted with a positive KIR mismatch (KIR-MM), but no advantage in overall survival was observed (Kaplan-Meier estimated 2-year OS of 21% with KIR-MM *vs.* 40% without KIR-MM; P=0.21) in the overall patient population (Figure 2E).

In the subgroup of patients with AML (n=38), we observed an association between KIR-MM and an impaired survival (Kaplan-Meier estimated 2-year OS of 19% with KIR-MM *vs.* 65% without KIR-MM; P=0.01).

Chimerism

Complete donor chimerism was reached in 54 patients after a median of 15 days after HHSCT (range 7-124); 7 patients died before reaching a full donor chimerism. Prior engraftment of neutrophil full chimerism was documented in 12 of 60 patients. Figure 3A shows chimerism data of the first year after transplant. In 20 of 61 patients, T-cell chimerism was monitored separately (Figure 3B). Complete T-cell chimerism was reached after a median of 20 days (range 20-100).

Donor lymphocyte infusions

Eleven patients received $CD3^+$ T lymphocytes (n=1-4 infusions) as DLI: 10 patients due to relapse (n=6) or mixed chimerism (n=4), and one patient as antigen-specific T cells because of virus reactivation. Infusions ranged from 2×10^4 to 3×10^8/kg at varying time points after haplo-HSCT: Day +0-100, n=4; Day +100-200, n=6; Day +200-300, n=4; Day +300-600, n=4. Two patients developed aGVHD after receipt of DLI. Seven patients died due to relapse, 2 because of infections, and 2 patients receiving DLI for mixed chimerism are alive and in CR.

Risk factors for survival and non-relapse mortality

A univariate analysis of risk factors for OS, EFS and NRM identifies several possible factors (Table 3). Improved OS and EFS were associated with the presence of cGVHD (P=0.019 and P=0.010, respectively). In multivariate Cox's regression analysis adjusted for competing risk relapse/progression, male gender was associated with a decreased hazard ratio (HR) for NRM (HR 0.4, P=0.046) while age 55 years or over was associated with an increased HR of 2.8 (P=0.030).

Discussion

The present study evaluated a new approach for haplo-

HSCT aimed at improving engraftment even with grafts containing lower numbers of CD34+ stem cells, and allowing the use of an RIC regimen. The regimen proved to have a low toxicity profile enabling it to be used even in an older or heavily pre-treated patient population, including patients who had received prior allogeneic or autologous HSCT. Various other groups have confirmed the feasibility and low toxicity of this RIC for allogeneic HSCT.[30-33] Consequently, the median age of the CD3/19 patients reported here is approximately a decade older than that previously reported in adult patients receiving haplo-HSCT with CD34 selected grafts.[5,34]

The significant influence of graft composition and conditioning regimen on engraftment is illustrated by the fast engraftment kinetics observed. This also translated into low transfusion requirements. Although these engraftment kinetics are similar to data reported by Aversa *et al.* after haplo-HSCT with CD34-selected grafts (median 11 days to ANC>1×10^9/L and 15 days to PLT>25×10^9/L),[5] it is remarkable that our cohort received a much lower median CD34 dose with 7.0×10^6 CD34+/kg *versus* 13.8×10^6 CD34+/kg in the Aversa study.[5] We saw sustained engraftment with CD34 doses of as low as 3.2×10^6 CD34+ cells/kg.

Evaluation of the immune reconstitution shows fast reconstitution of NK cells. This might be directly related to the high NK-cell content of CD3/CD19 depleted grafts. NK cells play an important role in the defense of bacterial, viral and fungal infections.[35,36] Furthermore, NK cells may significantly facilitate engraftment and promote graft *versus* tumor effects after haplo-HSCT, especially in the setting of KIR mismatch. Ruggeri *et al.* showed a significant positive impact of the presence of a KIR-ligand mismatch on engraftment and survival.[29,37] However, we could not confirm a survival advantage for patients transplanted from an NK-alloreactive donor in our study. Apart from the limited patient number, this could be due to the different kind of graft manipulation with more residual T cells

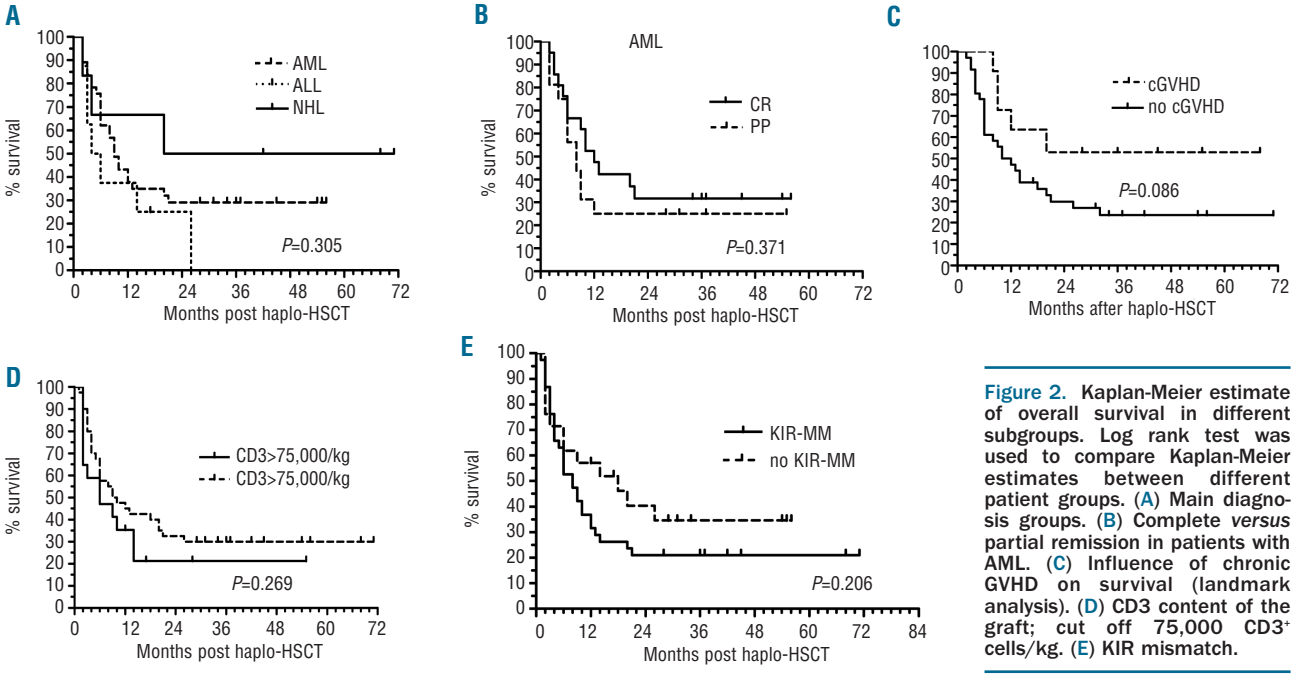

Figure 2. Kaplan-Meier estimate of overall survival in different subgroups. Log rank test was used to compare Kaplan-Meier estimates between different patient groups. (A) Main diagnosis groups. (B) Complete *versus* partial remission in patients with AML. (C) Influence of chronic GVHD on survival (landmark analysis). (D) CD3 content of the graft; cut off 75,000 CD3+ cells/kg. (E) KIR mismatch.

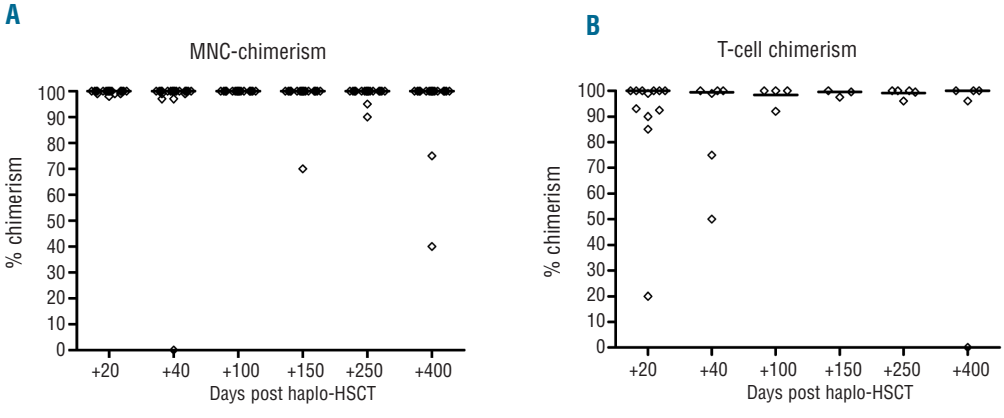

Figure 3. Donor chimerism after haplo-HSC. (A) Percentage of donor chimerism on different days after haplo-HSCT (n=55). Seven patients died before reaching complete chimerism. (B) Percentage of T-cell chimerism (n=20) analyzed by flow cytometry.

in the graft and the RIC used in our cohort. Cooley *et al.* hypothesized that T cells within the graft may affect NK-cell function and KIR expression and thereby reduce the impact of KIR-ligand mismatch on outcome.[38] Brunstein *et al.* have seen a negative impact of KIR-ligand mismatch on outcome of cord blood transplantation (CBT) after RIC.[39]

Using CD3/CD19 depleted grafts and RIC, T-cell reconstitution appears to be faster than that reported in published data with CD34 selected grafts.[40,41] Nevertheless, T- and B-cell reconstitution was still significantly delayed reflecting the low numbers of residual T and B cells in the graft. In the pediatric population, a faster T-cell reconstitution was seen after CD3/CD19 depleted haplo-HSCT.[42] This may be explained by the presence of a still functional thymus in children. Comparing the immune reconstitution of patients transplanted with CD3/CD19 depleted *versus* CD34 selected grafts, one has to consider the different conditioning regimens used. RIC, as used in this study, may allow faster immune recovery. A prospective study comparing both methods of graft manipulation for haplo-HSCT after a similar conditioning regimen would be needed to allow definite conclusions to be drawn.

We observed a higher incidence and degree of GVHD after haplo-HSCT with CD3/CD19 depleted grafts compared to the reported low incidence of less than 10% in patients receiving haplo-HSCT with CD34 selected grafts.[5] This may be related to the higher median T-cell dose transplanted in our patients. Observed aGVHD was primarily moderate skin GVHD that responded well to steroid therapy. With this regimen, doses of less than 5×10^4 $CD3^+$ cells/kg seem safe even without GVHD prophylaxis; doses of more than 5×10^4 $CD3^+$ cells/kg require GVHD prophylaxis such as MMF. Doses of more than 15×10^4 $CD3^+$ cells/kg should be avoided. Above 7.5×10^4 $CD3^+$ cells/kg, the incidence of GVHD was significantly increased.

NRM adjusted for relapse as competing risk was 23% on Day 100 and 42% at two years. This may seem to be high for an RIC, but the median EBMT risk score in the study population was 6 which is associated with an estimated NRM of approximately 45%.[26,27] Our NRM is comparable to the data of Aversa *et al.*[5] with CD34-selected grafts who reported 37% for patients transplanted in remission and 44% for patients transplanted in relapse. Ciceri *et al.* reported NRM ranging from 36% to 66% depending on disease status at haplo-HSCT.[43] Although these previous NRM rates were seen after myeloablative conditioning, our patients were approximately a decade older and almost all had advanced disease. Furthermore, most of the NRM was related to infections and GVHD, and not to the toxicity of the conditioning regimen.

In our study, the estimated OS of 41% at one year and 28% at two years is promising, given the high-risk profile of the patients treated. Those who received the greatest benefit from the approach described here were patients with NHL and AML in remission, with a 2-year survival of 50% and 32%, respectively. Even AML patients not in CR at time of haplo-HSCT had a 2-year OS of 25%, which might be related to additional NK-alloreactivity. Aversa *et al.* reported an EFS of 48% at two years for patients with AML in remission at time of haplo-HSCT but only 4% for patients transplanted in relapse.[5] Ciceri *et al.*, summarizing the EBMT experience on haplo-HSCT with CD34-selected grafts after myeloablative conditioning, described a 2-year leukemia free survival in AML of 1% for advanced patients and 21% in patients in CR2.[43] Furthermore, any comparison between these studies and our own data has to consider the several factors in our cohort that had a potential negative impact on outcome or risk of relapse. In fact, in our study, RIC was used in a patient population that was more than 10 years older. Our patients were more heavily pre-treated, with a median of 4 lines of chemotherapy, and one-third of the patients had previously undergone autologous or allogeneic HSCT. Only one of our patients was in CR1 but with high cytogenetic risk.

Various strategies are currently being investigated to improve immune reconstitution after haplo-HSCT using T-cell repleted[44,45] and depleted grafts. Strategies to enhance immune reconstitution after T-cell depleted haplo-HSCT include DLI,[28] suicide-gene-engineered DLIs,[46] photodynamically purged DLI,[47] but also the use of donor regulatory T cells (Tregs) early after haplo-HSCT.[48] Our group is currently evaluating selective depletion of αβ-T-lymphocytes[49] for haplo-HSCT. In a pilot study, we observed that the residual δγ-T-cells in the graft may enhance T-cell recovery and induce graft-*versus*-leukemia (GVL) effects without GVHD. A prospective multicenter study with this approach is currently in preparation.

Table 3. Risk factors for overall survival, event free survival and non-relapse mortality.

Variable (risk)	N under risk	Univariate log rank test *P*	Cox's regression Hazard ratio	95% CI	*P*
Overall survival					
Male gender	38	0.773	0.82	0.42-1.62	0.564
Age ≥ 55	18	0.280	1.56	0.79-3.11	0.202
Status (PR *vs.* CR)	31	0.509	1.44	0.78-2.66	0.240
Preceding Tx	30	0.255	1.46	0.75-2.82	0.263
Acute GVHD≥ 2	28	0.211	0.83	0.43-1.61	0.581
Limited/extensive cGVHD	11	0.019	0.38	0.14-1.07	0.068
Event free survival					
Male gender	38	0.647	0.85	0.43-1.67	0.630
Age ≥ 55	18	0.204	1.63	0.83-3.20	0.154
Status (PR *vs.* CR)	31	0.316	1.60	0.88-2.94	0.125
Preceding Tx	30	0.349	1.45	0.75-2.79	0.274
Acute GVHD≥ 2	28	0.148	0.81	0.42-1.58	0.531
Limited/extensive cGVHD	11	0.010	0.35	0.12-0.98	0.046
Non-relapse mortality					
Male gender	38		0.40	0.16-0.98	0.046
Age ≥ 55	18		2.81	1.10-7.16	0.030
Status (PR *vs.* CR)	31		1.12	0.49-2.55	0.786
Preceding Tx	30		1.77	0.72-4.36	0.212
Acute GVHD≥ 2	28		0.52	0.20-1.35	0.178
Limited/extensive cGVHD	11		0.57	0.14-2.39	0.439

Tx: therapy.

Conclusions

This study shows that haplo-HSCT with CD3/CD19 depleted grafts and RIC is feasible. The regimen described allows haploidentical HSCT in an older and heavily pre-treated patient population and results in long-term disease free survival.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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References

1. Martin PJ. Overview of Marrow Transplantation Immunology. In: Blume KG, Forman SJ, Appelbaum FR, eds. Thomas' Hematopoietic cell transplantation. Malden, MA: Blackwell Science, Inc., 2004:16-30.
2. Anasetti C, Beatty PG, Storb R, Martin PJ, Mori M, Sanders JE, et al. Effect of HLA incompatibility on graft-versus-host disease, relapse, and survival after marrow transplantation for patients with leukemia or lymphoma. Hum Immunol. 1990;29(2):79-91.
3. Aversa F, Tabilio A, Velardi A, Cunningham I, Terenzi A, Falzetti F, et al. Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. N Engl J Med. 1998;339(17):1186-93.
4. Aversa F, Tabilio A, Terenzi A, Velardi A, Falzetti F, Giannoni C, et al. Successful engraftment of T-cell-depleted haploidentical "three-loci" incompatible transplants in leukemia patients by addition of recombinant human granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells to bone marrow inoculum. Blood. 1994;84(11):3948-55.
5. Aversa F, Terenzi A, Tabilio A, Falzetti F, Carotti A, Ballanti S, et al. Full haplotype-mismatched hematopoietic stem-cell transplantation: a phase II study in patients with acute leukemia at high risk of relapse. J Clin Oncol. 2005;23(15):3447-54.
6. Koh LP, Rizzieri DA, Chao NJ. Allogeneic hematopoietic stem cell transplant using mismatched/haploidentical donors. Biol Blood Marrow Transplant. 2007;13(11): 1249-67.
7. Bethge WA, Faul C, Bornhauser M, Stuhler G, Beelen DW, Lang P, et al. Haploidentical allogeneic hematopoietic cell transplantation in adults using CD3/CD19 depletion and reduced intensity conditioning: an update. Blood Cell Mol Dis. 2008;40(1):13-9.
8. Lang P, Bader P, Schumm M, Feuchtinger T, Einsele H, Fuhrer M, et al. Transplantation of a combination of CD133+ and CD34+ selected progenitor cells from alternative donors. Br J Haematol. 2004;124(1):72-9.
9. Barfield RC, Otto M, Houston J, Holladay M, Geiger T, Martin J, et al. A one-step large-scale method for T- and B-cell depletion of mobilized PBSC for allogeneic transplantation. Cytotherapy. 2004;6(1):1-6.
10. Hale G, Kimberly K, Lovins R, Woodard P, Leung W, Yusuf U, et al. CD3 Depleted Hematopoietic Peripheral Blood Stem Cell Grafts in Children with Refractory Hematologic Malignancies Undergoing Transplantation from Mismatched Related Donors. Blood. 2005;106(11), Abstract #5458.
11. Ruggeri L, Capanni M, Casucci M, Volpi I, Tosti A, Perruccio K, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. Blood. 1999;94(1):333-9.
12. Bornhauser M, Thiede C, Brendel C, Geissler G, Oelschlagel U, Neubauer A, et al. Stable engraftment after megadose blood stem cell transplantation across the HLA barrier: the case for natural killer cells as graft-facilitating cells. Transplantation. 1999;68(1):87-8.
13. Fugier-Vivier IJ, Rezzoug F, Huang Y, Graul-Layman AJ, Schanie CL, Xu H, et al. Plasmacytoid precursor dendritic cells facilitate allogeneic hematopoietic stem cell engraftment. J Exp Med. 2005;201(3):373-83.
14. Grimes HL, Schanie CL, Huang Y, Cramer D, Rezzoug F, Fugier-Vivier I, et al. Graft facilitating cells are derived from hematopoietic stem cells and functionally require CD3, but are distinct from T lymphocytes. Exp Hematol. 2004;32(10):946-54.
15. Jacquet EG, Schanie CL, Fugier-Vivier I, Willer SS, Ildstad ST. Facilitating cells as a venue to establish mixed chimerism and tolerance. Pediatr Transplant. 2003;7(5): 348-57.
16. Tanaka J, Imamura M, Kasai M, Asaka M, Torok-Storb B. The role of accessory cells in allogeneic peripheral blood stem cell transplantation. Int J Hematol. 1999;69(2):70-4.
17. Bethge WA, Haegele M, Faul C, Lang P, Schumm M, Bornhauser M, et al. Haploidentical allogeneic hematopoietic cell transplantation in adults with reduced-intensity conditioning and CD3/CD19 depletion: fast engraftment and low toxicity. Exp Hematol. 2006;34(12):1746-52.
18. Leuner S, Arland M, Kahl C, Jentsch-Ullrich K, Franke A, Hoffkes HG. Enumeration of CD34-positive hematopoietic progenitor cells by flow cytometry: comparison of a volumetric assay and the ISHAGE gating strategy. Bone Marrow Transplant. 1998;22(7):699-706.
19. Schumm M, Lang P, Taylor G, Kuci S, Klingebiel T, Buhring HJ, et al. Isolation of highly purified autologous and allogeneic peripheral CD34+ cells using the CliniMACS device. J Hematother. 1999;8(2):209-18.
20. Bader P, Beck J, Frey A, Schlegel PG, Hebarth H, Handgretinger R, et al. Serial and quantitative analysis of mixed hematopoietic chimerism by PCR in patients with acute leukemias allows the prediction of relapse after allogeneic BMT. Bone Marrow Transplant. 1998;21(5):487-95.
21. Schumm M, Feuchtinger T, Pfeiffer M, Hoelle W, Bethge W, Ebinger M, et al. Flow cytometry with anti HLA-antibodies: a simple but highly sensitive method for monitoring chimerism and minimal residual disease after HLA-mismatched stem cell transplantation. Bone Marrow Transplant. 2007;39(12):767-73.
22. Cheson BD, Pfistner B, Juweid ME, Gascoyne RD, Specht L, Horning SJ, et al. Revised response criteria for malignant lymphoma. J Clin Oncol. 2007;25(5):579-86.
23. Sullivan KM. Graft-versus-host-disease. In: Blume KG, Forman SJ, Appelbaum FR, eds. Thomas' Hematopoietic Cell Transplantation. Blackwell Science Ltd., 2004:635-64.
24. Cheng SC, Fine JP, Wei LJ. Prediction of cumulative incidence function under the proportional hazards model. Biometrics. 1998;54(1):219-28.
25. Anderson JR, Cain KC, Gelber RD. Analysis of survival by tumor response. J Clin Oncol. 1983;1(11):710-9.
26. Gratwohl A, Stern M, Brand R, Apperley J, Baldomero H, de Witte T, et al. Risk score for outcome after allogeneic hematopoietic stem cell transplantation: a retrospective analysis. Cancer. 2009;115(20):4715-26.
27. Gratwohl A. The EBMT risk score. Bone Marrow Transplant. 2012;47(6):749-56.
28. Federmann B, Hagele M, Pfeiffer M, Wirths S, Schumm M, Faul C, et al. Immune reconstitution after haploidentical hematopoietic cell transplantation: impact of reduced intensity conditioning and CD3/CD19 depleted grafts. Leukemia. 2011;25(1):121-9.
29. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science. 2002;295(5562):2097-100.
30. Ciurea SO, Saliba R, Rondon G, Pesoa S, Cano P, Fernandez-Vina M, et al. Reduced-intensity conditioning using fludarabine, melphalan and thiotepa for adult patients undergoing haploidentical SCT. Bone Marrow Transplant. 2009;45(3):429-36.
31. Cesaro S, Gazzola MV, Marson P, Calore E, Caenazzo L, Destro R, et al. Successful engraftment and stable full donor chimerism after myeloablation with thiotepa, fludarabine, and melphalan and CD34-selected peripheral allogeneic stem cell transplantation in hemophagocytic lymphohistiocytosis. Am J Hematol. 2003; 72(2):143-6.
32. Majolino I, Davoli M, Carnevalli E, Locasciulli A, Di Bartolomeo P, Scime R, et al. Reduced intensity conditioning with thiotepa, fludarabine, and melphalan is effective in advanced multiple myeloma. Leuk Lymphoma. 2007;48(4):759-66.
33. Schwarer AP, Bollard G, Kapuscinski M, Muirhead J, Diviney M, Hart C, et al. Long-term follow-up of a pilot study using a chemotherapy-alone protocol for killer Ig-like receptor-ligand-mismatched haploidentical haematopoietic SCT. Bone Marrow Transplant. 2011;46(10):1331-8.
34. Nguyen S, Dhedin N, Vernant JP, Kuentz M, Al Jijakli A, Rouas-Freiss N, et al. NK-cell reconstitution after haploidentical hematopoietic stem-cell transplantations: immaturity of NK cells and inhibitory effect of NKG2A override GvL effect. Blood. 2005;105(10):4135-42.
35. Cook M, Briggs D, Craddock C, Mahendra P, Milligan D, Fegan C, et al. Donor KIR genotype has a major influence on the rate of cytomegalovirus reactivation following

T-cell replete stem cell transplantation. Blood. 2005;107(3):1230-2.
36. Morrison BE, Park SJ, Mooney JM, Mehrad B. Chemokine-mediated recruitment of NK cells is a critical host defense mechanism in invasive aspergillosis. J Clin Invest. 2003;112(12):1862-70.
37. Ruggeri L, Mancusi A, Capanni M, Urbani E, Carotti A, Aloisi T, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. Blood. 2007;110(1):433-40.
38. Cooley S, McCullar V, Wangen R, Bergemann TL, Spellman S, Weisdorf DJ, et al. KIR reconstitution is altered by T cells in the graft and correlates with clinical outcomes after unrelated donor transplantation. Blood. 2005;106(13):4370-6.
39. Brunstein CG, Wagner JE, Weisdorf DJ, Cooley S, Noreen H, Barker JN, et al. Negative effect of KIR alloreactivity in recipients of umbilical cord blood transplant depends on transplantation conditioning intensity. Blood. 2009;113(22):5628-34.
40. Eyrich M, Lang P, Lal S, Bader P, Handgretinger R, Klingebiel T, et al. A prospective analysis of the pattern of immune reconstitution in a paediatric cohort following transplantation of positively selected human leucocyte antigen-disparate haematopoietic stem cells from parental donors. Br J Haematol. 2001;114(2):422-32.
41. Shaffer J, Villard J, Means TK, Alexander S, Dombkowski D, Dey BR, et al. Regulatory T-cell recovery in recipients of haploidentical nonmyeloablative hematopoietic cell transplantation with a humanized anti-CD2 mAb, MEDI-507, with or without fludarabine. Exp Hematol. 2007;35(7):1140-52.
42. Lang P, Schumm M, Greil J, Bader P, Klingebiel T, Muller I, et al. A comparison between three graft manipulation methods for haploidentical stem cell transplantation in pediatric patients: preliminary results of a pilot study. Klin Padiatr. 2005;217(6):334-8.
43. Ciceri F, Labopin M, Aversa F, Rowe JM, Bunjes D, Lewalle P, et al. A survey of fully haploidentical hematopoietic stem cell transplantation in adults with high-risk acute leukemia: a risk factor analysis of outcomes for patients in remission at transplantation. Blood. 2008;112(9):3574-81.
44. Huang XJ, Liu DH, Liu KY, Xu LP, Chen H, Han W, et al. Treatment of acute leukemia with unmanipulated HLA-mismatched/haploidentical blood and bone marrow transplantation. Biol Blood Marrow Transplant. 2009;15(2):257-65.
45. Luznik L, O'Donnell PV, Symons HJ, Chen AR, Leffell MS, Zahurak M, et al. HLA-haploidentical bone marrow transplantation for hematologic malignancies using nonmyeloablative conditioning and high-dose, posttransplantation cyclophosphamide. Biol Blood Marrow Transplant. 2008;14(6):641-50.
46. Ciceri F, Bonini C, Stanghellini MT, Bondanza A, Traversari C, Salomoni M, et al. Infusion of suicide-gene-engineered donor lymphocytes after family haploidentical haemopoietic stem-cell transplantation for leukaemia (the TK007 trial): a non-randomised phase I-II study. Lancet Oncol. 2009;10(5):489-500.
47. Perruccio K, Topini F, Tosti A, Carotti A, Aloisi T, Aversa F, et al. Photodynamic purging of alloreactive T cells for adoptive immunotherapy after haploidentical stem cell transplantation. Blood Cells Mol Dis. 2008;40(1):76-83.
48. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. Blood. 2011;117(14):3921-8.
49. Chaleff S, Otto M, Barfield RC, Leimig T, Iyengar R, Martin J, et al. A large-scale method for the selective depletion of alpha-beta T lymphocytes from PBSC for allogeneic transplantation. Cytotherapy. 2007;9(8):746-54.