CLINICAL RESEARCH

Partial T Cell-Depleted Allogeneic Stem Cell Transplantation following Reduced-Intensity Conditioning Creates a Platform for Immunotherapy with Donor Lymphocyte Infusion and Recipient Dendritic Cell Vaccination in Multiple Myeloma

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Allogeneic stem cell transplantation (SCT) in multiple myeloma (MM) may induce a curative graft-versus-myeloma (GVM) effect. Major drawback in unmanipulated reduced-intensity conditioning (RIC) SCT is the risk of severe and longstanding graft-versus-host-disease (GVHD). This study demonstrates that transplantation with a partial T cell-depleted graft creates a platform for boosting GVM immunity by preemptive donor lymphocyte infusion (DLI) and recipient dendritic cell (DC) vaccination, with limited GVHD. All 20 MM patients engrafted successfully. Chimerism analysis in 19 patients evaluable at 3 months revealed that 7 patients were complete donor, whereas 12 patients were mixed chimeric. Grade II acute GVHD (aGVHD) occurred in 7 patients (35%) and only 4 patients (21%) developed chronic GVHD (cGVHD). Fourteen patients received posttransplantation immunotherapy, 8 preemptive DLI, 5 patients both DLI and DC vaccination, and 1 patient DC vaccination only. DC vaccination was associated with limited toxicity, and none of these patients developed GVHD. Importantly, overall treatment-related mortality (TRM) at 1 year was low (10%). Moreover, the overall survival (OS) is 84% with median follow-up of 27 months, and none of the patients died from progressive disease. These findings illustrate that this novel approach is associated with limited GVHD and mortality, thus creating an ideal platform for adjuvant immunotherapy.

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KEY WORDS: Multiple myeloma, Reduced-intensity conditioning, Donor lymphocyte infusion, Dendritic cell vaccination, Graft-versus-myeloma

INTRODUCTION

Allogeneic stem cell transplantation (SCT) may cure patients with multiple myeloma (MM) because of a graft-versus-myeloma (GVM) effect. Myeloablative (MA) conditioning has been limited by a high treatment-related mortality (TRM), and at present, reduced-intensity conditioning SCT (RIC-SCT) following autologous SCT seems a promising approach. Importantly, TRM following RIC-SCT is reduced from 30% to 40% to 10% to 20% [1]. However, 3 prospective trials comparing autologous transplantation followed by RIC-SCT versus double autologous SCT showed contradictory results in clinical outcome [2-4]. The study by Bruno et al. [2] showed a superior overall survival (OS) for autologous SCT followed by allogeneic RIC-SCT. In line with this study, Rosiñol et al. [4] observed a trend toward a longer progression-free survival (PFS) for patients treated with auto/RIC-SCT, but no significant differences in event-free survival (EFS) and OS. In contrast, the Intergroupe Francophone du Myelome (IFM) observed no differences in EFS and OS comparing double autologous SCT versus auto/RIC-SCT in high-risk patients. Although these differences in outcome may be explained by different inclusion criteria and treatment schedules, they illustrate that improvement of the

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GVM effect, without the toxicity and morbidity of graft-versus-host disease (GVHD) after allogeneic RIC-SCT, is a prerequisite to further establish this therapeutic approach.

Previously, we showed that partial T cell-depleted allogeneic SCT followed by preemptive donor lymphocyte infusion (DLI) resulted in long-term complete remission (CR) in about one-third of MM patients [5]. In this cohort of 24 patients, 1-year TRM after MA conditioning was 29%. But a continuous CR in 7 MM patients after preemptive DLI with a median follow-up of 8.6 years encouraged us to investigate partial T cell-depleted allogeneic SCT in the RIC setting, combined with preemptive immunotherapy with DLI. The major advantage of T cell-depleted grafts is reduction of severe and prolonged GVHD, but effective posttransplantation immunotherapy is essential to overcome the higher rate of relapse. As a novel approach we incorporated recipient-derived dendritic cell (DC) vaccination in the posttransplantation strategy for patients with residual disease after two preemptive DLI dosages.

DC are the professional antigen-presenting cells (APCs) of the immune system, and are essential for the induction of antigen-specific T cell immunity. In the setting of allogeneic SCT and DLI, alloreactive T cell responses targeting minor histocompatibility antigens (MiHA) on malignant cells of the recipient can be induced directly by recipient-derived DC and indirectly by donor-derived DC because of crosspresentation [6]. Boosting GVM immunity by vaccination with donor-derived DC loaded with hematopoiesis-restricted MiHA seems most ideal, but this approach is hampered by the limited number of known MM-expressed MiHA. Studies in mouse models demonstrated that recipient DC play a pivotal role in the initiation of alloreactive $CD8^+$ T cell-mediated immunity against leukemia [7,8]. Moreover, the presence of recipient DC in the setting of mixed chimerism has a positive impact on the effectiveness of DLI [9]. Because recipient DC and myeloma tumor cells are both derived from the hematopoietic system, immune responses induced by recipient-derived DC may enhance GVM with limited GVHD in other tissues, like mucosa, liver, and skin.

Here, we show the results of partial T cell-depleted RIC-SCT after autologous transplant for MM, with limited GVHD and a low 1-year TRM of 10%. Furthermore, we investigated the feasibility of generating mature recipient-derived DC from cryopreserved apheresis products, the immunogenicity of the vaccine, and the toxicity of recipient-derived DC vaccination. Our study indicates that partial T cell-depleted RIC-SCT is feasible, results in excellent engraftment, and offers opportunities for posttransplantation cellular immunotherapy with DLI in some patients combined with DC vaccination. Importantly, our approach keeps open the treatment with novel agents (bortezomib and lenalidomide) in case of progressive or relapsed disease even in combination with DLI.

MATERIALS AND METHODS

Transplantation Procedure

From January 2006 to May 2008, 20 patients have been included in a pilot study of partial T cell-depleted, allogeneic RIC-SCT for MM. All patients were pretreated for symptomatic MM with induction chemotherapy and high-dose melphalan (HDM), followed by autologous SCT (conform HOVON-50 or HOVON-65 studies or standard induction scheme at that time) [10]. Patients <65 years with an HLA-identical sibling donor were offered upfront allogeneic RIC-SCT within 6 months after autologous transplant, regardless of risk factors or disease status. Before RIC-SCT, autologous PBMC were collected by apheresis, washed to deplete platelets, and cryopreserved for posttransplant DC vaccination (Figure 1). The conditioning regimen consisted of cyclophosphamide (Cy) 1200 mg/m^2 .v. in combination with fludarabine (Flu) 30 mg/m^2 on each of 4 consecutive days (days −5, −4, −3, and −2 before SCT). Donor stem cell grafts were depleted from T and B cells by anti-CD3 and anti-CD19 immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Following depletion, $CD3^+$ T cells were added back to generate a stem cell graft containing a fixed number of 0.5×10^6 T cells/kg body weight of recipient. GVHD prophylaxis consisted of Cyclosporine A (CsA) 3 mg/kg/day .v. starting on day −1 until CsA could be taken orally. CsA was administered orally at a dose of 6 mg/kg/day until 8-10 weeks after SCT followed by a gradually tapering off in 4 weeks. Acute and chronic graft-versus-host disease (aGVHD, cGVHD) were classified grade I-IV and limited or extensive, respectively, according to the criteria described by Glucksberg [11] and Shulman [12].

Evaluation of Response and chimerism Analysis

Responses were evaluated according to the response criteria for MM described by Durie et al. in 2006 [13]. Bone marrow (BM) aspirates during posttransplantation immunotherapy were performed in patients receiving DC vaccination. Lambda free light chains were measured using the serum free light chain (FLC) assay (Freelite, Birmingham, UK). For measuring kappa free light chains, we used the ELISA assay, as described by Lamers et al. [14]. This ELISA was shown to parallel FLC kappa assay, with lower absolute values. To define CR, the FLC ratio was measured with the Freelite assay for both lambda and kappa free light chains. The data were analyzed in December 2008.

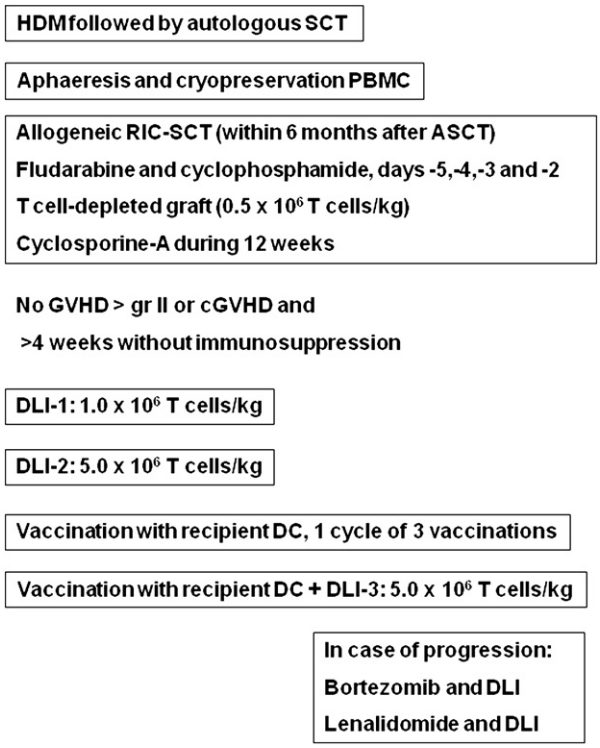

Figure 1. Flow chart of the tandem autologous SCT followed by RIC-SCT and posttransplantation cellular immunotherapy.

Molecular remission was defined as a negative patient-specific IgH-polymerase chain reaction (PCR) [15]. The sensitivity of the PCR is 1×10^{-5}. The patient-specific IgH-PCR did not play a role in the decision on the treatment schedule, because molecular analysis of disease load in BM was performed retrospectively.

Real-time quantitative PCR of single nucleotide polymorphisms (SNP) and/or the SMCY gene was used for chimerism analysis as described previously [16-18]. Briefly, recipient/donor pairs were screened for discriminating SNPs. Quantification was based on real-time PCR with allele-specific primers for DNA-sequences containing the discriminating SNP and target DNA-specific probes.

Posttransplantation Immunotherapy: Treatment Schedule

Patients without aGVHD grade >II and without cGVHD after RIC-SCT, were candidates for preemptive DLI 4 weeks after discontinuation of immunosuppression. The first DLI dose consisted of 1.0×10^6 T cells/kg body weight and the second dose 2 months later of 5.0×10^6 T cells/kg body weight (Figure 1). Patients with residual disease after 2 DLIs were eligible for recipient-derived DC vaccination. Exclusion criteria for vaccination were progressive disease (PD), extensive or uncontrolled GVHD, recent use of immunosuppressive drugs, and active infections. Vaccinations were administered 3 times at 2-week intervals. The DC dose was maximal at 30×10^6 cells i.v. as a bolus injection and 15×10^6 DCs intradermally (i.d.) in the upper leg near the inguinal lymph node region. If the yield of mature DC was too low, then only i.v. vaccination was given. Blood samples were taken from these patients before vaccination, on day 14 (after first vaccination), on day 28 (after second vaccination), on day 42 (after third vaccination), and on day 56 (28 days after the third vaccination). If no GVHD was induced, residual disease persisted and sufficient DC were cryopreserved, a second series of vaccinations was started using the combination of DC vaccination with DLI 5.0×10^6 T cells/kg body weight.

The study was approved by the Local Ethics Committee of Radboud University Nijmegen Medical Centre.

Posttransplantation Immunotherapy: Generation of DC Vaccine

DC vaccines were generated under good manufacturing practice conditions in a clean room facility. Before the conditioning for RIC-SCT, patient peripheral blood mononuclear cells (PBMCs) were collected by leukapheresis of 9 liters of blood using the Cobe Spectra aphaeresis system (Gambro BCT, Breda, The Netherlands). PBMC were washed with CliniMACS buffer containing 5% human serum albumin (HSA) to deplete from platelets, cryopreserved in HSA plus 10% DMSO and stored in liquid nitrogen until use. For culturing DC, PBMC were rapidly thawed at 37°C and resuspended in CliniMACS buffer containing 100 U/mL Pulmozyme (Roche, Woerden, The Netherlands), 3 mM $MgCl_2$, and 5% HSA. After a 30-minute incubation, PBMC were centrifugated, washed, and resuspended in XVIVO-15 medium (Cambrex Bio Sciences, Verviers, Belgium) plus 2% heat-inactivated virus-free human serum (HS). Monocytes were isolated by plastic adherence and cultured in XVIVO-15/2% HS supplemented with 800 U/mL GM-CSF and 500 U/mL IL-4 (CellGenix, Freiburg, Germany). Cells were harvested at day 3, counted and cultured at 0.5×10^6 cells/mL in 6-well plates in XVIVO-15/2% HS containing granulocyte macrophage-colony stimulating factor (GM-CSF) (800 U/mL), IL-4 (500 U/mL), and 50 μg/mL keyhole limpet hemocyanin (KLH) subunits (Biosyn Arzneimittel GmbH, Fellbach, Germany). Two days before harvesting, KLH-loaded DC were maturated in XVIVO-15/2% HS containing GM-CSF (800 U/mL), IL-4 (500 U/mL), IL-1β (5 ng/mL), IL-6 (15 ng/mL), TNF-α (20 ng/mL) (all CellGenix Freiburg, Germany), and prostaglandin E2 (PGE_2; Pharmacia & Upjohn, Puurs, Belgium, 1 μg/mL). Mature DC were harvested at day 9 and tested for microbial and phenotypic analysis. One-third of the cells were used for immediate injection and remaining cells were cryopreserved for subsequent vaccinations.

Immunologic Monitoring

T cell responses against KLH were measured using the ^{3}H-thymidine incorporation assay with PBMC of the patient before and after vaccination. Briefly, PBMC were restimulated in vitro with 50 μg/mL KLH subunits or 1 μg/mL PHA plus 100 U/mL IL-2 (ie, positive control). At day 7 of incubation, T cell proliferation was determined by ^{3}H-thymidine incorporation. The stimulation index was calculated as the counts ratio between stimulated and nonstimulated PBMC. Antibodies against KLH were measured in the serum of vaccinated patients by ELISA as described by De Vries et al. [19] A positive signal at a 400× dilution of the patient's serum was considered positive.

Alloreactive CD8^{+} T cell responses against recipient MiHA were determined by major histocompatibility complex (MHC) tetramer staining. Therefore, patients and donors were first genotyped for known MiHA-mismatches using allele-specific PCR assays as described previously [20,21]. In case of MiHA mismatches, PBMCs were incubated with the appropriate phycoerythrin (PE)-labeled MHC tetramer complex for 20 minutes at room temperature. After washing with PBS/0.5% BSA, cells were labeled with the appropriate concentration anti-CD8-FITC (ProImmune, Oxford, UK), anti-CD3-PECy7, and anti-CD45-ECD (Beckman Coulter, Fullerton, CA) for 15 minutes at 4°C. After washing, cells were resuspended in PBS/0.5%BSA and 7-amino-actinomycin D (7AAD; Sigma, St. Louis, MO, USA) was added. Cells were analyzed using the Coulter FC500 flow cytometer (Beckman Coulter).

RESULTS

Patient Characteristics

Twenty MM patients received upfront allogeneic RIC-SCT after autologous SCT between January 2006 and May 2008 (Table 1). Median age of these patients was 57 years (range: 39-64 years) at the time of RIC-SCT and of these 20 patients 11 were male and 9 were female. Disease status following autologous SCT was complete response (CR) in 7 patients (35%), very good partial response (VGPR) in 2 (10%), partial response (PR) in 8 (40%), stable disease (SD) in 2 (10%), and PD in 1 patient (5%). These results are comparable to the outcome after autologous SCT in other studies [4].

Table 1. Characteristics of MM Patients

Characteristic	Number
Number of patients	20
Median age at transplantation, years (range)	57 (39-64)
Sex, no (%)	
Male	11 (55%)
Female	9 (45%)
Immunoglobulin class (%)	
IgG	11 (55%)
IgA	3 (15%)
Light chain	6 (30%)
Cytogenetics	17
Karyotypic analysis	
Normal	11
Deletion of chromosome 13*	1
Hyperdiploid/complex	5
Interphase FISH	
Deletion of chromosome 13	7
Beta2-microglobulin	
<3.5 mg/L	5
>3.5 mg/L and <5.5 mg/L	3
≥5.5 mg/L	1
Not done	11
Induction chemotherapy	
VAD	16
TAD	2
PAD	2

VAD indicates vincristine, doxorubicin, dexamethasone; TAD, thalidomide, doxorubicin, dexamethasone; PAD, Bortezomib, doxorubicin, dexamethasone; FISH, fluorescein in situ hybridization; MM, multiple myeloma.
*Deletion of chromosome 13 by metaphase cytogenetics.

RIC-SCT with Partial T Cell-Depleted Grafts Resulted in successful Engraftment with Limited GVHD

All 20 patients received PB stem cell (PBSC) grafts from HLA-identical sibling donors. Median number of infused CD34^{+} cells was 8.3×10^{6} per kg body weight of the recipient (range: 4.3-12.8 $\times 10^{6}$). Median number of infused CD3^{+} T cells was 0.50×10^{6} per kg (range: 0.31-0.77 $\times 10^{6}$). Furthermore, median number of infused B cells was 0.22×10^{6} per kg (range 0.05-0.75 $\times 10^{6}$) and median number of infused NK-cells was 34.5×10^{6} per kg (range: 7.9-97.1 $\times 10^{6}$). After RIC-SCT, median time to reach leukocyte counts $>1.0 \times 10^{9}$/l and platelet counts $>20 \times 10^{9}$/l was 13 days (range: 10-20 days) and 9 days (range: 0-11 days), respectively. In 3 patients platelet counts did not decline to below 20×10^{9}/l.

Nineteen patients could be evaluated for PB cell chimerism at 3 months after RIC-SCT, and 1 patient died 2.2 months after RIC-SCT. All 19 patients showed successful donor engraftment and there was no secondary graft failure. At 3 months, 7 patients were complete donor chimeric and 12 patients were mixed chimeric. In these 12 mixed chimeric patients, the median value of autologous PBSCs was 7% (range: 2%-27%). Two patients converted to complete donor chimerism after discontinuation of CsA at 5 and 6 months after RIC-SCT, respectively. At the last follow-up, 16 of the 19 patients were complete donor chimeric, and 3 patients were still mixed chimeric. These 3 patients included 1 patient in which the donor was unavailable for donating lymphocytes and 2 patients treated with pre-emptive DLI shortly before or after the last follow-up.

Importantly, none of the patients developed grade III or IV aGVHD. Grade II aGVHD occurred in 7 of 20 (35%) patients. Nineteen patients were evaluable for cGVHD, of whom only 4 (21%) developed cGVHD. The median duration of CsA treatment was 99 days (range: 58-230 days). In 9 patients, CsA could be discontinued within 100 days. Three patients with cGVHD received CsA for more than 5 months.

Clinical Response after Partial T Cell-Depleted RIC-SCT and Preemptive DLI

Nineteen patients could be evaluated for clinical response at 3 months after RIC-SCT. Eight patients (42%) were in CR, 1 (5%) patient in VGPR, 7 (37%) patients in PR, and 3 patients had SD after the auto/RIC-SCT tandem-procedure.

Twelve of the 19 patients (63%) received preemptive DLI of 1.0×10^6 T cells/kg after RIC-SCT, and 7 of these patients also received a second dose-escalated preemptive DLI of 5.0×10^6 T cells/kg. Details of these twelve patients are shown in Table 2. Only 1 of the 12 patients developed GVHD grade I after preemptive DLI.

Two patients (UPN2 and UPN17) reached CR after preemptive DLI in a dose of 5.0×10^6 T cells/kg (Figure 2A and B). For patient UPN2, a patient-specific IgH-PCR was developed and this PCR became negative after preemptive DLI indicating molecular remission. Furthermore, 3 patients (UPN1, UPN3, and UPN7) converted to complete donor chimerism after posttransplantation immunotherapy with DLI, 6, 22, and 25 months after SCT, respectively.

Seven of the 19 patients did not receive preemptive DLI, because of GVHD (n = 4), infections (n = 2), and donor unavailability in 1 patient.

Generation of Recipient-Derived DC Vaccines

Following preemptive DLI, DC vaccines were generated for 6 patients as part of posttransplantation immunotherapy. Therefore, the cryopreserved apheresis product collected just prior RIC-SCT was thawed and used to generate mature monocyte-derived DC of recipient origin. Preclinical investigations showed that mature DC could be generated from cryopreserved apheresis products of MM patients that efficiently stimulated allogeneic T cell proliferation in vitro (Figure 3). The yield of PBMC and $CD14^+$ monocytes postcryopreservation for the 6 patients was 45% to 90% and 25% to 57%, respectively (Figure 4A). DC culture from cryopreserved PBMC resulted in sufficient DC yield in 4 patients (ie, 9%-16% from $CD14^+$ monocytes), but for 2 patients only a limited number of DC could be obtained (yield <5% from $CD14^+$ monocytes). The final vaccine contained >95% viable DCs (Figure 4A). Furthermore, DC vaccines had a very mature phenotype with >85% expression of CD83 and the costimulatory molecules CD80, CD86, and CD40 (Figure 4B). Moreover, 58% to 95% of the DC in the vaccines expressed the lymph-node migration receptor CCR7. For 1 patient, we did not obtain good quality mature DC, and this

Table 2. Disease Status and Chimerism in Patients Receiving Preemptive DLI after RIC-SCT

UPN	M-protein	Disease Status 3 Months after RIC-SCT*	Chimerism 3 Months after RIC-SCT	Preemptive DLI: T Cells/kg Body Weight	Outcome after Preemptive DLI (after Second DLI)
1.	IgG-kappa	CR	2.1% recipient cells	1.0×10^6	CR, complete donor chimerism, Guillain-Barre syndrome, died 13 months after SCT from pneumonia
2.	IgG-kappa	PR	5.2% recipient cells	1.0×10^6 and 5.0×10^6	CR, complete donor chimerism, DC vaccination
3.	Light chain λ	PR	26.7% recipient cells	1.0×10^6 and 5.0×10^6	VGPR, 6.7% recipient cells, DC vaccination
4.	IgG-kappa	PR	Complete donor	1.0×10^6 and 5.0×10^6	PR, ongoing decline of M-protein
5.	IgG-κ	SD	Complete donor	1.0×10^6 and 5.0×10^6	SD, DC vaccination
7.	Light chain λ	CR	14.0% recipient cells	1.0×10^6 and 5.0×10^6	CR, 12.8% recipient cells, DC vaccination
8.	IgG-kappa	CR	Complete donor	No preemptive, but therapeutic DLI	VGPR after therapeutic DLI for relapse, DC-vaccination
10.	IgA-κ	PR	8.6% recipient cells	No DLI, donor not available	Not applicable, DC vaccination
12.	IgA-κ	PR	Complete donor	1.0×10^6 and 5.0×10^6	Relapse
13.	IgG- λ	PR	Complete donor	1.0×10^6 and 5.0×10^6	PR, DC vaccine did not fulfill quality criteria
14.	IgG- κ	SD	9.5% recipient cells	1.0×10^6	Relapse, 1.8% recipient cells
15.	Light chain λ	CR	2.2% recipient cells	1.0×10^6	Relapse, 1.1% recipient cells
17.	IgG-lambda	PR	6.9% recipient cells	5.0×10^6	CR, 1.0% recipient cells
19.	Light chain κ	CR	5.5% recipient cells	1.0×10^6	CR, second preemptive DLI is planned

CR indicates complete remission; VGPR, very good partial response; PR, partial response; SD, stable disease; RIC-SCT, reduced-intensity conditioning-stem cell transplantation; DLI, donor lymphocyte infusion; DC, dendritic cell.
*Response 3 months after RIC-SCT.

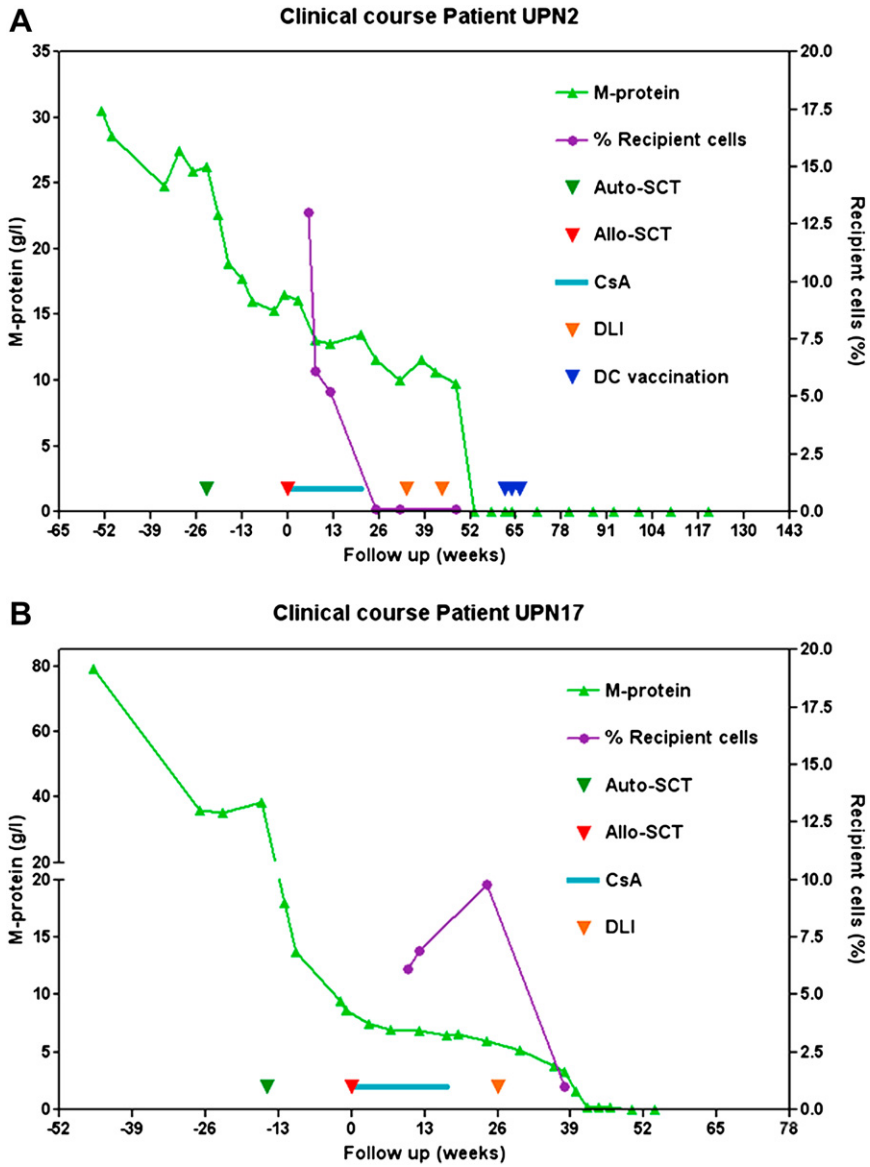

Figure 2. Clinical course of patient UPN2 (A) and patient UPN17 (B). The Y-axis on the left shows disease load as measured by serum FLCs or M-protein, and is shown with the green line. The Y-axis on the right shows the percentage recipient cells in peripheral blood and is shown with a purple line. Triangle in blue indicates autologous SCT, triangle in red indicates RIC-SCT, triangle in orange indicates DLI, and triple-triangle in blue indicates 1 cycle of DC-vaccinations.

vaccine was not administered (data not shown). Because of the variability in the yield of DC from thawed PBMC, the number of administered DC varied. Four patients received at least 1 maximum dose, 1 patient received 3 vaccinations each with total 10×10^6 cells (i.v. and i.d.) and 1 patient received 3 i.v. vaccinations each with 4×10^6 cells (Table 3).

Vaccination with Recipient-Derived DC Vaccines after RIC-SCT

Six MM patients were vaccinated with recipient-derived DC (Tables 2 and 3). The median time from RIC-SCT to DC vaccination was 11.6 (range: 8.1-24.4) months. Median interval from last DLI to DC vaccination was 4.2 (range: 3.5-4.9) months.

Four patients were vaccinated after 2 preemptive DLIs. In 1 patient, the donor was no longer available for donating lymphocytes, and she was treated with DC vaccination only as posttransplantation immunotherapy. The sixth patient was treated with DC vaccination following 2 therapeutic DLIs. None of these patients showed clinically active disease at the time of vaccination.

DC were loaded with the antigenic protein KLH as an adjuvant to provide $CD4^+$ T cell help and for boosting of alloreactive $CD8^+$ T cell responses as well as to analyze the induction of a primary immune response posttransplantation. All patients showed

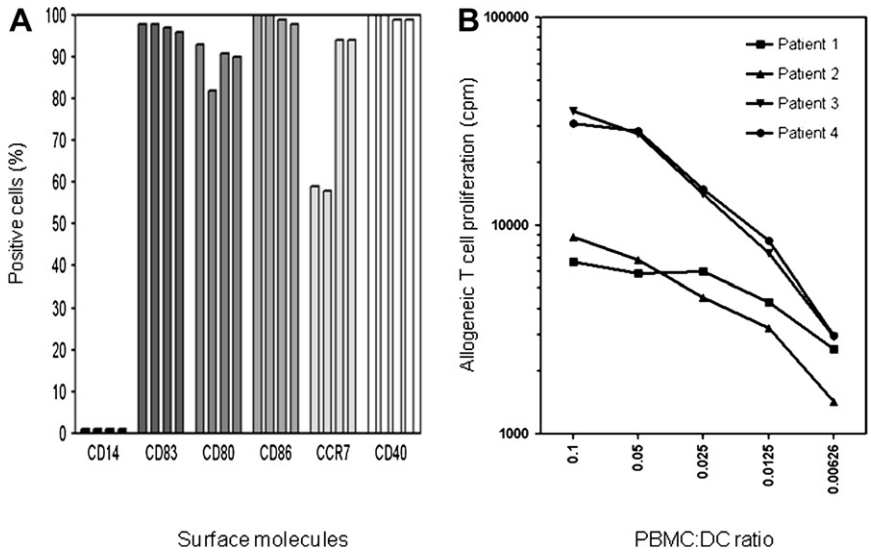

Figure 3. (A) Preclinical study of characteristics of mature DC generated from cryopreserved apheresis products from 4 MM patients. Apheresis was performed after autologous SCT. Cultured DC had a mature phenotype with high expression of CD83, CD80, and CD86. (B) In vitro stimulation capacity of mature DC generated from cryopreserved apheresis products. DC from patients 1 and 2 and patients 3 and 4 were tested with responder cells from different healthy donors.

a PB T cell proliferative response against KLH that could already be detected after 1 single DC vaccination (Table 3 and Figure 5A). However, the anti-KLH T cell proliferative response in some patients decreased following subsequent DC vaccinations (Figure 5A). Furthermore, antibody responses against KLH could not be detected (Table 3).

Five patients were evaluated for T cell recovery at the time of DC administration (Table 4). Median $CD3^+$ T cell count was 0.7×10^9/L, median $CD4^+$ T cell count 0.3×10^9/L, and median $CD8^+$ T cell count was 0.4×10^9/L, indicating a not completely recovered immune system, especially from the $CD4^+$ T cells at the time of vaccination. Natural killer (NK) cells were recovered to normal in 4 of the 5 patients with a median of 0.2×10^9/L $CD3^-CD16/56^+$ NK cells. CD4/CD8 ratios were still inversed in 3 of the 5 patients. Although $CD4^+$ and $CD8^+$ T cell counts were not completely recovered to normal levels, all 5 patients showed a strong *in vitro* polyclonal T cell proliferative response upon stimulation with PHA and IL-2 (Table 4).

Impact of DC-vaccination on regulatory T cells has been studied before and after each DC vaccination. Regulatory T cells were studied as the percentage of CD25 bright+ cells within the $CD3^+CD4^+$ population and are shown in Figure 5B. There is no trend in increasing percentages of $CD4^+$ CD25 $bright^+$ cells in DC vaccinated patients.

Characterization of T Cell Responses

For all 6 patients who received DC vaccination, we performed genomic typing of 12 previously described MiHA. In 2 patients, a mismatch against a known MiHA was found with the immunogenic allele in the recipient. Patient UPN2 was mismatched for MiHA HY and patient UPN5 for HA-8. PB samples (before and after DLI as well as before and after DC vaccination) of patient UPN2 were analyzed with tetramers against HLA-A2 and HLA-B7 restricted epitopes in the male-specific SMCY protein. However, in these samples no SMCY-tetramer-positive cells could be detected (data not shown). Samples of patient UPN5 (before DC vaccination and after each DC vaccination) were analyzed with tetramers against the HLA-A2 restricted HA-8 antigen. Also in this patient we found no HA-8 tetramer positive cells in PB samples (data not shown). Although we could not detect tetramer-positive T cells against known MiHA in DLI and DC-treated patients, this does not exclude the presence of MiHA-specific or tumor antigen-specific T cell responses in these patients.

Recipient DC Vaccination Did Not Induce Severe Toxicity and GVHD

Toxicity of recipient DC vaccination was limited to flu-like symptoms with mild fever and local induration at the injection site (Table 3). Importantly, none of the 6 patients developed clinical signs of GVHD, but 2 patients developed discrete dermal changes with folliculitis and eosinophilia in peripheral blood (PB).

At the time of the first DC vaccination, 3 patients were complete donor chimeric and 3 patients had still mixed chimerism with 6.7%, 12.8%, and 8.6% recipient cells, respectively (Table 2). DC vaccination alone did not induce conversion to complete donor chimerism. Patient UPN3 converted to complete donor chimerism after a second cycle of vaccinations in

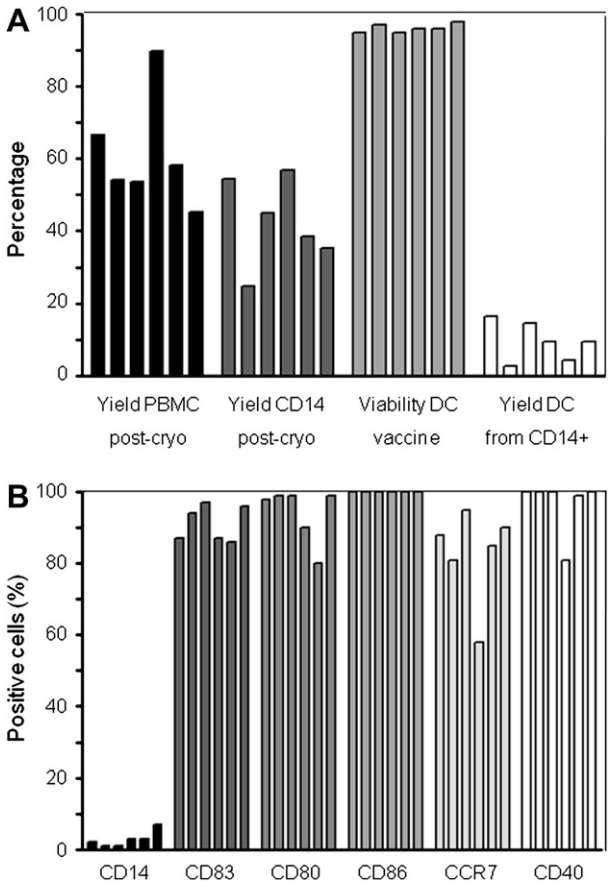

Figure 4. (A) Characteristics of DC vaccine from thawed apheresis products. Postcryopreservation, the yield of PBMC varied from 45% to 90% and yield of $CD14^+$ monocytes varied from 25% to 57%. Yield of mature DC from $CD14^+$ cells was sufficient in 4 patients (9%-16%); however, <5% in 2 patients. Viability of the vaccination product was >90% for all administered vaccines. (B) DC vaccine phenotype. All administered DC vaccines had a mature phenotype with high expression of the cell surface antigens CD83, CD80, CD86, and CCR7.

combination with DLI (Figure 6A). Patient UPN7 received a therapeutic DLI of 0.5×10^8 T cells/kg for increase of free light lambda chains, resulting in complete donor chimerism and GVHD grade II (Figure 6B).

Clinical Outcome of DC Vaccination after RIC-SCT

Fourteen of 20 patients were treated with posttransplantation immunotherapy (13 preemptive and 1 for relapse), including 8 patients with preemptive DLI alone, 5 patients received both DLI and DC vaccination and 1 patient DC vaccination only (Table 2).

DC vaccination in 6 patients did not result in induction of responses by itself. However, patient UPN3 showed a gradual decline of light chains after RIC-SCT during posttransplantation immunotherapy with DLI and DC vaccinations (Figure 6A). Two years after RIC-SCT, light chains started rising again without clinical symptoms. An escalating dose of DLI was administered, but the serum free light chains continued to rise. Presently, this patient is treated with the combination of lenalidomide and DLI.

Patient UPN7 showed a rise in FLC lambda 8 months after completion of DC vaccination (Figure 6B). Although this rise did not fulfill the criteria for PD, immunotherapy was continued because in our experience rise of FLCs predicts clinical relapse. He was treated with therapeutic DLI (0.5×10^8 T cells/kg) and developed GVHD grade II in combination with stabilization of free light lambda chains. Patient UPN5 and UPN10 did not respond to DC vaccination.

Patient UPN8 reached a VGPR after 2 therapeutic DLIs of 5.0 and 10.0×10^6 T cells/kg for relapsed MM (Figure 7). Because of the relapse, DC vaccination was combined with DLI in a dose of 10.0×10^6 T cells/kg. At the last follow-up, immunofixation remains positive but the M-protein cannot be quantified.

OS and PFS after RIC-SCT

With a median follow-up for surviving patients of 27 months (range: 8.9-34.9 months) the OS is 84% (Figure 8A). TRM was 5% at 100 days, and 10% at 1 year. One patient died from sepsis and cardiac failure

Table 3. Immune Responses and Toxicity after recipient DC Vaccination

	Number of Vaccinated DC*							Anti-KLH response	
UPN	Vac I	Vac II	Vac III	DLI†	Induration	Fever	GVHD	T cell§	Ab¶
3.	30/15	30/15	29/13	no	yes	yes	no	+++	–
	6.5/3.5	6.5/3.5	5/2.5	DLI (5.0×10^6 /kg)	no	no	no		
5.	4/0	4/0	3/0	No	na‡	no	no	+++	–
7.	30/15	33/15	34/17	No	Yes	yes	no	+++	–
10.	28/14	27/13	27/13	No	Yes	yes	no	+++	–
2.	7/3	7/3	6.5/3.5	No	Yes	yes	no	++	–
8.	30/15	18/9	18/9	DLI (10.0×10^6 /kg)	No	no	no	+	–

DLI inidcates donor lymphocyte infusion; MM, multiple myeloma; GVHD, graft-versus-host disease; DC, dendritic cell.
*Cell numbers are given in 10^6, intravenously/intradermally.
†Two patients received the combination of DLI and DC vaccination. UPN3 received a second cycle of vaccinations in combination with DLI on the day of vaccination I. UPN 8 relapsed after RIC-SCT, and responded to therapeutic DLI. Because of relapsed MM, this patient received DC-vaccination in combination with DLI. DLI was coinfused with DC-vaccination II.
‡Local induration was not applicable to this patient.
§KLH-specific proliferation of PBMC after vaccination is depicted as stimulation index (SI): + SI > 2 < 10; ++ SI ≥ 10 < 100; +++ SI ≥ 100.
¶KLH-specific antibody titers in serum after vaccination: – no Ab or <1:400; + Ab titer ≥1:400.

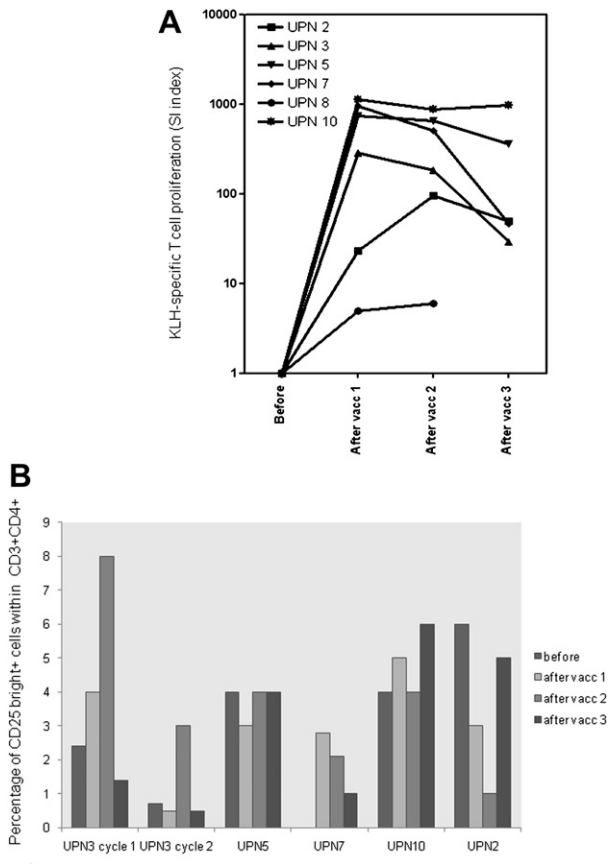

Figure 5. (A) KLH specific T cell proliferation indicated as stimulation index. (B) Regulatory T cells (CD25 bright+ cells within the $CD3^+CD4^+$ population) before and after each DC vaccination in 5 patients treated with DC vaccination.

2.2 months after RIC-SCT and 1 patient from pulmonary cGVHD 9 months after RIC-SCT. One additional patient died in CR 13 months after RIC-SCT from the complications of pneumonia during recovery from a Guillain-Barré syndrome. None of the patients died from relapsed or progressive multiple myeloma.

At the last follow-up in December 2008, 6 patients were in CR, 1 patient reached a VGPR, and 2 patients with PR were still receiving immunotherapy. Eight patients had started with systemic therapy (bortezomib, thalidomide, or lenalidomide) for PD after RIC-SCT (3 from SD, 2 with PR, and 1 with VGPR) or relapse from CR (2 patients). The current PFS is shown in Figure 8B.

DISCUSSION

Here, we report on the feasibility of Flu-Cy RIC-SCT in combination with a partial T cell-depleted graft for MM patients following induction chemotherapy and autologous transplant with HDM. One-year TRM was reduced to 10% in a cohort of 20 patients with a median follow-up of surviving patients of 27 months. This TRM is in line with other studies using RIC-SCT for MM [1,4]. OS rate is comparable with other studies in patients undergoing autologous SCT followed by RIC-SCT. In our study, OS was 84% at 2 years. In the ECOG-study the actuarial survival rate at 2 years was 78% and OS was 78% at 20 months in the study from Maloney et al. [1,22]. All patients showed donor engraftment and no late graft failures occurred. Importantly, aGVHD was limited to grade I and II, and could be managed with CsA and corticosteroids. The prevalence of cGVHD was 21%, including 1 patient suffering from pulmonary disease, probably related to GVHD. The duration of immunosuppressive therapy with CsA was relatively short for a RIC regimen with a median of 99 days, 3 patients were treated with CsA for more than 5 months because of cGVHD.

Preemptive DLI resulted in conversion from PR to CR in 2 patients. At the last follow-up, 6 of the 17 evaluable patients were in CR and 3 of them have received preemptive DLI. However, durability of these responses with a median follow-up of 27 months has to be shown by a longer follow-up. DC-vaccination alone did not induce GVM responses after RIC-SCT. However, this study was designed to analyze the feasibility, immunogenicity, and toxicity of recipient-derived DC vaccination. Important questions concerning the optimal dose of DC, route of administration, and combination with DLI have not been studied yet in the setting of allogeneic SCT.

Table 4. T cell Recovery at Time of DC Vaccination

UPN	Interval SCT-DC Vaccination (Months)	$CD3^+ \times 10^9$/L	$CD3^-$ $CD16/56^+ \times 10^9$/L	$CD19^+ \times 10^9$/L	$CD4^+ \times 10^9$/L	$CD8^+ \times 10^9$/L	PHA/IL2 Response*
Normal (5-95 percentile)		1.2 (0.7-2.1)	0.3 (0.09-0.6)	0.2 (0.1-0.5)	0.7 (0.3-1.4)	0.4 (0.3-1.4)	
3.	11.8	0.7	0.23	0.2	0.4	0.3	+++
		1.5	0.64	0.07	1.6	0.7	
5.	11.3	0.8	0.2	0.0	0.2	0.5	++++
7.	11.3	0.8	0.2	0.05	0.4	0.4	++++
10.	8.1	0.3	0.12	0.08	0.2	0.1	++++
2.	14.4	0.7	0.06	0.22	0.2	0.6	++++

DC indicates dendritic cell; SCT, stem cell transplantation.
*PHA/IL2 induced proliferation of PBMC before vaccination is depicted as stimulation index (SI): + SI > 2 < 10; ++ SI ≥ 10 < 100; +++ SI ≥ 100 < 500; ++++ SI ≥ 500.

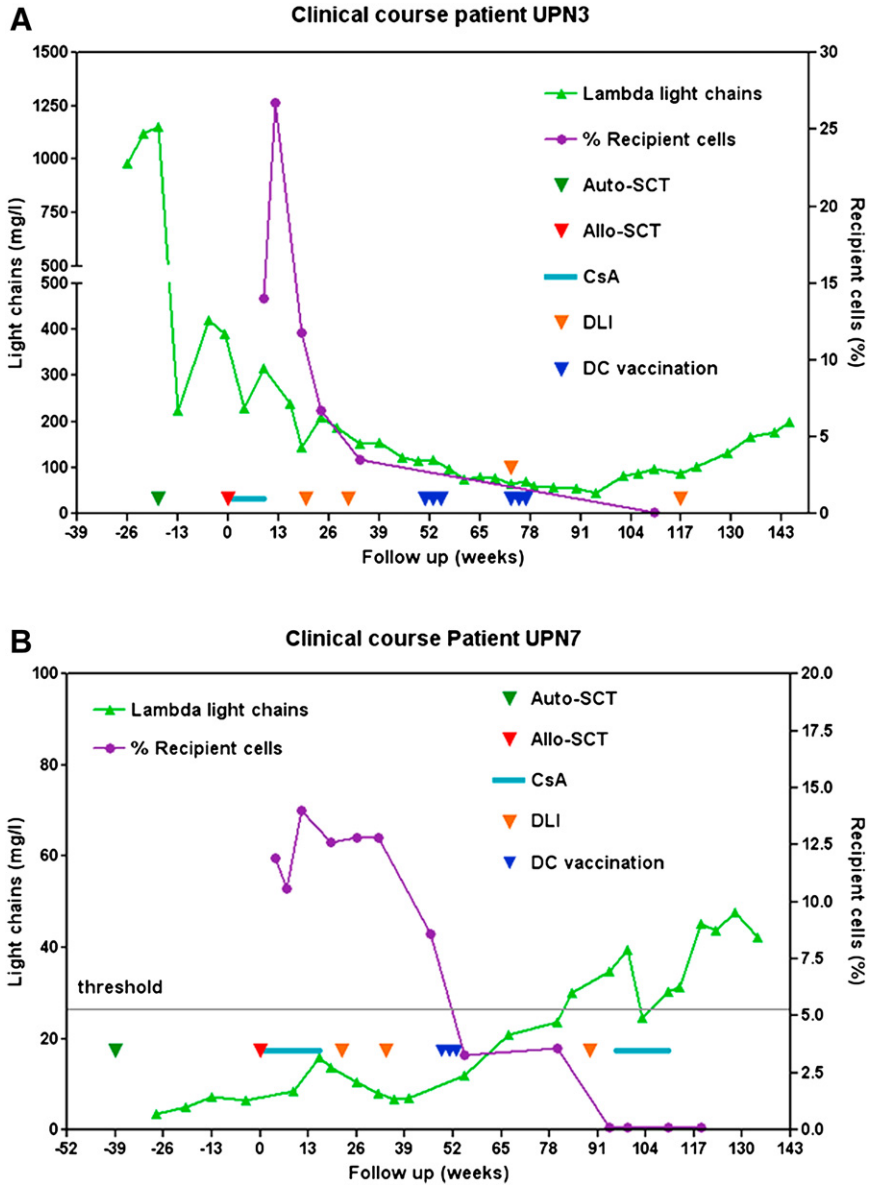

Figure 6. Clinical course of patient UPN3 (A) and patient UPN7 (B). The Y-axis on the left shows disease load as measured by serum FLCs or M-protein, and is shown with the green line. The Y-axis on the right shows the percentage recipient cells in peripheral blood and is shown with a purple line. Triangle in blue indicates autologous SCT, triangle in red indicates RIC-SCT, triangle in orange indicates DLI, and triple-triangle in blue indicates 1 cycle of DC vaccinations.

Our data indicate that partial T cell-depleted RIC-SCT creates a platform for posttransplantation cellular immunotherapy with preemptive DLI and DC vaccination, given the low incidence and severity of GVHD and the short duration of immunosuppressive therapy. DLI has proved to be effective in MM as preemptive immunotherapy; however, the optimal dose and timing is not known [23]. In this study, preemptive DLI started with a low dose of 1.0×10^6 T cell/kg 4 weeks after discontinuation of CsA followed by a second dose of 5.0×10^6 T cells/kg 2 months later. The lowest dose of 1.0×10^6 T cell/kg did not result in GVM reactivity, or in GVHD. Conversion of chimerism was observed in only 1 patient after this low-dose DLI. The dose of 5.0×10^6 T cells/kg resulted in CR in 2 patients without GVHD. Therefore, we apply a starting DLI dose of 5.0×10^6 T cells/kg in the current protocols.

Repeated DLIs were only given if a patient did not reach CR. Importantly, most responses to DLI were seen after the first or second DLI. Only 1 patient developed GVHD and decreasing FLCs after the third DLI. Repeated DLIs were given to patients with relapsed MM after RIC-SCT who responded to systemic therapy with Bortezomib or Lenalidomide (data not shown). These repeated DLIs did not result in long-term remissions. We consider most patients not responding to the first or second DLI resistant to

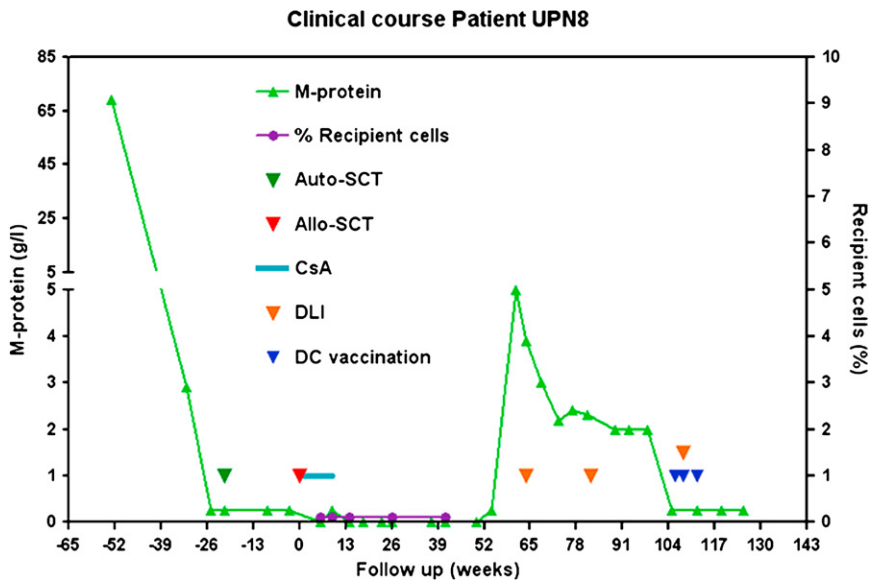

Figure 7. Clinical course of patient UPN8. The Y-axis on the left shows disease load as measured by serum FLCs or M-protein, and is shown with the green line. The Y-axis on the right shows the percentage recipient cells in PB and is shown with a purple line. Triangle in blue indicates autologous SCT, triangle in red indicates RIC-SCT, triangle in orange indicate DLI and triple-triangle in blue indicate 1 cycle of DC vaccinations.

DLI. Prerequisites for the induction of a GVM effect by DLI are effective antigen presentation and costimulation in conjunction with sufficient inflammation. In this study, we have been focusing on the antigen presentation, and introduced DC-vaccination as post-transplantation immunotherapy. Another strategy to further boost GVM immunity after DLI could be blockade of negative regulatory mechanisms. For example, strategies aimed at reducing regulatory T cells or blocking of the T cell inhibitory PD-1/PD-L1 pathway.

Although preemptive DLI is effective, other therapies are needed to further improve the GVM effect of RIC-SCT. Both regulatory T cells and host APCs have been implicated in GVHD and graft-versus-leukemia (GVL) reactivity after DLI. The requirement of recipient APC for the induction of GVL has been clearly demonstrated by Mapara et al. [9]. Moreover, Xia et al. [24] have shown that in the long-term, complete chimeras loss of DLI-induced GVL can be restored by infusion of host DC. In this study, we analyzed the feasibility of generating recipient-derived mature DC and the toxicity of vaccination with these DC.

To generate recipient-derived mature DC several months after allogeneic SCT, we used cryopreserved patient apheresis products obtained after autologous SCT and shortly before RIC-SCT. DC with a mature phenotype and sufficient CD80, CD83, CD86, and CCR7 expression could be generated from the cryopreserved PBMC from 6 of 7 patients. The administered vaccine products all fulfilled the quality-criteria as described by Figdor et al. [25]. The generated DC of 1 patient did not have a mature phenotype. Comparative studies with immature and mature DC have demonstrated that only mature DC stimulate T cell in vivo and it has been shown that immature DC can silence immune responses [19]. Therefore, this not fully mature DC vaccine was not administered.

Induction of a primary immune response was measured by T cell responses against KLH. We showed that recipient-derived mature DCs loaded with KLH induce a potent primary T cell response after the first vaccination. However, the PB T cell proliferative response against KLH after the second and third DC injection decreased in most patients. DCs were not extra loaded with antigens because recipient-derived DCs are able to directly present recipient-specific MiHA to donor T cells. In this setting of using unloaded recipient-derived DC, we aimed at the induction of MiHA-specific donor T cell responses against known and unknown MiHA. Genotyping for known MiHA in the vaccinated recipients and their donors did identify MiHA-mismatches in some patients that could be involved in GVM and GVHD (data not shown). However, we were unable to detect antigen-specific T cells against known MiHA using tetramer staining. Currently, we are analyzing whether recipient-derived DC did boost or induce T cell responses against unknown MiHA by functional characterization of alloreactive T cell lines generated from vaccinated patients.

Toxicity of recipient-derived DC was limited to fever the evening after the second and third vaccination and local induration at the injection site. Such toxicity is known from other vaccination studies, and probably results from immune responses against KLH [26]. None of the 6 patients developed GVHD

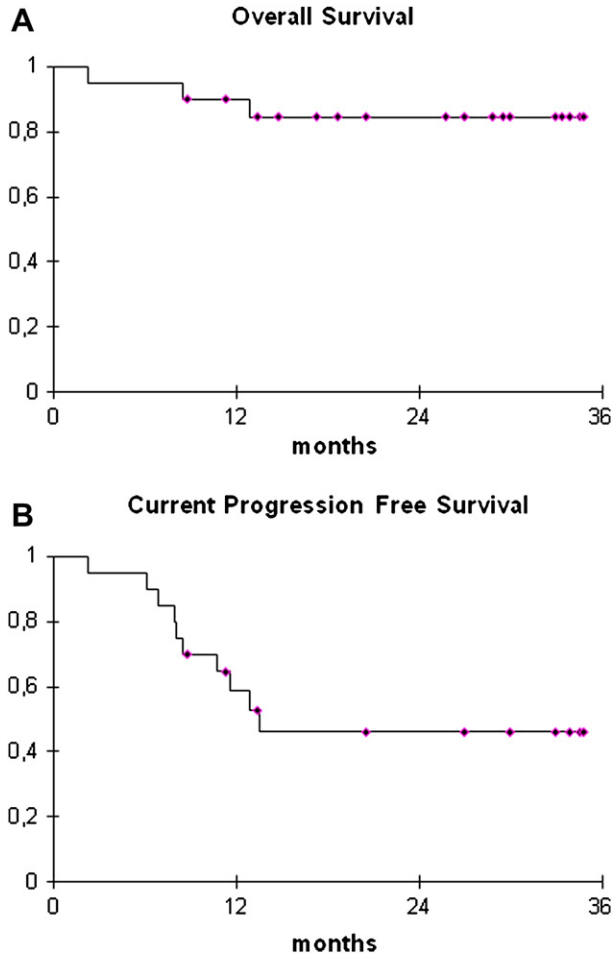

Figure 8. OS of 20 patients after RIC-SCT with a median follow-up of 27 months. (A) Current PFS of 20 patients after RIC-SCT. Progression was noted when systemic therapy was started.

after recipient-derived DC-vaccination, although 2 patients reported discrete skin changes.

This is the first study that applied recipient-derived DC vaccines after allogeneic SCT. One major limitation of recipient-derived DC vaccines after allogeneic SCT is the requisite to collect and to cryopreserve PBMC before SCT for generation of mature monocyte-derived DC several months later. Alternatively, donor DC loaded with recipient-specific MiHA may also induce alloreactive T cell responses after allogeneic SCT and the use of donor-derived DC for vaccination circumvents the obstacle of cryopreservation. To explore donor-derived DC vaccines for the induction of MiHA-specific immune responses after SCT, a set of hematopoietic-restricted MiHA with expression on tumor cells must be available for loading of donor-derived DC. Until now, the number of identified hematopoietic-restricted MiHA with expression on MM tumor cells was limited, and therefore hampered the application of this strategy. However, the proof of principle may be explored clinically by using MiHA HA-1 and LRH-1, which have been shown to be functionally expressed by MM tumor cells [27,28].

Vaccination with donor-derived DC, loaded with tumor lysate or tumor-associated antigens, after SCT has been published before in 3 reports. In the first report, DC were cultured from granulocyte colony-stimulating factor (G-CSF) mobilized PB stem cells from the donor [29]. Donor-derived DC pulsed with irradiated tumor cells and primed T cells were injected in 4 patients with relapse after SCT. In the second report, DC cultured from PB cells from the donor and pulsed with tumor lysate were given to a patient transplanted for metastatic renal cell carcinoma [30]. Vaccination with donor-derived DC appeared to be safe in this single patient, but did not induce graft-versus-tumor reactivity. In the third report, a patient with relapsed acute myelogenous leukemia (AML) after SCT was vaccinated with WT1 peptide and KLH-pulsed donor-derived DC [31]. Immune responses were induced to the immunogenic antigen KLH, however T cell responses against WT1 were not detected and the relapsed leukemia did not respond.

New options to salvage patients with relapsed or PD after allogeneic SCT are thalidomide, bortezomib, and lenalidomide. El-Cheikh et al. [32] reported 37 patients treated with bortezomib as salvage treatment for relapse or progression following RIC-SCT. An objective disease response (including CR, VGPR, and PR) was achieved in 27 patients (73%). Lenalidomide treatment for relapse MM was reported by Minnema et al. [33]. Lenalidomide alone or in combination with dexamethasone resulted in a response rate of 87.5%. Importantly, some patients developed aGVHD when lenalidomide was given as monotherapy within months after SCT or DLI. These studies are performed in patients with relapsed or PD; however, the role of new agents in combination with cell therapies for residual disease after RIC-SCT has not been explored yet.

In conclusion, partial T cell-depleted RIC-SCT has the advantage of a low 1-year TRM of 10%, limited severe GVHD, and sustained GVM reactivity. This strategy opens the possibility for posttransplantation immunotherapy, alone or in combination with new agents. Because of the low incidence of aGVHD and cGVHD, the quality of life in these patients remains good and consequently preemptive immunotherapy can be performed in a majority of patients.

Therefore, in the era of RIC-SCT for MM, posttransplantation strategies have become more important than before. We show that vaccination with recipient-derived DC is feasible, safe, immunogenic, and most importantly, does not induce GVHD. The potential of this approach to induce GVM reactivity is not yet fully exploited. Further studies on the coinfusion of donor lymphocytes with recipient-derived DC or MiHA-loaded donor-derived DC are needed.

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