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Separation and Further Characterization of Hematopoietic Cell Populations Based on Phenotypic and Biophysical Properties

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Rapid and effective CD3 T-cell depletion with a magnetic cell sorting program to produce peripheral blood progenitor cell products for haploidentical transplantation in children and adults

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BACKGROUND: Effective T-cell depletion is a prerequisite for haploidentical peripheral blood progenitor cell (PBPC) transplantation. This study was performed to investigate the performance of magnetic cell sorting–based direct large-scale T-cell depletion, which is an attractive alternative to standard PBPC enrichment procedures.

STUDY DESIGN AND METHODS: PBPCs were harvested from 11 human leukocyte antigen (HLA)-haploidentical donors. T cells labeled with anti-CD3–coated beads were depleted with a commercially available magnetic separation unit (CliniMACS, Miltenyi Biotec) with either the Depletion 2.1 (D2.1, n = 11) or the novel Depletion 3.1 (D3.1, n = 12) program. If indicated, additional CD34+ selections were performed (n = 6). Eleven patients received T-cell-depleted grafts after reduced-intensity conditioning.

RESULTS: The median log T-cell depletion was better with the D2.1 compared to the D3.1 (log 3.6 vs. log 2.3, $p < 0.05$) and was further improved by introducing an immunoglobulin G (IgG)-blocking step (log 4.5 and log 3.4, respectively). The D3.1 was superior to the D2.1 ($p < 0.05$) in median recovery of CD34+ cells (90% vs. 78%) and in median recovery of CD3– cells (87% vs. 76%). The median processing times per 10^{10} total cells were 0.90 hours (D2.1) and 0.35 hours (D3.1). The transplanted grafts (directly T-cell–depleted products with or without positively selected CD34+ cells) contained a median of 10.5×10^6 per kg CD34+, 0.93×10^5 per kg CD3+, and 11.6×10^6 per kg CD56+. Rapid engraftment was achieved in 10 patients. The incidences of acute graft-versus-host disease were less than 10 percent (Grade I/II) and 0 percent (Grade III/IV).

CONCLUSION: The novel D3.1 program with IgG blocking enables highly effective, time-saving large-scale T-cell depletion. Combining direct depletion techniques with standard CD34+ selection enables the composition of grafts optimized to the specific requirements of the patients.

In hematopoietic stem cell (HSC) transplantation, the occurrence of severe graft-versus-host disease (GVHD) is related to the degree of donor-recipient human leukocyte antigen (HLA) disparity.[1,2] The depletion of T lymphocytes from the graft is an effective method for preventing severe GVHD in HLA-mismatched transplantation and is an absolute prerequisite in three-loci haploidentical transplantation.[3,4]

Indirect T-cell depletion, based on the positive selection of CD34+ peripheral blood progenitor cells (PBPCs), has been the standard method for obtaining efficiently T-cell–depleted grafts containing high doses of purified CD34+ cells.[5,6] The positive selection technique, however, excludes a variety of immunocompetent cells that may play an important role in improving the outcome of HSC transplantation. The profound depletion of all lymphoid

ABBREVIATIONS: D2.1 = Depletion 2.1; D3.1 = Depletion 3.1; DTS = depletion tubing set; LSTS = large-scale tubing set; NK = natural killer; TNC(s) = total nucleated cell(s).

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cells results in a considerably delayed immune reconstitution leading to a high rate of infectious complications.[7] Furthermore, increasing knowledge on the role of alloreactive natural killer (NK) cells in exerting antileukemic[8] and engraftment-facilitating effects[9] suggests the advantage of a direct T-cell depletion strategy, retaining potentially important cells in the graft.

Previously available T-cell depletion techniques, primarily based on complement-mediated lysis[10] or elimination of unwanted cells by agglutination,[11] do not adequately deplete PBPCs of T cells and are, furthermore, time- and labor-intensive. Also, these methods do not meet up to Good Manufacturing Practice (GMP) standards. An immunomagnetic technique (CliniMACS system, Miltenyi Biotec, Bergisch-Gladbach, Germany) for efficient direct depletion of T cells from PBPC has recently been introduced[12] and further optimized aiming for a time-effective, best-possible large-scale cell separation, with the newly released Depletion 3.1 (D3.1) program representing the latest development in the field.

In the current study, we investigated the performance of two CliniMACS direct T-cell depletion programs, Depletion 2.1 (D2.1) and D3.1, and utilized both direct and indirect T-cell depletion techniques, aiming to provide the best possible PBPC graft suitable for haploidentical transplantation in children and adults.

MATERIALS AND METHODS

Mobilization and collection of PBPCs

Between March 2005 and May 2006, PBPCs of 11 haploidentical family donors were mobilized by administration of granulocyte–colony-stimulating factor (G-CSF; 10 μg/kg/day; Neupogen; Amgen, Thousand Oaks, CA) as previously described.[13] Large-volume leukapheresis was performed with a commercially available apheresis system (COBE Spectra, Gambro BCT, Lakewood, CO) and its mononuclear cell (MNC) program, Version 7.0, starting on Day 4 of G-CSF administration. Peripheral blood CD34+ cell counts were monitored daily during leukapheresis.

Cell preparation

PBPCs were prepared and separated with the CliniMACS system according to the manufacturer's protocol. PBPCs were stored overnight (maximum 24 hr) at room temperature. The cells were concentrated to a maximum volume of 200 mL and washed once with CliniMACS buffer (phosphate-buffered saline–ethylenediaminetetraacetate; Miltenyi Biotec) supplemented with 0.5 percent human serum albumin (albumin Behring, ZLB Behring, Marburg, Germany) to remove platelets (PLTs). The cells were incubated with either anti-CD3 (OKT3) or anti-CD34, conjugated to magnetic microbeads (Miltenyi Biotec). One vial (7.5 mL) of CD3 beads per 4×10^{10} total cells or one vial of CD34 beads (7.5 mL) per 6×10^{10} total cells was used. The cells were incubated under continuous agitation at room temperature for 30 minutes, washed once (anti-CD3 protocol) or twice (anti-CD34 protocol) with CliniMACS buffer, and resuspended in 150 mL of buffer per 4×10^{10} total cells (anti-CD3 protocol) or per 6×10^{10} total cells (anti-CD34 protocol). The manufacturer's original protocol was eventually modified by addition of 0.15 percent human immunoglobulin G (IgG; Gammagard S/D, N.V. Baxter, S.A., Lessines, Belgium) before anti-CD3 microbead incubation, for blocking of nonspecific binding.

Cell processing

The cells were processed with the fully automated CliniMACS device (Software Version 2.40, Miltenyi Biotec). Direct T-cell depletion was performed with one of two different procedures, the D2.1 program with the large-scale tubing set (LSTS, 162-01) separation column (labeled cell capacity, 2×10^{10}; maximum processing time, 5 hr) or the D3.1 program with the depletion tubing set (DTS, 261-01) separation column (labeled cell capacity, 4×10^{10}; maximum processing time, 2.5 hr). Both separation programs automatically load the cells onto the separation column in adequately sized fractions ("staged loading"), not to exceed the binding capacity of the column. The difference between the programs lies in the number of cells that are loaded onto the column per loading stage as well as the number of sequential loading stages, that is, higher numbers of cells per loading stage and less frequent loading for the D3.1 compared to the D2.1 program. Furthermore, the D3.1 program performs a second "sensitive loading stage" after the first round of rapid bulk T-cell depletion to deplete any remaining labeled cells. Positive selection was performed with the LSTS (162-01) with the CD34 Selection 2 program.

Flow cytometric analysis

Aliquots were taken before and after cell separation for blood cell counts, with an automated hematology analyzer (Sysmex KX-21N; Sysmex, Kungsbacka, Sweden) and for flow cytometric analysis of CD3, CD34, and CD56 based on the manufacturer's protocol (Miltenyi Biotec), with a four-color flow cytometer (FACSCalibur, BD, Beckton Dickinson, San Jose, CA). A total of 50,000 events were acquired for all antigens except for the CD3 in the depleted fraction. Here, 1×10^6 events were acquired from two separated samples for T-cell analysis. Fluorochrome-labeled monoclonal antibodies were used as follows: anti-CD34–phycoerythrin (PE) and anti-CD45–fluorescein isothiocyanate (FITC) for the CD34+ cell determination; anti-CD3–FITC, anti-CD45–peridinin

chlorophyll protein, and the combination of the PE-labeled antibodies anti-CD19, anti-CD14 (all BD), and anti-CD15 (Becton Dickinson, PharMingen, San Diego, CA) for CD3+ cell determination; and anti-CD56 FITC (BD) and anti-CD3 PE (DakoCytomation Denmark A/S, Glostrup, Denmark) for CD56+ cell determination. Antibodies for detection and selection-depletion were chosen such that they bind to different epitopes and thus the selection-depletion antibody did not interfere with the analysis of the final product. In CD34+ selection, the microbeads are conjugated to the anti-CD34 QBEND 10 (Miltenyi Biotec), whereas Clone 8G12 (BD) is chosen for detection of CD34+ cells.[14] For CD3 determination the *SK7* clone (BD) was used to minimize interference with the microbead-conjugated OKT3 antibody (Miltenyi Biotech) used in the direct T-cell depletion procedures.[12] A sequential T-cell gating strategy was employed for analysis of the PBPC product before (Fig. 1AI) and after (Fig. 1B) depletion, respectively.

Transplantation procedures

Eleven patients received haploidentical grafts after reduced-intensity conditioning (Table 1). Separated PBPCs were infused, fresh or after cryopreservation, with a target cell dose of at least 5×10^6 CD34+ cells and not more than 1×10^5 CD3 + cells per kilogram of recipient body weight. Chimerism was assessed utilizing short tandem repeat–polymerase chain reaction analysis of PBPC subsets (T, B, and myeloid cells).

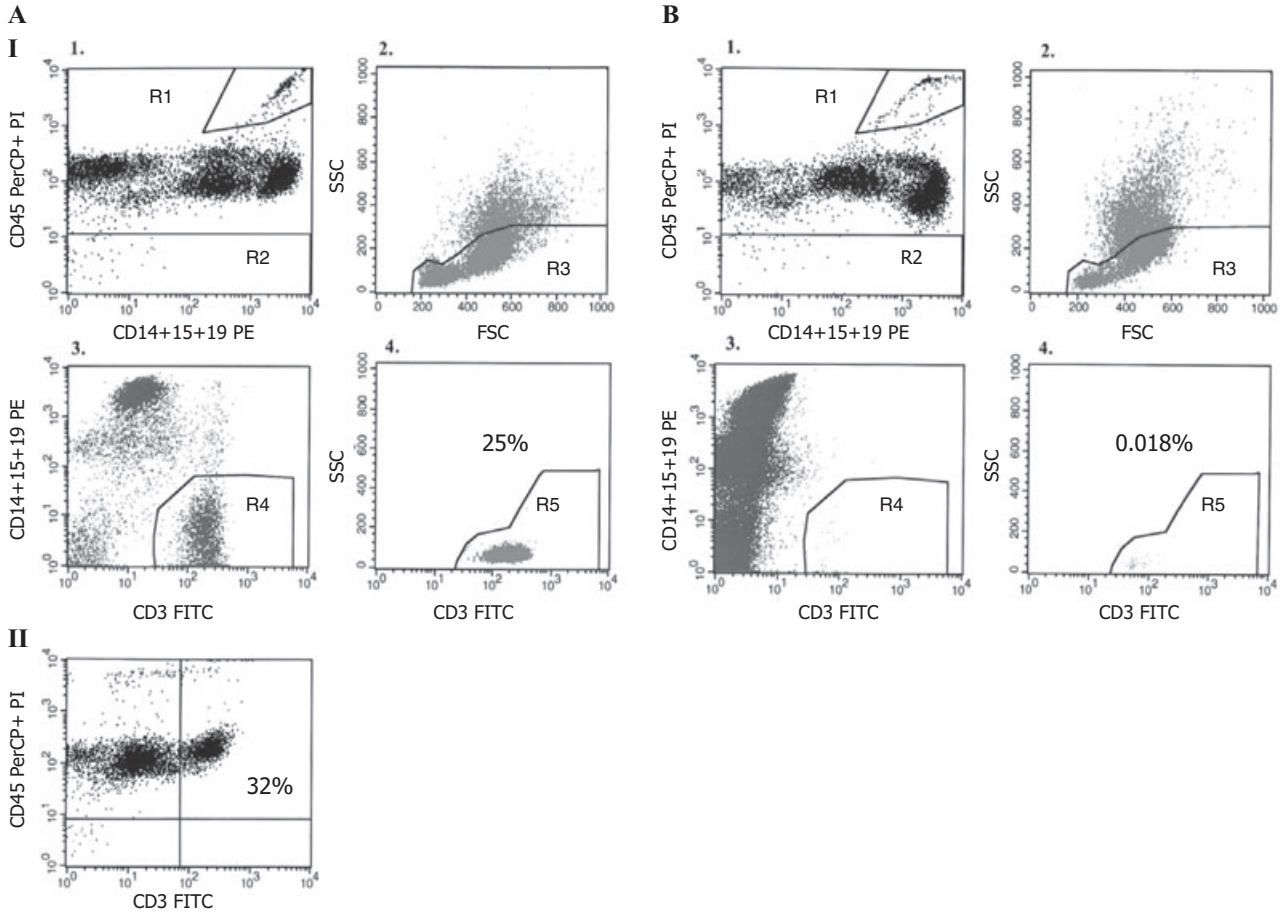

Fig. 1. (AI) CD3 determination of the PBPC product before depletion, with a sequential T-cell gating strategy. The exclusion dye propidium iodide (PI) is used to exclude dead cells. (Plot 1) PI+ dead cells (R1) and CD45– (R2) non –white blood cells (WBCs) are and excluded from analysis. (Plot 2) Viable WBCs are displayed, lymphocytes and monocytes (R3) are gated based on scatter properties. (Plot 3) Viable lymphocytes and monocytes are displayed, the T-cell marker (CD3) is plotted against markers for monocytes (CD14), granulocytes (CD15), and B cells (CD19). (Plot 4) The CD3+, low side scatter (SSC) population is further defined and enumerated (25% CD3+ cells). (AII) Ungated CD3 analysis, not excluding dead cells and other unspecifically stained cells, results in false-positive higher CD3 counts (32% CD3+ cells). (B) For CD3 determination of the T-cell–depleted product, the T-cell gating strategy described in (A) is applied (0.018% CD3+ cells). PerCP = peridinin chlorophyll protein.

TABLE 1. Patient data and transplantation outcome*

UPN	Age (years)	Diagnosis and status at transplantation	Donor	Engraftment	Acute GVHD
1	56	AML (M4), CR 2	Child	Sustained	No
2	40	MDS, not treated	Sibling	Sustained	No
3	11	Neuroblastoma, Stage IV	Parent	Sustained	No
4	7	Neuroblastoma, Stage IV	Parent	Rejection, regraft failure	No
5	8	Neuroblastoma, Stage IV	Parent	Sustained	No
6	5	Neuroblastoma, Stage IV	Parent	Sustained	No
7	7	Neuroblastoma, Stage IV	Parent	Sustained	No
8	13	AML, second relapse	Parent	Sustained	No
9	8	High risk T-ALL, CR 2	Parent	Sustained	No
10	50	AML, third relapse	Child	Sustained	Grade I
11	43	CML, accelerated phase	Sibling	Nonengraftment, regraft failure	No

* Pediatric patients (UPNs 3-9) received a reduced-intensity conditioning (fludarabine, thiotepa, and melphalan), and a single dose of rituximab.
Orthoclone OKT3, mycophenolate mofetil, and steroids were given. Neuroblastoma patients received high-dose radionuclide therapy, with a single intravenous infusion of 10,000 MBq [^{131}I]metaiodobenzylguanidine. Adult patients (UPNs 1, 2, 10, 11) received a reduced-intensity conditioning (fludarabine, cytarabine, melphalan) and a single dose of rituximab. Thymoglobulin, orthoclone OKT3, and mycophenolate mofetil were given.
Abbreviations: AML = acute myeloid leukemia; CML = chronic myeloid leukemia; CR = complete remission; MDS = myelodysplastic syndrome; T-ALL = T-cell acute lymphoblastic leukemia; UPN = unique patient number.

Presentation of results and statistical analysis

Data are presented as median (range) or as mean ± standard deviation (SD). Statistical analysis was performed with the Wilcoxon log rank sum test, and a p value of less than 0.05 was considered significant.

RESULTS

PBPC collection

A total of 26 leukapheresis procedures were performed on 11 donors. Sufficient numbers of PBPCs for grafting were obtained by one (n = 2) or two leukapheresis procedures performed on consecutive days (n = 8). One donor had to be remobilized for a third harvest to obtain sufficient numbers of PBPCs. One donor underwent an additional harvest without prior mobilization aiming to increase the number of T cells in the final graft. Three donors were remobilized and harvested by one (n = 2) or two (n = 1) leukapheresis procedures on consecutive days, to obtain cells for PBPC and immune boost in 1 patient and for a second transplant in 2 patients, respectively. The volume of blood processed in each procedure was 14.8 ± 2 L (mean ± SD). A median count of 56.4 circulating CD34+ cells per μL at the time of harvest allowed the collection of 9.8×10^6 CD34+ cells (median) per kilogram of recipient body weight by a single apheresis procedure (Table 2).

TABLE 2. PBPC mobilization and collection*

Donor peripheral blood (before apheresis)	
TNCs ($\times 10^9$/L)	44.7 (28.1-59.1)
CD34+ cells/μL	56.4 (14.7-113.4)
PLTs ($\times 10^9$/L)	168 (84-289)
PBPC product	
TNCs ($\times 10^{10}$)	6.4 (2.7-8.6)
MNCs (%)	66 (40-85)
CD34+ cells (%)	0.68 (0.19-2.18)
CD34+ cells/μL	1128 (314-3460)
CD34+ cells/kg recipient ($\times 10^6$)	9.8 (2.6-37.9)
CD3+ cells (%)	24 (11-44)
CD3+ cells/kg recipient ($\times 10^6$)	324 (120-1266)
PLTs ($\times 10^9$)	565 (109-1094)

* Data are presented as median (range) of 25 leukapheresis procedures.

PBPC processing

PBPCs from 25 of 26 apheresis products were processed within 24 hours after collection, according to the manufacturer's recommendation. Depending on cell content before processing and expected yield, apheresis products were either processed in one run on the CliniMACS device or split in fractions and run separately. This resulted in a total of 23 direct T-cell depletion procedures, with either the D2.1 (n = 11) or the D3.1 program (n = 12) and an additional six CD34+ selection procedures. Human IgG (Gammagard) blocking of unspecific binding was introduced in the later D2.1 (n = 5/11) and D3.1 (n = 8/12) procedures.

Cell preparation before depletion-selection. The characteristics of the apheresis products before and after preparation for the depletion and selection procedures are given in Table 3. In general, cell viability before and after cell preparation was good with a median viability of approximately 95 percent. The recovery of total nucleated cells (TNCs) and CD34+ cells reached a median of more than 90 percent in the PBPC products. PLTs were more efficiently depleted (80% vs. 94%, $p < 0.05$) in the PBPC products prepared for CD34+ selection, which is due to the additional washing step that is included in the protocol.

TABLE 3. PBPC preparation before separation*

Product	Cell preparation before CD3+ D2.1/D3.1 (n = 23)	Cell preparation before CD34+ selection (n = 6)
Collected PBPC product†		
Viability (%)‡	95.0 (87.4-97.9)	94.2 (85.4-96.0)
TNCs ($\times 10^{10}$)	4.5 (1.8-7.5)	5.6 (4.0-7.7)
PLTs ($\times 10^9$)	433 (118-1080)	608 (325-822)
Washed PBPC product§		
Viability (%)	94.6 (82.4-98.2)	94.8 (87.3-96.8)
Recovery of TNCs (%)	91 (76-100)	96 (88-97)
Recovery of CD34+ cells (%)	100 (32-100)	95 (84-100)
PLTs ($\times 10^9$)	74 (0.2-250)	39 (6-69)
PLT depletion (%)	80 (72-100)	94 (90-98)

* Data are presented as median (range) of a total of 25 apheresis products.
† Measurements were performed on harvested PBPC products after overnight storage (maximum, 24 hr).
‡ Viability was determined with PI in flow cytometry analysis.
§ Measurements were performed on PBPC products immediately before depletion-selection, that is, after all necessary washing and incubation steps.

Depletion of CD3+ T cells. Apheresis products were directly T-cell–depleted with the D2.1 or the D3.1 program on the CliniMACS device. The median log T-cell depletion rate was significantly better with the D2.1 compared to the D3.1 (log 3.6 vs. log 2.3, $p < 0.05$) as shown in Table 4. Introducing the use of a blocking step to prevent unspecific binding significantly ($p < 0.05$) improved the performance of both depletion programs, with median log T-cell depletion rates of 4.5 (D2.1) and 3.4 (D3.1). The number of residual T cells after depletion was below the clinical limit of 1×10^5 CD3+ per kg in all but one of the D2.1 runs without blocking and in all of the D2.1 runs including blocking. This threshold was, however, exceeded in all of the D3.1 runs that were performed without prior blocking (median, 27.2×10^5/kg; range, 12.1×10^5-61.3×10^5/kg). In contrast, blocking increased T-cell depletion efficacy in D3.1 depletion such that in only one of the eight runs were residual T cells above the threshold of 1×10^5 CD3+ per kg. Although in this particular run, the product was intended for a retransplantation and, thus, the clinical limit of residual T-cells was set higher. The median TNC count before D3.1 depletion without blocking was significantly higher ($p < 0.05$), however, compared to the D3.1 depletion runs including blocking (6.2×10^{10} vs. 3.4×10^{10}). The same holds true for the median number of PLTs (136×10^9 vs. 36×10^9) and, certainly, this must be taken into consideration when comparing the effectiveness of the procedures. Noteworthy, PLT counts (250×10^9) were also very high in the case of the D2.1 run that failed to deplete the CD3 cells below the limit despite prior blocking (Table 4).

Recovery of CD34+, CD3–, and NK cells. The D3.1 program was superior ($p < 0.05$) compared to the D2.1 as well as compared to CD34+ selection with regard to the median recovery of CD34+ PBPCs (90, 78, and 61%, respectively). Furthermore, the D3.1 program had a better median recovery of TNCs than the D2.1 (87% vs. 76%). No significant differences were observed for NK-cell recovery rates, although there was a tendency in favor of the D3.1 over the D2.1 (71% vs. 68%). Recovery parameters were not influenced by the use of blocking (Table 4).

Processing time. The processing time on the CliniMACS device differs with the specific separation program used and is dependent on the number of cells processed. For D2.1, D3.1, and CD34+ selection procedures, the median processing time per 10^{10} TNCs was 00.90 hours (range, 0.68-1.05 hr), 0.35 hours (range, 0.29-0.42 hr), and 0.17 hours (range, 0.12-0.31 hr), respectively.

Processing of PBPCs after prolonged storage. For logistical reasons, fractions of two apheresis products and one additional apheresis product (1/26) were stored and processed on the second day after collection, that is, exceeding the maximum 24-hour storage recommended by the manufacturer. The TNC viability after prolonged storage was poor (43%-77%), and the influence of cell viability on performance was reflected in poor depletion results, with log T-cell depletion rates as low as 1.5.

Graft composition

Fourteen grafts, consisting of 28 CliniMACS processed PBPC products, were infused. The grafts were composed of directly T-cell–depleted cells (D2.1, D3.1), plus an additional fraction of positively selected CD34+ cells in 7 cases (Fig. 2). The median infused cell doses per kilogram of recipient body weight were as follows: CD34+, 10.5×10^6 per kg (range, 5.5×10^6-22.0×10^6/kg); CD3+, 0.93×10^5 per kg (range, 0.14×10^5-2.94×10^5/kg), and CD56+, 11.6×10^6 per kg (range, 0.2×10^5-77.0×10^6/kg). Positively selected CD34+ cells were added to the grafts when the directly T-cell–depleted products 1) contained insufficient numbers of CD34+ cells or 2) could be transplanted only in part due to excessive numbers of residual T cells. Four of 32 CliniMACS-processed PBPC products (3 D3.1, 1 D2.1) were not used as grafts, but cryopreserved and stored for future possible infusion.

Transplantation outcome

All patients but 1 achieved primary engraftment with a median of 13 days (range, 10-20 days) to a neutrophil count of more than 0.5×10^9 per L and a complete donor chimerism in the peripheral blood myeloid cell subset at 14 days. A second patient rejected the graft. In 3 patients, a mixed chimerism was observed and successfully

TABLE 4. Separation procedures*

PBPCs	D2.1 (LSTS) Without Gammagard	D2.1 (LSTS) With Gammagard	D2.1 (LSTS) All	D3.1 (DTS) Without Gammagard	D3.1 (DTS) With Gammagard	D3.1 (DTS) All	CD34+ selection
Washed PBPCs							
Viability (%)	94 (82-98)	95 (90-96)		95 (84-96)	95 (90-98)		95 (87-97)
TNCs ($\times 10^{10}$)	4.7 (1.6-5.9)	3.1 (2.4-7.2)	3.7	6.2 (5.9-6.9)	3.4 (2.9-6.1)	4.4	5.3 (3.5-7.3)
CD3+ cells (%)	26 (13-42)	21 (16-34)		26 (20-45)	25 (16-34)		33 (11-37)
CD34+ cells (%)	1.07 (0.18-2-30)	1.07 (0.40-1.62)		0.85 (0.44-1.18)	0.61 (0.35-1.62)		1.12 (0.72-1.47)
PLTs ($\times 10^9$)	96 (23-250)	31 (20-39)		136 (121-173)	36 (0.2-99)		39 (6-69)
T-cell–depleted PBPC							
Viability (%)	95 (88-97)	94 (87-96)		94 (89-95)	94 (90-98)		99 (99-99)
Recovery							
CD34+ cells (%)	82 (66-94)	78 (58-84)	78†	92 (87-97)	89 (84-100)	90†	61 (52-75)
TNCs (%)	80 (69-90)	69 (59-82)	76†	87 (77-93)	88 (78-92)	87†	NE‡
CD56+ cells (%)	64 (51-99)	68 (54-70)	68†	70 (12-85)	71 (49-85)	71†	NE
T-cells							
Log depletion	3.6 (2.0-4.3)	4.5 (4.1-5.1)		2.3 (2.2-2.7)	3.4 (3.0-3.6)		5.0 (4.6-5.3)
CD3+ cells (%)	0.013 (0.001-0.385)	0.001 (0.0002-0.003)		0.279 (0.061-0.357)	0.018 (0.011-0.024)		0.034 (0.021-0.079)
CD3+ cells/kg ($\times 10^5$)	0.6 (0.3-40.1)	0.03 (0.01-0.10)		27.2 (12.1-61.3)	0.6 (0.3-2.9)		0.05 (0.03-0.11)
Purity							
CD34+ cells (%)	1.37 (0.30-3.31)	1.42 (0.52-2.48)		1.38 (0.57-2.02)	0.86 (0.46-2.21)		91 (85-98)

* Data are presented as median (range).
† Recovery of PBPCs (CD34+), CD3– cells (TNCs), and NK cells (CD56+) were not influenced by the use of blocking.
‡ NE = not evaluated.

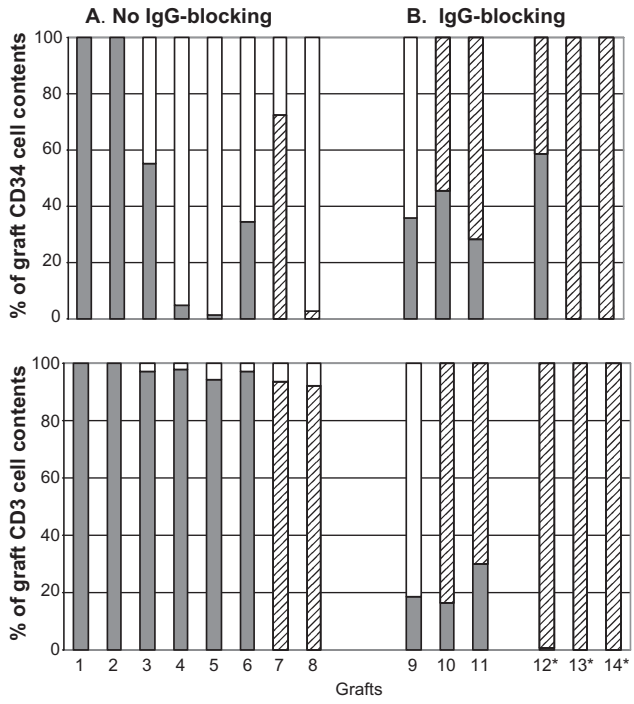

Fig. 2. The relative contribution (%) of directly (■, D2.1; ▨, D3.1) and indirectly depleted (□, CD34+-selected) PBPC products to graft CD34+ and CD3+ cell contents, respectively. (A) Before the introduction of the IgG-blocking step, most grafts were only partly composed of directly T-cell–depleted PBPC products to stay below the clinical limit of CD3+ cells. (B) After the introduction of the IgG-blocking step, the majority of grafts were exclusively composed of directly T-cell–depleted PBPC products. *Grafts used for PBPC and immune boost or for a second transplant.

reversed by giving donor lymphocyte infusions. Thus, sustained engraftment, that is, a stable myeloid complete donor chimerism, was achieved in 82 percent (9/11) of the patients, with a median follow-up of 12 months (range, 4-22 months). The median time to achieve more than 100 CD3+ and CD4+ cells per µL was after 57 days (range, 49-85 days) and 70 days (range, 42-119 days), respectively. The median time to achieve more than 300 CD56+/16+ cells per µL was after 21 days (range, 20-35 days). CD19+ cells were not detectable during the first 3 months after HSC transplantation due to in vivo B-cell depletion with rituximab. One patient experienced acute GVHD Grade I. Viral activation (cytomegalovirus in 8, adenovirus in 4, BK/JC virus in 6, and herpes zoster virus in 1) necessitated preemptive therapy in all patients. Epstein-Barr virus infection did not occur (Table 1).

DISCUSSION

The major obstacles of rejection and severe GVHD in HLA-mismatched transplantation can be largely

overcome by high numbers of PBPCs combined with a profound depletion of donor T cells.[15-17] Adopting the approach of direct T-cell depletion in haploidentical graft engineering, thereby preserving an array of immunocompetent cells that are unavoidably lost when applying indirect T-cell depletion methods, may help to improve upon the current major problems of transplant-related mortality (TRM), that is, disease relapse and infection.[18] Based on recently published protocols for patients at high risk of TRM,[19,20] we have developed a haploidentical HSC transplantation protocol with reduced-intensity conditioning regimen and transplantation of directly T-cell–depleted grafts.

Herein, we report on our results of direct T-cell depletion with the CliniMACS device. In addition to the D2.1 program (LSTS separation column), we have evaluated the recently released D3.1 program (DTS separation column) that was developed for processing of higher cell numbers in shorter processing time while retaining the depletion efficiency of the D2.1. In our hands the D3.1 was indeed superior with regard to processing time and also with regard to the recovery of CD34+ cells (90%) and TNCs (87%). This helped to minimize PBPC loss and to retain a substantial number of effector cells in the graft, that is, NK cells with possible antileukemic[8] and engraftment-facilitating[9] potential. Furthermore, monocytes, granulocytes, and T-cell precursors are not removed by the procedure, which may help in preventing infections and hasten immune reconstitution. The T-cell depletion performance of the D3.1 was much less efficient (log 2.3) than what has been observed by us and others with the D2.1 program.[12,21] The introduction of a blocking step, however, which was not part of the original protocol, improved T-cell depletion significantly (log 3.4), indicating that unspecific binding of cells to anti-CD3 microbeads considerably impaired depletion. Unspecific CD3 staining (clone SK7) is generally evident in flow cytometric analysis of PBPC samples because sequential T-cell gating gives lower CD3 counts compared to ungated CD3+ events, which also include dead cells (Fig. 1AII). In the CliniMACS depletion procedure, an underestimation of the actual number of anti-CD3 microbead-binding cells to be processed could possibly result in exceeding the capacity of the separation column. Furthermore, unspecific binding of cells to anti-CD3 microbeads may leave an insufficient number of beads available for specific binding, allowing unlabeled or weakly labeled T cells to escape the depletion process. With the D2.1, cells are processed at a slower rate thus allowing a wider safety margin regarding a possible overloading of the column. It also gives more time for weakly labeled T cells to be retained to the column, resulting in a more efficient T-cell depletion (log 3.6). Adding the blocking step to the D2.1 program results in a highly effective method for direct T-cell depletion (log 4.5), which can be useful in situations where T-cell numbers in the final graft may be limiting, for example, when depleting a second or third PBPC product, aiming to increase CD34+ cells numbers in the final graft, or in case of large donor-recipient weight differences (adult donor, pediatric recipient).

PLT contamination has been reported to have a negative impact on CD34+ cell recovery in CD34+ selection.[22] In our study, high numbers of PLTs, generally seen in PBPC products harvested with the COBE Spectra MNC program,[23] were efficiently depleted by manual cell wash according to the manufacturer's protocol. Notwithstanding, all PBPC products that were suboptimally T-cell–depleted were relatively rich in PLTs before cell separation, which may indicate a negative impact of PLT contamination also on T-cell depletion performance. As indicated in our study, an additional washing step in the cell preparation process may improve PLT depletion without affecting TNC and CD34+ cell recovery. Another important observation in our study was the impact of poor cell viability on the outcome of cell selection, which translated into insufficient T-cell depletion in D2.1 and D3.1 procedures.

Our current strategy for haploidentical graft engineering is to primarily apply a direct depletion technique. The choice between the D3.1 and D2.1 programs is made considering the specific requirements of the patient and the estimated numbers of CD3+ cells per kg and CD34+ cells per kg in the target fraction, which are based on the cell counts of the start product, the weight of the recipient, and the expected performance of the available methods. A blocking reagent is used in all direct depletion procedures and PBPC products are processed within a maximum of 24 hours.

Sustained primary engraftment was observed in 82 percent (9/11) of the patients. The one patient who did not achieve primary engraftment was transplanted in an accelerated phase of chronic myeloid leukemia, which per se is a risk factor for graft rejection. The incidence of acute GVHD was low and no severe acute GVHD was observed. The high TRM usually observed in haploidentical transplants is primarily linked to delayed immune recovery and impaired response to pathogens.[15-18,24] In adults, T-cell reconstitution after transplantation depends for months on peripheral expansion of mature T cells in the graft because thymic output of new T cells is extremely poor.[25] Consequently, patients who receive T-cell–depleted grafts are highly susceptible to viral infections. In this study immune reconstitution was rapid and compared favorably to previous reports with directly T-cell–depleted grafts.[7,20] This may be attributed to the remaining facilitating effector cells in the graft. Indeed, studies comparing CD34+ selection to CD3/CD19 depletion in haploidentical transplantation indicate that the latter is advantageous with regard to immunrecovery.[7]

The impact of CD3+ depletion on specific T-cell subsets in the graft and in T-cell reconstitution after transplantation is an important question. The CD4+ CD25+ FOXP3+ regulatory T-cell population, which may be an important player in exerting graft-versus-leukemia effects, is of particular interest for further studies.[26,27]

We conclude that the recently available D3.1 program offers a large-scale, time-saving method for direct T-cell depletion, with excellent recovery of CD34+ cells and, with the addition of a blocking reagent, an efficient reduction of T-cell numbers. The flexibility of the CliniMACS technology enables the composition of grafts according to the specific requirements of the patient.

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