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Transplantation of highly purified peripheral blood CD34$^+$ cells from HLA-mismatched parental donors in 14 children: evaluation of early monitoring of engraftment

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HLA-mismatched family members may represent an important cell source for patients that require stem cell transplantation but lack both a matched sibling donor and a closely matched unrelated donor. We report the outcome of 19 transplantations from HLA two- or three- loci mismatched parental donors in which 14 pediatric patients with hematological malignancies or other disorders, received a median of 21.5 × 10^6 (range, 5.4–58) highly purified CD34$^+$ peripheral blood stem cells (PBSC), as well as 4.7 × 10^4 (range, 0.4–12) donor T cells per kg body weight. T cell depletion was performed using a two-step CD34-positive selection on two different magnetic beads devices. Ten of 14 patients presented with rapid myeloid engraftment. The four patients who presented with graft failure (two non-engraftments, two rejections) received a second stem cell graft and one a third. Graft rejection was detected early by polymerase chain reaction (PCR) analysis of FACS-sorted T cells. Eight of the 14 patients are still alive after a median observation period of 15.6 months (range, 3–31.3) with full donor chimerism in all hematopoietic cell lineages. No acute organ graft-versus-host disease (GVHD) and no chronic GVHD have occurred. One patient experienced relapse of leukemia. We conclude that transplantation of allogeneic PBSC from haploidentical donors will open new perspectives for pediatric patients for whom an HLA-matched stem cell graft is not available. Close monitoring of recipient and donor hematopoiesis might be of clinical value, to recognize early engraftment or rejection.
Keywords: PBSCT; mismatched; magnetic beads; engraftment; chimerism; rejection

Introduction

Various hematopoietic disorders and malignancies can be cured by transplantation of allogeneic hematopoietic stem and progenitor cells contained in bone marrow (BM), cord blood or peripheral blood (PB). Over the last years, the use of the latter cell source has increased for several reasons: (1) The majority of PBSC is less committed than their BM counterparts;[1] (2) the engraftment kinetics were found superior after transplantation of recombinant human (rh) granulocyte colony-stimulating factor (G-CSF)-mobilized PBSC;[2] and (3) both the short-term side-effects and the collection procedure seem acceptable for healthy donors.[3] Only 25–30% of patients eligible for allogeneic stem cell transplantation (SCT) have an HLA-matched sibling donor, and only half of the remainders find an HLA-matched unrelated donor. *In vivo* and/or *in vitro* T cell depletion has been applied widely to reduce complications from acute or chronic graft-versus-host disease (GVHD).[4,5] This procedure has shown to be associated with a higher risk of graft failure,[6] with a reduced graft-versus-leukemia (GVL) effect in patients with malignancies, and with long-lasting immune deficiency.[7] Animal studies have demonstrated that a dramatic increase of stem cell dose might overcome HLA barriers.[8] In man, the combination of T cell-depleted BM with PBSC from G-CSF-mobilized haploidentical donors led to sustained engraftment in patients suffering from advanced leukemia.[9] To reduce the risk of rejection and relapse, these patients were treated with highly myeloablative and immunosuppressive chemotherapy including single dose total body irradiation (TBI) which might have adverse long-term effects in young children. Our aim was to apply conditioning regimens that were sufficiently cyto- and immunotoxic to control malignant disease on the one hand and enable engraftment of highly T cell-depleted PBSC on the other hand. We wanted to gain information on hematopoietic reconstitution by very close monitoring of the donor/patient chimerism, with particular emphasis on early detection of rejection or non-engraftment, because long-lasting aplasia raises the risk of severe infections.

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Materials and methods

Patient characteristics

Between September 1996 and January 1999, 14 children (11 males, three females) underwent allogeneic transplantation with PBSC from HLA-mismatched parental donors. All patients lacked an HLA-identical family donor, a matched unrelated donor and a suitable cord blood specimen. The patients were treated for different disorders including chronic myeloid leukemia (CML) in 3rd chronic phase (n = 2), acute myeloid leukemia/myelodysplastic syndrome (AML/MDS) (n = 2), high risk (HR) acute lymphoblastic leukemia (ALL) in 2nd and 3rd remission after very early bone marrow relapse (n = 3), ALL relapse after allogeneic bone marrow transplantation (BMT) from an unrelated donor (n = 1), ALL (t9;22) in 1st remission after non-engraftment of allogeneic BM from an unrelated donor (n = 1), very severe aplastic anemia (vSAA, n = 1), familial hemophagocytic lymphohistiocytosis (FHL, n = 2), Kostmann syndrome (KS, n = 1) non-responsive to rhG-CSF treatment and Fanconi anemia (FA, n = 1). The patients' age at the time of transplantation ranged from 1.7 to 18 years (median 5.1 years), the median body weight was 27.6 kg (range, 13.5–98.8). The median interval between diagnosis and PBSC transplantation (PBSCT) was 21.8 months (range, 3.8–84.6). The median duration of follow-up was 15.6 months (range, 3–31.3) (Table 1). Eleven patients had received different courses of chemotherapy before conditioning for SCT, nine of them were heavily pre-treated, particularly those with

Table 1 Patient characteristics

Patient No. Sex/No. of transplant-ations	*Patient age (years)*	*Diagnosis*	*Conditioning regimen total dose, mg/kg*	*DLI-infusion: No. of T cells, day after last TX*	*Stable engraftment*	*aGVHD highest overall grade*	*Clinical complications*	*Outcome: survival/ in days/status of disease/causes of death*
1/m/1	6.4	CML, 3rd CP	TBI, CY 120, ATG 60		no	0	rejection	rejection
1/m/2	6.5		Thio 10, Mel 4.6, ALG 30	1×10^4/kg day 68	yes	I*	—	779/CR
2/m/1	2.5	CML, 3rd CP	BU 16, Thio 10, CY 120, ATG 60		yes	0	lung transplantation	died day 119/CR CMV-pneumonia
3/m/1	18	Sec. AML after MDS; PD	BU 16, Thio 10, CY 120, ATG 60		yes	I	—	940/CR
4/m/1	3.6	AML, 2nd CR	BU 16, Thio 10, CY 120, ALG 30		no	0	—	no engraftment
4/m/2	3.7		Flu 200 mg/m^2, OKT3 3.63	2.5×10^4/kg day 50	yes	0	—	120, CR
5/f/1	4.0	ALL, 2nd CR after very early relapse	TBI, Thio 10, CY 120, ATG 60	1×10^4/kg day 100	yes	I*	relapse day +376	597/3rd CR
6/m/2	5.0	ALL, 3rd CR after MUD-BMT	BU 14, Thio 10, CY 120, ALG 30	5×10^4/kg day 49	yes	0	enterococci sepsis	died day 69/CR/ MOF, DIC
7m/1	11	ALL, 3rd CR	BU 16, Thio 10, CY 120, ATG 60		yes	I	legionella pneumonia	died/CR/ARDS
8/f/1	4.9	ALL, 2nd CR	TBI, Thio 10, CY 120, ALG 30		no	0	—	non-engraftment
8/f/2	5.0		OKT3 4.45		no	0	Candida sepsis	rejection
8/f/3	5.1		TLI, Flu 200 mg/m^2, ATG 60		yes	II	CMV infection renal toxicity IV	240/CR
9/m/2	8.6	ALL (t9;22), CR1, non-engraftment after MUD-BMT	Flu 220 mg/m^2, OKT3 2.45	2.5×10^4/kg day 43 5×10^4/kg day 65	yes	II*	CMV viraemia	121/CR
10/m/1	16.4	vSAA	TLI, No. Flu 100 mg/m^2, CY 100, ALG 30		yes	0	Parinfluenza pneumonia, *E. coli*-sepsis HSV-pneumonia	died MOF day 74
11/m/1	2.3	FHL	BU 16, Thio 10, CY 200, ATG 60		yes	I	—	632/NED
12/m/1	2.8	FHL	BU 16, Thio 10, CY 200, ATG 60		yes	0	—	450/NED
13/m/1	1.7	KS	BU 16, Thio 10, CY 200, ATG 60		yes	0	thiotepa toxicity III	died ARDS day 110
14/f/1	5.8	FA	TLI, CY 80, ATG 60,		no	0	pseudomonas sepsis	rejection
14/f/2	5.8		Thio 10, Mel 3,3, ALG 30		yes	0	thiotepa-toxicity IV	died MOF day 126

FHL, familial hemophagocytic lymphohistiocytosis; KS, Kostmann syndrome, congenital agranulocytosis; vSAA, very severe aplastic anemia; FA, Fanconi anemia; ARDS, acute respiratory distress syndrome: MOF, multiple organ failure; DIC, disseminated intravascular coagulation; MUD-BMT, matched unrelated donor-bone marrow transplantation; CR, complete remission; CP, chronic phase; PD, progressive disease; NED, no evidence of disease; ALG, rabbit antilymphocyte globulin; ATG, equine antithymocyte globulin; CY, cyclophosphamide; BU, busulphan; Flu, fludarabine; Mel, L-PAM, melphalan; Thio, thiotepa; TBI, total body irradiation (fractionated); TLI, total lymphoid irradiation; VP16, etoposide.
No. scheduled doses (Flu: 200 mg/m^2; CY: 200 mg/kg) reduced due to renal failure during *E-coli* sepsis.
*After donor lymphocyte infusion.

advanced leukemia or due to prior conditioning regimen for allogeneic SCT.

GVHD prophylaxis

Patients with leukemia did not receive any pharmacological GVHD prophylaxis. All other patients received 1.5 mg/kg cyclosporine A (CsA) intravenously twice daily commencing on day −1. When applicable, oral administration was preferred (3 mg/kg/day) and was tapered down if no GVHD occurred after T cell engraftment.

Conditioning regimens

Antithymocyte globulin (ATG) was administered daily from days −3 to −1. Nine patients received horse ATG at a daily dose of 20 mg/kg body weight (Lymphoglobulin, Pasteur-Merieux, Lyon, France). Five patients were treated with rabbit ATG, the daily dose was 5–10 mg/kg body weight (Thymoglobulin; Pasteur-Merieux). In a previous escalation study the administration of both ATG preparations (horse or rabbit) proved to be safe and effective.[10] All patients received additional myeloablative and immunosuppressive treatment with busulphan (BU) or fractionated total body irradiation (FTBI, 6 MV linear accelerator, 8×1.5 Gy on 4 consecutive days, dose rate 50 cGy/min, lung shielding at 10 Gy). 1 mg/kg BU was given orally every 6 h for 4 days (total dose 16 mg/kg). The patients with vSAA and FA received total lymphoid irradiation (TLI) (2×2.5 Gy within 12 h) instead of FTBI or BU. IN addition, 2×5 mg/kg thiotepa and 2×50–60 mg/kg cyclophosphamide (CY) were administered (Table 1). Conditioning after rejection in two patients consisted of rabbit ATG at a daily dose of 10 mg/kg given on days −4, −3 and −2, two doses of 5 mg/kg thiotepa on day −4 and 100 mg/m^2 melphalan on day −1 before the second PBSCT. Two patients who presented with non-engraftment received a murine monoclonal antibody to human CD3$^+$ cells (Orthoclone OKT3; Janssen-Cilag, Saunderton, UK) before the second transplantation. One of these patients experienced rejection of the second graft and was conditioned with TLI and fludarabine.

Supportive care

All patients were nursed in laminar air flow rooms with high-efficiency particular air filtration. They received non- absorbable antibiotics (paramomycin, vancomycin) for complete gut decontamination and oral amphotericin B. Furthermore, trimethoprime/sulfamethoxazole was given for *Pneumocystis carinii* prophylaxis. All patients had a double lumen Hickman line to enable infusion support and blood sampling. Intravenous immunoglobulin was given every second week. All blood products were irradiated (30 Gy) and filtered. Platelets were derived from cytomegalovirus (CMV)-negative single donors. All patients with myeloablative therapy received nasogastric tubes on day −1 to facilitate enteral nutrition and oral decontamination. In addition, all patients received parenteral nutrition until sufficient oral intake was possible. Acyclovir was given for herpes simplex prophylaxis from day −7 until discharge. Those patients with CMV-PCR evidence received ganciclovir instead of acyclovir. Hematopoietic growth factors were not administered prophylactically.

Donors

Parents were assessed for the degree of mismatch by HLA typing and mixed lymphocyte cultures. Class I HLA-A and HLA-B typing was performed by microcytotoxicity assays. Class HLA-II alleles were determined by PCR-sequence-specific oligonucleotide probe (PCR-SSOP) typing, including DRB1, DRB3 and DQB. The differing HLA loci and the donor characteristics are summarized in Table 2.

In 11 cases the father served as stem cell donor and the mother in three. The donor age ranged from 23 to 47 years (median 35.5 years). CMV serology was negative in six donors and positive in eight. After approval by a local ethical committee, consent to cytokine mobilization and apheresis procedure was obtained. All donors were stimulated with 10 μg/kg rhG-CSF (Filgrastim; Amgen, Thousand Oaks, CA, USA) beginning 4 days before the first apheresis. One to three leukaphereses were performed via peripheral venous access. The CS 3000+ (Baxter, Irvine, CA, USA) or the Cobe Spectra (Cobe BCT, Lakewood, CO, USA) were used for cell separating. The entire blood volume was processed three to five times for each collection, resulting in a total cell yield of median 5.3×10^{10} nucleated cells (NC, range 1.7–8.9). If a minimum of 20×10^6 CD34$^+$ cells/kg of recipient's body weight could not be obtained, stimulation was continued and additional leukapheres were performed. All donors were monitored carefully after the aphereses by evaluating blood and infection parameters.

CD34-positive selection

Positive selection to enrich CD34$^+$ cells was started immediately after leukapheresis and performed separately for each buffy using the Baxter Isolex 300i (Baxter, Irvine, CA, USA). The cells obtained from this selection procedure (10^8–10^9 NC in 100 ml) were purified further on the Super-MACS device (Miltenyi, Bergisch Gladbach, Germany).[11] After narrowing down by centrifugation at 400 *g*, the cells were incubated at a concentration of 3.3×10^8 cells per ml, with CD34-coupled microbeads (100 μl per 10^8 NC) for 30 min at room temperature. After one washing step in calcium- and magnesium-free phosphate-buffered saline (supplemented with 1% human serum albumin and 0.5% sodium citrate) to remove unbound monoclonal antibodies, the cells were resuspended in 50–100 ml of the same buffer and transferred to a 50 ml or a 120 ml syringe (Ivac, Newark, NJ, USA). A perfusor pump (Ivac) set at a speed of 500 ml/h was used to provide a constant flow over a large-size (xs+) column (Miltenyi). The latter was placed within the Super-MACS magnetic field and connected to a second syringe to collect unbound cells. Application of the cell suspension was followed by 50 ml of washing buffer before the column was removed from the magnet. Another pair of 50 ml syringes was used to flush the selected target cells out of the column. Quantitative flow cytometric (FACS) analyses were performed on the original material as well as the positive and negative cell fractions assessing all leukocyte subtypes. All FACS data were evaluated before transfusion of the CD34$^+$ cells. Upon processing the highly purified PBSC, a total volume of 50 to 100 ml was transfused.

Table 2 Donor characteristics, HLA constellation of donor/recipient, graft composition

Patient No. TX No.	*Patient/Donor relation donor age (years)*	*HLA donor*	*HLA recipient*	*CD34+ (×10⁶/kg recipient's weight)*	*CD3+ (×10⁴/kg recipient's weight)*	*CD19+ (×10⁴/kg recipient's weight)*
1/1	father/39	A3, A24/B13,B56/Cwl,Cw6/DR7,DR11	A24,A28/B58(17),B56/Cw1,Cw3/DR2,DR11 DRB1*15021,DRB1*1101/DRB3*0202/DRB5*0102/ DQB1*0601,DQB1*0301	28	7	2.8
1/2	father			58	12	8.7
2	father/31	A2,A11/B52(5),B18/Cw.,Cw./DR15(2),DR15(2)/ DR51,DR51/DQ6(1),DQ6(1)/ DRB1*1501,DRB1*1502/ DRB5*0101,DRB5*0102/DQB1*0601	A11,A11/B52,B70/Cw.,Cw./DR15(2),DR4/ DR53,DR51/DQ6(1),DQ7(3)/ DRB1*1502,DRB1*0403/DRB4*0103/ DRB5*0102/DQB1*0601,DQB1*0301	35.1	6.0	0
3	father/47	A2,A25/B18,B35/Cw4/DR2/DRB1*15,DRB1*16/ DRB5/DQB1*01	A2,A3/B18,B35/Cw4,Cw6/DR2/ DRB1*1501,DRB1*1601/ DRB5*0101,DRB5*0201/02/DQB1*0502, DQB1*0602	7.2	5	ND
4/1	mother/40	A3,A23(9)/B35,B44(12)/Cw4/DR7,DR13(6)/ DRB1*07,DRB1*13/DRB3*0202/DRB4/ DQB1*01,DQB1*02	A23(9)/B44(12)/Cw4/DR7,DR6/ DRB1*0701,DRB1*1302/DRB3*0301/DRB4/ DQB1*0604,DQB1*0202	7.5	0.4	0.2
4/2	father/33	A2,A23/B44,B51/DRB7/9/DRB13a3		38.8	9.0	1.2
5	mother/41	A*0202,A*3601/B*5301/C*0401/ DRB1*13,DRB1*15/DRB3*0301/DRB5/DQB1*01	A*0201,A*0202/B*4101,B*5301/C*04,C*17/ DRB1*07,DRB1*15/DRB4/DRB5/DQB1*01, DQB1*03	12.9	6.9	ND
6	father/40	A2,A24/B35,B61/Cw4/ DRB1*07,DRB1*11/DRB3/DRB4/DQB1*03	A2/B14,B61/Cw8/ DRB1*1101,DRB1*0701/DRB3*0202/DRB4/ DQB1*0301,DQB1*0201/02	22.2	3	ND
7	father/38	A*02,A*23/B*13,B*49/Cw*06,Cw*07/ DRB1*07,DRB1*15/DRB4/DRB5/DQB1*01, DQB1*02	A*02,A*23/B*38,B*49/Cw*07Cw*12/ DRB1*07,DRB1*16/DRB4/DRB5/DQB1*01, DQB1*02	16.4	4.7	0.4
8/1	mother/42	A1,A2/B62(15),B35/Cw3,Cw4/DR4,DR8/ DR53/DQ7(3),DQ8(3)/ DRB1*04,DRB1*0801/DRB4*0103/ DQB1*0301,DQB1*0302	A1,A1/B63(15),B35/Cw4,Cw7/DR16(2),DR8/DR51/ DQ5(1),DQ7(3)/ DRB1*0801,DRB1*160/DRB5*0202/ DQB1*0301,DQB1*0502	5.4	6.5	0.1
8/2	mother			14.8	3.3	2.6
8/3	father/33	A1,A2/B63(15),B60(40)/Cw3,Cw7/DR16(2),DR13(6)/ DR51,DR52/DQ5(1),DQ6(1)/ DRB1*1601,DRB1*1301/DRB3*0101/DRB5*0202/ DQB1*0502,DQB1*0603		21.5	11.0	ND
9	father/30	A2,A24(9)/B15,B60(40)/Cw7/DR15(2),DR8/ DRB1*08,DRB1*15/DRB5/DQB1*01	A2,A11/B60(40)/Cw7/DR8/ DRB1*0803/DQB1*0601	16.4	0.6	0.2
10	father/43	A1,A3/B7,B8/Cw7,Cw./DR15(2),DR17(3)/ DR51,DR52/DQ6(1),DQ7(3)/ DRB1*1501,DRB1*0301/DRB3*0101/ DRB5*01011/DQB1*0602,DQB1*0201	A1,A1/B8,B8/Cw7,Cw./DR13(6),DR17(3)/ DR52/DQ2,DQ7(3)/ DRB1*0301,DRB1*1303/DRB3*0101/ DQB1*0201,DQB1*0301	29.2	0.9	0.8
11	father/29	A2,A24/B35,56/Cw1,Cw4/DR7,DR6/ DRB1*13,DRB1*07/DRB3/DRB4/ DQB1*01,DQB1*02	A2,A24/B44,B56/Cw1,Cw5/DR11,DR7/ DRB1*07,DRB1*11/DRB3/DRB4/DQB1*02, DQB1*03	20.9	7.5	0.4
12	father/23	A11,A24/B35,B56/Cw1,Cw4/DR2,DR6/ DRB1*15,DRB1*13/DRB3/DRB5/DQB1*01	A23,24/B7,B35/Cw4,Cw7/DR4,DR6/ DRB1*04,DRB1*13/DRB3/DRB4/ DQB1*01,DQB1*03	32.5	1.1	2.0
13	father/31	A1.A23/B8,B49/Cw7/DR4,DR11/ DRB1*04/DRB1*11/DRB3/DRB4/ DQB1*03	A1,A2/B7,B8/Cw7/DR4,DR6/ DRB1*04;DRB1*13/DRB3/DRB4/ DQB1*01,DQB1*03	26.0	0.5	ND
14/1	father/44	A11,A19(33)/B18,B35/Cw4,Cw5/DR3	A24(9),A33(19)/B18,62(15)/Cw3,Cw5/DR3,DR7 DRB1*03011,DRB1*0701/DRB3*0202/DRB4/DQB1*02	24.3	0.6	ND
14/2	father			21.4	4.0	0.7

ND, not detectable.

Engraftment monitoring

Blood samples were analyzed daily between infusion of the transplant and hematopoietic reconstitution, using a routine three- or four-color flow cytometric (FACS) approach to quantify circulating leukocytes in PB of myeloablated patients (<100 NC/μl). Cell preparation for FACS analysis was performed as described elsewhere.[12] Chimerism analysis was performed by PCR amplification of highly polymorphic microsatellite markers (STR-PCR). DNA was extracted from samples containing 2×10^1–5×10^5 NC, by standard techniques, with minor modifications and subjected to PCR analysis with primers encompassing an informative short tandem repeat (STR) locus. After enzymatic amplification for 40 cycles under standard conditions, PCR products were separated on 12% polyacrylamide gels and were visualized using ethidium bromide or SYBR green staining (Molecular Probes, Leiden, The Netherlands). Mixed chimerism was assessed by optical methods.

Assessment of engraftment, toxicity and GVHD

Neutrophil recovery was defined as the first of 3 consecutive days with an absolute neutrophil count (ANC) $>0.5 \times 10^9$/l. Platelet recovery was defined as the 3rd day the platelet count exceeded 20×10^9/l, with no requirement for platelet transfusions within the following week. Diagnosis of GVHD was based on clinical and histological criteria. Grading of GVHD was performed according to the consensus criteria.[13] Treatment-related toxicity was scored as described elsewhere.[14]

Immunologic analysis

At selected time intervals, mononuclear cells were isolated from heparinized blood, phenotyped by standard direct immunofluorescence and functionally evaluated in microtiter proliferation assays (100 000 cells/well in 200 μl of X-VIVO 15 culture medium) following a 3-day stimulation course with phytohemagglutinin A (PHA, 1 μg/ml), a mixture of staphylococcal enterotoxin A and B (SEA/SEB, 100 ng/ml), or monoclonal antibodies recognizing CD2, CD2R and CD28 (OKT11 + VIT13, Leu28; 2.5 μg/ml and 0.5 μg/ml, respectively). The microtiter cultures were labeled with 0.5 μCi ^{3}H-thymidine and harvested 16 h later (proliferation assays), or supernatants were collected on day 3 and analyzed by sandwich ELISA for IL-2, IL4, IL10 and IFNγ content.

Donor lymphocyte infusion

Five patients with advanced leukemia received donor lymphocyte infusions (DLI) between days 43 and 100. We aimed at infusing 1–5×10^4 T cells per kg of recipient's body weight, and we used fresh unmanipulated citrated donor blood. At the time of DLI, no peripheral lymphocytes were detectable in the blood and no GVHD was apparent.

Results

Donors

After the first stimulation with 10 μg/kg rhG-CSF, a rapid increment of the ANC was observed in PB, but sufficient CD34$^+$ numbers were not mobilized before day 4. Side-effects included mild bone pain (66%), headache (66%), fatigue (22%) and itching (11%), but did not exceed WHO toxicity grade II. None of the donors took analgesics or discontinued filgrastim application. All leukaphereses were performed by peripheral venous access in an outpatient setting, and no severe side-effects were observed. Three parental donors insisted on a second stimulation with rhG-CSF after occurrence of non-engraftment or rejection. The second stimulation was as effective as the first course, and side-effects were comparably low. In two patients with graft failure the father was taken as PBSC donor instead of the mother. No delayed adverse effects of cytokine application have been detected so far.

Stem cell products

A median of 5.3×10^{10} NC (1.7–8.9) were collected in each apheresis. The percentage of CD34$^+$ leukocytes ranged from 0.27% to 2.0% (median 0.9%). After CD34-positive selection on the Isolex 300i, the median purity of CD34$^+$ cells was 89.6% (range, 57–95.4). The calculated recovery was 50.8% (range, 38.8–66.4). The median percentage of CD34$^+$ cells dropped from 32% (range, 16.5–43) to 2.1% (range, 0.7–7). After the second-step CD34-positive selection on the MACS device, the median purity of CD34$^+$ cells was 99.1% (range, 97.5–99.6). The final product contained a median of 21.5×10^6 (range, 5.4–58) CD34$^+$ and a median of 4.7×10^4 (range, 0.4–12) CD3$^+$ per kg of recipient's body weight.

Hematopoietic engraftment, immunologic reconstitution, relapse of leukemia

The appearance of monocytes and granulocytes in patients' blood indicated the beginning of hematopoietic engraftment. These cells always displayed donor genotype as identified by PCR analysis of FACS-sorted cells. The first day of a stable leukocyte count >200/μl was between days 8 and 15 (median day 10), and an ANC count >500/μl blood was reached between days 10 and 24 (median day 12). All patients required blood cell support, after engraftment transfusion independence for red cells was between days 6 and 83 (median day 25), and for platelets between days 6 and 72 (median day 23) (Table 3). The appearance of donor T cells varied between day 12 and day 144 (median day 22). Reconstitution of cellular immunity was monitored by *in vitro* stimulation of isolated mononuclear cells with mitogens, superantigens and monoclonal antibodies at several time points. Three patients had substantial numbers of circulating CD3$^+$ cells as early as day 100, which responded with vigorous proliferation and IL-2 production to mitogenic costimulation. The remaining evaluable patients reconstituted functionally between days 180 and 360.

One patient with ALL experienced leukemic relapse 376 days after haploidentical PBSCT, to date. She underwent chemotherapy and two courses of donor lymphocyte infusions from the original stem cell donor and is currently in complete remission.

All patients are complete chimeras in all hematopoietic cell lines as demonstrated by PCR analysis of FACS-sorted leukocytes.

Table 3 Engraftment kinetics, immune reconstitution

Patient No. TX No.	*ANC >500/μl day after PBST*	*Monocytes >50/μl day*	*NK cells >50/μl day*	*CD3+ >50/μl day*	*CD4+ >50/μl day*	*CD8+ >50/μl day*	*CD19+ >50/μl day*	*Platelets >20000/μl day*	*HGB >8 g/dl day*
1/1	NA	10	NA	NA	NA	NA	NA	NA	NA
1/2	17	16	20	144	60	128	56	72	83
2	24	18	22	79	NA	NA	49	33	23
3	12	8	19	50	144	144	34	12	12
4/1	NA	NA	NA	NA	NA	NA	NA	NA	NA
4/2	11	11	21	56	70	56	42	15	12
5	15	13	15	22	22	22	41	10	6
6	13	11	18	60	NA	NA	41	NA	NA
7	11	9	13	21	NA	NA	60	10	11
8/1	NA	NA	NA	NA	NA	NA	NA	NA	NA
8/2	23	10	ND	NA	NA	ND	NA	25	29
8/3	10	9	103	14	67	14	NA	54	55
9	11	10	35	84	84	84	57	98	17
10	11	11	18	NA	NA	NA	NA	NA	NA
11	10	6	13	13	22	22	48	34	29
12	12	13	13	22	13	17	78	6	30
13	16	12	19	12	19	28	40	NA	NA
14/1	12	9	NA	NA	NA	NA	NA	NA	NA
14/2	15	8	28	NA	NA	NA	NA	NA	NA

NA, not achieved; ND, not done.

Non-engraftment, rejection

Two patients who had received relatively low numbers of maternal PBSCs (both mothers mobilized few amounts of $CD34^+$ cells into the peripheral blood) had no detectable increment of donor hematopoiesis until days 20 and 30, respectively. In one patient (No. 8) we gave a PBSC boost after application of OKT3, as T cells of recipient phenotype were detectable. The mother of the second patient refused another stem cell harvest and we therefore had to transplant paternal stem cells. As we had to cross a different HLA barrier we added fludarabine to OKT3 and the patient achieved sustained engraftment.

Three patients rejected the graft on day 11, 14 and 34 after PCR-proven occurrence of donor myelopoiesis. The T cells detected by flow cytometry were of recipient genotype as documented by STR-PCR on sorted cells. The majority was positive for $CD8^+$. During the following days, the donor monocytes, granulocytes and reticulocytes disappeared from PB. PCR analysis of FACS-sorted BM aspirates confirmed the rejection. Two patients underwent a second conditioning regimen and were re-transplanted with purified PBSCs from the same donors. The second transplantation was successful in one case, leading to a stable tri-lineage engraftment, whereas the second patient died from multiple organ failure after myeloid engraftment (Table 1).

The third patient who rejected the second graft from her mother received TLI, CY, immunosuppression and PBSCs from her haploidentical father and finally achieved sustained engraftment.

Transplantation-associated complications

Although some of the patients had been heavily pre-treated, the conditioning regimens were generally well tolerated. In general, fever, skin rash and elevated CRP levels were observed during ATG treatment. All children developed oropharyngeal mucositis grade I–III and required systemic analgesia. Diarrhea was common during the first 2 weeks after PBSCT. Two patients developed generalized skin erythema and edema followed by epidermal desquamation (grade III and IV, respectively), as described after thiotepa application.[15] The onset time of toxicity was days 5 and 11, respectively, after thiotepa exposition and was a major contribution to the fatal course in our patient with FA (see below).

Six patients did not experience any severe infection during the early or late transplantation course. Five patients developed CMV antigenemia as demonstrated by virus nucleic acid (VNA)-PCR, and therefore received ganciclovir. One of these patients experienced life-threatening CMV pneumonia and was treated with extracorporeal membrane oxygenation and lung transplantation. Despite temporary clearance of CMV the new organ became infected and the patient eventually died due to respiratory failure. The patient with FA developed life-threatening enterosepsis with *Pseudomonas aeruginosa* on day 2, with necrosis on the labium major. She received a combination therapy of broad-spectrum antibiotics and in addition, leukocyte transfusions from prednisone-stimulated volunteer donors. In response to this treatment, she showed complete resolution of the infection with demarcation of the necrosis. However, she subsequently rejected the first graft and had to undergo a second transplantation which was complicated by severe skin toxicity from thiotepa, by capillary leakage and by renal failure. Although myeloid engraftment was evident, she died from multiple organ failure. One patient developed enterococci sepsis and succumbed due to multiple organ failure. The patient with vSAA not responding to any immunosuppression (ie ATG, CsA and prednisone), experienced life threatening parainfluenza-induced pneumonia and gram-negative sepsis with renal and pulmonary failure. He underwent PBSCT from his haploidentical father on the intensive care unit. Patient No. 8, who underwent three PBSCTs, developed candida sepsis after the second and CMV viremia after the third PBSCT. As she had proven resistance to ganciclovir, she received foscarnet and cidofovir and developed reversible renal failure.

Seven patients have not developed any signs of acute or

chronic GVHD so far. Four patients developed skin rash clinical stage ++ (overall grade I) and one patient stage +++ (overall grade II) when the first donor T cells became detectable in the circulation. The histology of skin biopsies was compatible with the features of acute GVHD, but not entirely conclusive. Nevertheless, erythema and maculopapular rash disappeared after treatment with prednisone. Three patients with leukemia developed acute skin GVHD clinical stage + or ++ between 32 and 46 days after infusion of $1–5 \times 10^4$ $CD3^+$ cells per kg of recipient's body weight as supplied prophylactically against leukemia relapse. It cannot definitely be proven that the mild skin GVHD was an effect of the initial SCT or if it was initiated by the DLI, since the DLI was performed relatively early in order to hinder an early relapse before T cell engraftment in patients with high relapse risk for leukemia. No liver or gut GVHD occurred in any patient within the observation period. None of the patients demonstrate any signs of chronic GVHD.

Discussion

Despite the increasing availability of unrelated volunteer BM donors, a substantial number of patients cannot find a suitable stem cell graft within an acceptable time. Although the transplantation of T cell-depleted, partially matched BM from relatives has a relatively long history,[16] it has been associated with a higher incidence of graft failure and severe infectious complications.[17] To overcome this obstacle the number of infused hematopoietic precursor cells was increased by adding PBSC from the same donor[18] to the BM grafts. For the donor, this procedure is associated not only with adverse effects such as stem cell mobilization by growth factors, but also with potential complications and discomfort during general anesthesia and BM harvesting. On the other hand, it was demonstrated that the transplantation of allogeneic PBSC from HLA-identical donors, whether T cell depleted or not, is associated with sustained engraftment and with decreased transfusion demand, but not with a higher incidence of acute GVHD compared to BM.[19] Therefore, our intention was to confirm that a trilineage engraftment is possible by transplanting exclusively PBSC obtained from haploidentical parental donors.[20]

Only three of all parents had a favorable HLA constellation (two HLA loci mismatched), and the criteria for donor selection (father or mother) were therefore CMV serology, younger age, higher body weight and the feasibility of aphereses from peripheral veins. All donors were monitored during stem cell mobilization and aphereses, and after harvesting. In accordance with others,[21] the procedure was well tolerated in all instances, and no severe side-effects occurred. Nevertheless, long-term follow-up and careful evaluation of risk factors associated with cytokine application and aphereses are requested for the donors' safety.

T cell-depleted BM has been applied widely as a prophylactic measure against GVHD. T cell depletion by rosetting techniques[22] and by the use of monoclonal antibodies[23] for prevention of acute GVHD were performed. Particularly in BMTs from HLA-mismatched donors this goal was hard to meet. Especially rejection,[24] Epstein–Barr virus (EBV)-associated B cell lymphoproliferative disorders[25] and relapse of leukemia during the time of T cell reconstitution[26] were sometimes the price that had to be paid for an effective T cell reduction. In our study we accomplished a highly effective T cell depletion by combining two CD34-positive selection procedures and additionally, ATG was given for preconditioning. The $CD34^+$ cell fraction contained a median of 5×10^4 T cells per kg of the recipient's body weight as determined by FACS analysis. The application of high doses of horse or rabbit ATG were tolerated without severe side-effects and could contribute to an additional graft rejection and GVHD prophylactic effect. In fact, with the methods applied, incidence of GVHD was low. It needs to be investigated within a larger cohort of patients whether this regimen is able to reduce the occurrence of leukemic relapse. Furthermore, because of our early, very exact engraftment monitoring, we could document not only the first signs of myeloid engraftment, but also rapidly prove the definite presence of recipient $CD8^+$ cells due to the FACS-sorted lymphocytes. We achieved myeloid engraftment due to rapid re-conditioning and transplantation of parental PBSCs in all patients with graft failure. Alas, one patient with DNA fragility succumbed due to organ toxicity. Our data demonstrate that even without BM cells, the myeloid engraftment was comparably fast with ANC $>500/\mu$l on median day 12. In addition, the appearance of lymphocytes occurred within 12 and 144 days (Table 3). The time up to T cell engraftment was independent not only of the T cell numbers transplanted, but also of the HLA constellation, the CMV status and the conditioning regimen. This early reconstitution might be influenced by the young age of the recipients and the therefore enhanced thymic regeneration but also due to the absence of late acute or chronic GVHD.

The conditioning regimen used in our pilot protocol had to meet the following requirements: (1) to enable engraftment with highly purified stem cells from HLA-mismatched family members; (2) to eradicate residual malignant cells in leukemia patients; (3) to minimize toxicity in very young children; and (4) to be available within a short time period to avoid dangerous complications during the waiting intervals for irradiation. In our heterogeneous group of patients it was not possible to apply a uniform conditioning regimen. In all leukemia patients the regimen-related toxicity after first PBSCT was low, although the children had advanced disease and had been heavily pre-treated. When using alternative donors BU-based regimens have been associated with an increased risk of graft failure.[27] Others have published data showing successful engraftment with an irradiation-free conditioning regimen.[28] In our study, one patient, who received a BU-based regimen, had no engraftment. This was also true for another patient conditioned with a TBI-containing regimen. We speculate, that this was due to relatively low numbers of transplanted $CD34^+$ cells which might have been below a critical threshold. Rejection occurred in two patients not conditioned with thiotepa. This drug seems to play an important role in conditioning for patients who receive an extensively T cell-depleted transplant.[29,30] After rejection both patients experienced sustained engraftment receiving melphalan and thiotepa but the toxicity of the second conditioning was fatal in one patient with FA.

Infectious complications were the most common cause of mortality in the post-transplant period in our patients. It is of interest that none of our patients died during the phase of agranulocytosis. Most problems occurred between days 30 and 80 when lymphocyte reconstitution was not evident. These observations suggest the need for long-term immunophenotypic monitoring as well as prolonged infection surveillance and prophylaxis.

As described in other publications[31] the relapse risk is impressively low during the observed period, despite the highly effective T cell depletion. Possibly, the pre-emptive DLIs given at a later period contributed to this. An additional

speculation is that of a higher GVL effect in the setting of major HLA disparity.

In summary, we could demonstrate that in our unselected group of patients, transplantation of high doses of purified PBSCs obtained from HLA-mismatched parents, enables rapid myeloid engraftment without severe acute or chronic GVHD. The conditioning regimen applied induced remission of leukemia refractory to chemotherapy in nine patients. We could also show that an irradiation-free conditioning regimen enables complete engraftment after haploidentical PBSCT. Using very close monitoring of the donor/patient chimerism, it was possible to detect graft failure at a very early stage and carry out the appropriate re-conditioning and subsequent SCT. Our study indicates that the methods introduced justify further investigations to optimize SCT from HLA-mismatched family donors. We suggest that this transplantation model should be further developed, since it is readily available for most patients who lack an HLA-matched donor and is possibly more economic in comparison to transplantations from unrelated donors.

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