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ORIGINAL ARTICLE

Brigitte Kircher · Dietger Niederwieser · Anne Gächter · Karla Lätzer · Günther Eibl · Günther Gastl · David Nachbaur

No predictive value of cytotoxic or helper T-cell precursor frequencies for outcome when analyzed from the graft after stem cell transplantation

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Abstract The predictive value of limiting dilution analyses (LDA) measuring cytotoxic and helper T-lymphocyte precursor (CTLp and HTLp) frequencies for outcome after stem cell transplantation (SCT) is still a matter of debate. One reason may be that CTLp and HTLp frequencies are determined in peripheral blood mononuclear cells (PBMC) and this responder cell population does not reflect the cell type composition of the graft. We assessed whether CTLp and HTLp LDA can predict complications after human leukocyte antigen (HLA)-identical SCT when CTLp and HTLp frequencies are analyzed in PBMC of the respective stem cell graft [bone marrow (BMMC) or granulocyte colony-stimulating factor (G-CSF)-mobilized PBMC] and compared to PBMC of PB. Host-specific CTLp frequencies measured in 25 patients and HTLp frequencies analyzed in 6 patients were low in all responder cell sources. CTLp and HTLp frequencies seen against HLA-mismatched unrelated third-party cells were high, but third-party-specific CTLp and HTLp frequencies were lower in G-CSF-PBMC than in PBMC (p=0.047 for CTLp frequencies). Host-specific CTLp frequencies analyzed in all responder cell sources did not predict acute or chronic graft-versus-host disease (GVHD). Lower CTLp frequencies were detected in all responder cell sources from patients who relapsed after SCT than in patients without relapse, but the differences between both groups were statistically significant only in PBMC. In conclusion, a significant correlation was detected only between relapse and CTLp frequencies measured in PBMC. The lower frequency of third-party-specific cells in G-CSF-PBMC indicates that the mobilization procedure with G-CSF itself may influence results.

Keywords CTLp frequencies · HTLp frequencies · GVHD · SCT · G-CSF

Abbreviations BMT: Bone marrow transplantation · CTLp: Cytotoxic T-lymphocyte precursor · G-CSF: Granulocyte colony-stimulating factor · GVHD: Graft-versus-host disease · HTLp: Helper T-lymphocyte precursor · LDA: Limiting dilution analysis · PBMC: Peripheral blood mononuclear cells · PBSC: Peripheral blood stem cells · SCT: Stem cell transplantation

B. Kircher (✉) · D. Niederwieser · A. Gächter · K. Lätzer · G. Gastl · D. Nachbaur
Laboratory for Tumor and Immunobiology and Bone Marrow Transplantation Unit, Department of Hematology & Oncology, Innsbruck Medical University,
Anichstrasse 35,
6020 Innsbruck, Austria
e-mail: brigitte.kircher@uklibk.ac.at
Tel.: +43-512-50423337
Fax: +43-512-50423338

D. Niederwieser
Division of Hematology & Oncology, Department of Internal Medicine II, Leipzig University,
Leipzig, Germany

G. Eibl
Department of Biostatistics and Documentation, Innsbruck Medical University,
Innsbruck, Austria

Introduction

Although human leukocyte antigen (HLA) typing methods have been improved, graft-versus-host disease (GVHD) remains a serious complication after hematopoietic stem cell transplantation (SCT) [1]. GVHD may be induced by cryptic epitopes [2] or minor histocompatibility antigens [3]. On the other hand, HLA mismatches may be permissive and the patients do not develop GVHD.

Limiting dilution analyses (LDA) measuring cytotoxic T-lymphocyte precursor (CTLp) [4–6] and helper T-lymphocyte precursor (HTLp) [7–12] frequencies have been developed and described as predictive assays for incidence and severity of acute GVHD after human bone marrow transplantation (BMT). However, a large number of publications are inconsistent on the predictive value of LDA [13–16]. Furthermore, it was recently shown that HTLp LDA, although predictive for the incidence and severity of acute GVHD after HLA-identical BMT [8], is

not predictive for outcome after transplantation of peripheral blood stem cells (PBSC) [17].

One reason for the discrepancy may be that all functional studies published to date were performed with peripheral blood mononuclear cells (PBMC) from the donor as the responder cell population. Our study is the first to assess whether CTLp and HTLp LDA can predict outcome after HLA-identical sibling and unrelated SCT when PBMC from peripheral blood without granulocyte colony-stimulating factor (G-CSF) mobilization (PBMC), bone marrow (BMMC), or G-CSF in vivo-primed PBMC (G-CSF-PBMC) are used as responder cells.

Patients and methods

Twenty-five adult patients receiving allogeneic stem cell transplants were enrolled in the analysis. Twenty-one patients received stem cell grafts from their HLA-identical sibling and four patients from HLA-matched volunteer unrelated donors. HLA class I typing (HLA-A, B, Cw) and class II typing (DR, DR51–53, DQ) were performed according to the manufacturers' instructions using commercially available serological typing kits and polymerase chain reaction (PCR) sequence-specific primer (SSP) typing kits for nucleic acid analysis, respectively.

Only one patient was treated with busulfan (8 mg/kg)/fludarabine (90 mg/m^2)-based reduced-intensity conditioning; the other patients received standard myeloablative conditioning consisting of either cyclophosphamide (120 mg/kg) plus fractionated total body irradiation (12 Gy) or busulfan (16 mg/kg)/cyclophosphamide (120 mg/kg). Patients transplanted with an unrelated stem cell graft also underwent in vivo T-cell depletion with low-dose antithymocyte globulin, as recently published [18].

As GVHD prophylaxis, 15 of the patients received cyclosporin A and methotrexate according to the Seattle protocol [19]. Acute and chronic GVHD were diagnosed from clinical symptoms and/or biopsies from skin, oral mucosa, liver, and gut and classified according to the previously published standard Seattle criteria [20, 21]. Detailed patient characteristics are listed in Table 1.

Preparation of cells PBMC from PB, BM, and apheresis samples were collected from patients, donors, and unrelated HLA-mismatched healthy individuals (third-party cells) who gave written informed consent. The study was approved by the local ethics committee. Cells were isolated by density gradient centrifugation (Lymphoprep, Axis Shield PoC AS, Oslo, Norway) and stored in liquid nitrogen until use.

Culture medium RPMI-1640 (Biochrom, Berlin, Germany) supplemented with 2 mM L-glutamine (Biochrom), 100 μg/ml streptomycin (Grünenthal GmbH, Stolberg, Austria), 100 U/ml penicillin (Biochemie GmbH, Vienna, Austria), and 10% heat-inactivated pooled human AB serum.

Establishment of phytohemagglutinin (PHA) blasts PBMC were adjusted in culture medium to 1×10^6/ml and stimulated with 1% PHA-P (Difco, Detroit, Mich., USA) in a humidified 5% CO_2/95% air atmosphere at 37°C for 72 h. For further growth, cells were cultured in culture medium supplemented with 20 U/ml recombinant interleukin (IL)-2 (Proleukin, Fresenius AG, Bad Homburg, Germany) and fed three times a week.

CTLL cell maintenance The murine cell line CTLL was used as indicator cell line for the IL-2 bioassay. The cell line was grown in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10^{-5} M 2-mercaptoethanol (Sigma, St Louis, Mo., USA), antibiotics, and 10 U/ml human rIL-2. The culture medium was refreshed three times a week. For evaluation of IL-2 production of the LD cultures, the CTLL cells were cultured for 48 h without

Table 1 Patient characteristics. *AML* acute myeloid leukemia, *ALL* acute lymphatic leukemia, *ATG* antithymocyte globulin, *CyA* cyclosporin A, *MMF* mycophenolate mofetil, *MTX* methotrexate

		BMT patients	PBSCT patients
Diagnosis	Acute leukemia (AML or ALL)	*n*=7	*n*=7
	Chronic myeloid leukemia	*n*=1	*n*=2
	Myeloproliferative syndrome	*n*=1	*n*=1
	Plasma cell disease	–	*n*=2
	Lymphoma	–	*n*=1
	Chronic lymphatic leukemia	–	*n*=1
	Bone marrow aplasia	–	*n*=1
	Embryonal carcinoma	–	*n*=1
Median patient age (range)		25 (19–46) years	39 (6–60) years
Donor	HLA-identical sibling	*n*=8	*n*=13
	HLA-identical unrelated	–	*n*=3
	HLA-mismatched unrelated	*n*=1	–
GVHD prophylaxis	CyA	–	*n*=2
	CyA + MTX	*n*=8	*n*=7
	CyA + MTX + ATG	*n*=1	*n*=1
	CyA + ATG	–	*n*=1
	CyA + MMF	–	*n*=2
	No prophylaxis	–	*n*=3
Acute GVHD	No acute GVHD	*n*=5	*n*=11
	GVHD grades II–IV	*n*=4	*n*=5
Chronic GVHD	No chronic GVHD	*n*=7	*n*=10
	Limited to extensive	*n*=2	*n*=6
Relapse	No relapse	*n*=6	*n*=14
	Relapse	*n*=3	*n*=2

addition of IL-2 and washed four times in IL-2-free medium.

Limiting dilution analysis (LDA) All assays were performed prospectively in the graft-versus-host direction. When low patient or donor PBMC numbers were available, CTLp LDA was preferred to HTLp LDA.

In the cytotoxic LDA [4], graded numbers (25,000–390 cells) of responding donor PBMC from either cell source were co-cultured with 50,000 irradiated (30 Gy) stimulator cells (patient pre-SCT and in parallel unrelated third-party cells) in 100 μl culture medium. From every dilution step 24 replicates were assessed in U-bottomed microtiter plates (Greiner Bio-One, Frickenhausen, Germany). On days 3 and 6, a final concentration of 5 U/ml human rIL-2 was added to the cultures. After 10 days of culture at 37°C in a 5% CO_2/95% air atmosphere, each microculture was individually tested against PHA-stimulated Cr^{51}-labeled ($Na_2Cr^{51}O_4$, specific activity 300–500 Ci/g chromate, NEN, Dreieich, Germany) patient pre-SCT or third-party cells for cytolytic activity after 4 h. Of the supernatant 100 μl was harvested and measured in a liquid scintillation counter (Clinigamma, Pharmacia LKB, Bromma, Sweden). As control, each microculture was also tested against autologous responder cells. Only three patients (UPN 272, 293 and 388) showed cytolytic activity against autologous responder PBMC. No autoreactivity was observed with BMMC or G-CSF-PBMC as responder cells.

In the helper LDA [8] graded numbers (1–10×10^4 cells) of PBMC from the donor of either responder cell source were co-cultured with 10×10^4 irradiated (30 Gy) patient pre-SCT cells for 72 h at 37°C in a 5% CO_2/95% air atmosphere. For allogeneic control 1–0.125×10^4 responder cells were stimulated with 10×10^4 irradiated (30 Gy) third-party cells. Twenty-four replicate microcultures were set up in U-bottomed 96-well microtiter plates resuspended in 150 μl culture medium. Negative controls consisted of stimulator cells and responder cells alone. IL-2 production of these cultures was assessed by adding the supernatant to 1.5×10^4 CTLL cells per well. After 8 h of incubation, 2 μCi [^{3}H-methyl]thymidine (Amersham Inc., Arlington Heights, Ill., USA, 5 Ci/mM) was added to each well. After additional incubation for 18–24 h at 37°C, the cells were harvested onto glass paper filter and the uptake of ^{3}H was measured in a liquid scintillation counter (Beckmann LS 1801, Galway, Ireland). In a few patients, controls were performed with autologous stimulator cells. However, these cultures showed HTLp frequencies below the detection limit.

Statistical analysis Calculation of frequencies for both methods was based on Poisson distribution. The values for frequency, the 95% confidence limits for the frequencies, and the probability for single-hit kinetics were calculated by likelihood maximization and chi-square minimization, as described by Taswell [22]. The CTLp or HTLp frequencies are given as median and 25–75% percentile range. The Wilcoxon test was used to analyze statistical differences between the various responder cell sources. The relationship between CTLp and HTLp frequency and acute GVHD, chronic GVHD, and relapse was assessed by the Mann–Whitney U test (SPSS, Chicago, Ill., USA).

Table 2 CTLp frequencies. *BM* bone marrow, *n.d.* not determined, *SCT* stem cell transplantation, *TRM* treatment-related mortality

UPN	Host-specific CTLp frequencies (1/*x*)				Third-party-specific CTLp frequencies (1/*x*)				Stem cell source	Acute GVHD (grade)	Chronic GVHD	Outcome
	PBMC	*P*	Stem cell graft	*P*	PBMC	*P*	Stem cell graft	*P*				
185	*x*=55,049	0.50	*x*=59,398	0.20	*x*=1,753	0.61	*x*=3,072	0.72	PBSC	II	–	TRM
191	220,639	0.50	882,559	0.50	847	0.15	543	0.21	BM	II	–	Relapse (lost to follow-up)
193	882,559	0.50	882,559	0.50	1,351	0.62	544	0.24	BM	II	–	Alive
195	41,708	0.10	45,864	0.87	6,381	0.97	2,133	0.75	BM	–	–	TRM
208	435,245	0.68	91,262	0.51	866	0.86	1,996	0.86	BM	II	–	Alive
224	169,332	0.90	882,559	0.50	27,222	0.97	55,049	0.50	PBSC	–	–	TRM
263	82,313	0.74	17,518	0.53	27,159	0.68	35,774	0.22	PBSC	–	–	TRM
264	108,811	0.68	35,774	0.22	19,023	0.12	13,579	0.68	PBSC	–	Limited	Alive
272	29,406	0.86	122,751	0.92	1,074	0.77	803	0.35	BM	–	–	Alive
275	137,756	0.25	882,559	0.50	7,088	0.78	2,683	0.76	BM	–	Limited	Alive
282	21,389	0.20	26,659	0.71	5,775	0.07	2,284	0.55	BM	II	–	Alive
286	882,559	0.50	441,279	0.50	1,930	0.97	1,219	0.63	BM	–	–	Relapse
293	24,146	0.94	9,670	0.89	2,253	0.79	760	0.27	PBSC	–	–	TRM
299	55,049	0.50	84,660	0.90	2,901	0.52	13,579	0.68	BM	II	–	Alive
306	441,279	0.50	183,082	0.64	2,945	0.77	13,579	0.68	PBSC	–	–	TRM
361	42,084	0.28	882,559	0.50	3,718	0.64	7,418	0.55	PBSC	II	–	Alive
362	n.d.	n.d.	98,995	0.51	n.d.	n.d.	2,823	0.28	PBSC	II	Extensive	TRM
364	110,320	0.50	54,405	0.68	3,027	0.97	5,643	0.26	PBSC	–	Limited	Alive
370	338,664	0.90	882,559	0.50	11,472	0.96	24,900	0.70	PBSC	–	–	Alive
372	441,279	0.50	882,559	0.50	18,068	0.95	6,690	0.16	PBSC	II	Limited	Relapse (TRM after 2nd SCT)
379	110,320	0.50	882,559	0.50	3,358	0.55	8,621	0.91	PBSC	–	Extensive	Alive
387	143,096	0.22	110,320	0.50	9,006	0.60	27,524	0.50	PBSC	IV	–	TRM
388	23,959	0.99	23,376	0.93	6,351	0.85	3,663	0.74	PBSC	–	–	Alive
398	108,811	0.68	338,664	0.90	570	0.54	5,244	0.50	PBSC	–	–	TRM
399	183,082	0.64	301,436	0.85	3,011	0.63	17,590	0.54	PBSC	I–II	Limited	Relapse

Results

The host-specific CTLp frequencies obtained after 10 days incubation of responder PBMC with recipient PBMC from 25 patients were equally low in all responder cell sources (PBMC: median: 1/110,320, 25–75% percentiles: 45,325–309,158; BMMC: median 1/122,751, 25–75% percentiles: 65,262–882,559; G-CSF-PBMC: median 1/146,701, 25–75% percentiles: 40,432–882,559). Detailed CTLp frequencies are presented in Table 2.

In contrast, the CTLp frequencies against HLA-mismatched third-party cells were high in PBMC (median 1/3,193, 25–75% percentiles: 1,797–8,527) and BMMC (median 1/2,209, 25–75% percentiles: 907–3,903), but significantly lower in G-CSF-PBMC (median 1/8,020, 25–75% percentiles: 4,058–23,073, p=0.047 against PBMC; Fig. 1).

In six patients host-specific HTLp frequencies were determined that were equally low in all responder cell sources as compared to high third-party-specific HTLp frequencies. The third-party-specific HTLp frequencies of G-CSF-PBMC were again lower than in PBMC, but statistically not significant (data not shown).

Acute GVHD

Nine patients received BM from either sibling or unrelated donors. Acute GVHD (grades II–IV) was diagnosed in four patients. No significant differences were seen in host-specific or third-party-specific CTLp or HTLp frequencies between PBMC and BMMC in patients with or without GVHD (data not shown).

The majority of patients received G-CSF-mobilized PBSC (n=16). Whereas the host-specific CTLp frequencies did not differ between PBMC and G-CSF-PBMC, the third-party-specific CTLp frequencies were significantly lower in the G-CSF-PBMC responder cell population (median 1/8,020, 25–75% percentiles: 4,058–23,073) than in PBMC (median 1/3,718, 25–75% percentiles: 2,945–18,068, p=0.047; data not shown). Acute GVHD (grades II–IV) was diagnosed in 5 of 16 patients (31%). The host-specific CTLp frequencies measured in G-CSF-PBMC or the corresponding PBMC responder population could not discriminate between patients with and without acute GVHD. Lower third-party-specific CTLp frequencies in G-CSF-PBMC vs PBMC were again detected; however, the differences were significant only in patients without acute GVHD (p=0.041) and not in patients with acute GVHD (data not shown).

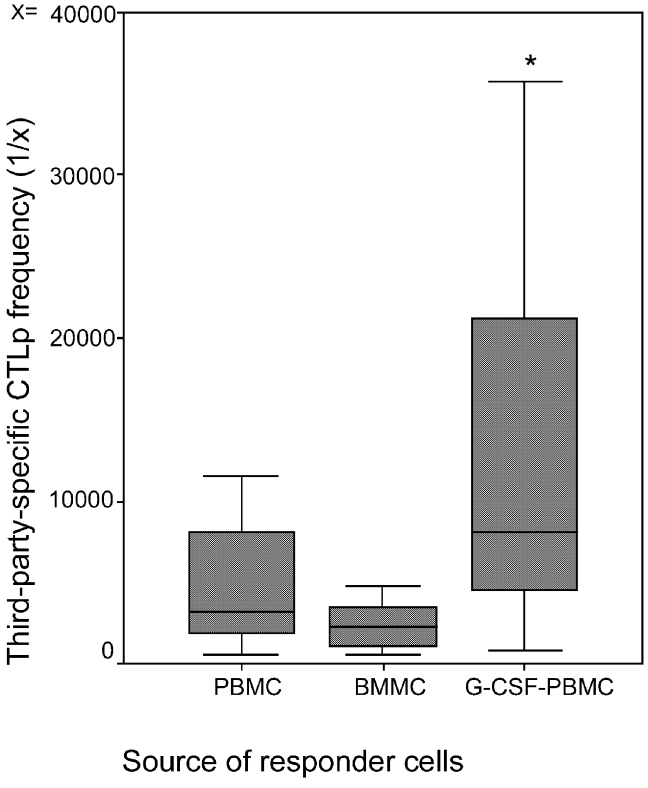

Fig. 1 Third-party-specific CTLp frequencies of 25 patients determined in various responder cell populations. The median ± 25 and 75% percentile is shown (* p=0.047 against PBMC)

Chronic GVHD

Chronic GVHD (limited or extensive) was detected in 8 of 25 patients (32%). No significant differences in host-specific or third-party-specific CTLp frequencies were measured in either responder cell population between patients who developed chronic GVHD and patients who did not (data not shown).

Relapse

Of 25 patients 5 (20%) suffered a relapse of their underlying disease. The mean host-specific CTLp frequency determined in PBMC from patients with relapse was significantly lower (median 1/441,279, 25–75% percentiles: 201,861–661,919) than the CTLp frequency for patients without relapse (median 1/108,811, 25–75% percentiles: 41,708–143,096, p=0.003; Fig. 2). Also, the mean host-specific CTLp frequency determined in BMMC and G-CSF-PBMC from patients with relapse was lower than for patients without relapse. However, the differences did not reach statistical significance (Fig. 2). The third-party-specific CTLp frequency did not significantly differ between responder cell sources and patients with or without relapse (data not shown).

Discussion

Limiting dilution analyses measuring the frequencies of CTLp and HTLp were used as functional assays to predict the incidence and severity of acute GVHD [4–12]. However, these results could not be confirmed by several investigators [13–17]. Also, this study detected mainly low host-specific CTLp and HTLp frequencies in each

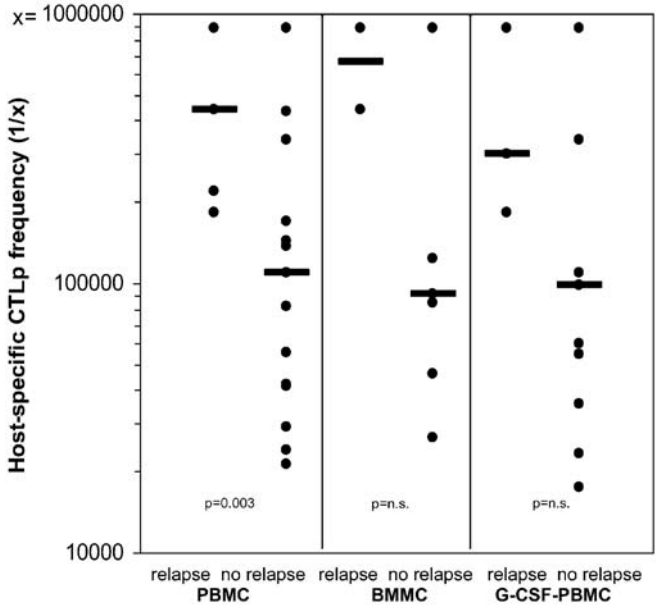

Fig. 2 Host-specific CTLp frequencies determined in various responder cell sources from patients with and without relapse. Each *dot* represents a single CTLp frequency

responder cell population, which were not predictive for the occurrence or severity of acute or chronic GVHD.

Although slightly modified protocols were used in the various laboratories, sensitivity problems were able to be excluded since all publications including this study showed high CTLp and HTLp frequencies against allogeneic HLA-mismatched third-party cells. However, the low number of patients might not allow detection of significant correlations between host-specific CTLp frequencies and acute or chronic GVHD, especially when the results for the various responder cells are correlated with the respective stem cell source. From this point of view, the difference between relapse and non-relapse patients in the PBMC responder cell population takes on even more significance.

Another explanation for the lack of predictive value might be the various conditioning and GVHD prophylaxis regimens used. However, e.g., the study using intravenous infusion of CAMPATH 1G [5] showed an association between CTLp frequency and acute GVHD, as did the study with ex vivo depletion of mature T-cells [6]. Wang et al. directly compared patients receiving cyclosporine alone and those receiving cyclosporine and methotrexate, as used in this study, and could not find a correlation between CTLp frequency and acute GVHD [16].

The study by Healey and Schwarer was the first to attempt to correlate HTLp frequencies and outcome of patients transplanted with G-CSF-stimulated PBSC [17], but determined HTLp frequencies in PB. This is the first study to directly measure CTLp and HTLp frequencies in the respective stem cell graft as responder population and compare them with CTLp and HTLp frequencies in PB. Therefore, the results were expressed as median CTLp and HTLp frequencies and were not divided into "low" and "high" frequencies, as done in the other studies.

The absence of any correlation for CTLp and HTLp frequencies when analyzed with G-CSF-PBMC as responder cells might, independently of the lack of association with PBMC or BMMC, also be explained by the G-CSF mobilization procedure itself. We analyzed in 13 patients differential counts of the mononuclear cell fraction used as responder cells. As expected, no significant differences were detected in PBMC or BMMC. In contrast, G-CSF-PBMC contained 54.3% lymphocytes and 43.5% monocytes vs 78.6% lymphocytes and 19.5% monocytes in PBMC (p=0.008 and p=0.008, respectively). These preliminary data, a comparison of marrow and blood cell yields from the same donor [23], and a report on the proportion and function of various cell types in apheresis products [24] further support this hypothesis. Accordingly, Hoffmann et al. measured equal or higher frequencies of minor histocompatibility antigen-reactive proliferating T-lymphocyte precursors in BM and PB [25].

The significantly lower third-party-specific CTLp (and HTLp) frequencies in G-CSF-PBMC compared to PBMC and BMMC, although in contrast to a previous report [26], suggest a diminished T-cell response. One explanation might be that L-selectin-positive T-cells that respond better to alloantigen than do L-selectin-negative T-cells are decreased after G-CSF administration [27]. Further explanations for downregulation of the alloreactive potential of G-CSF-primed PBSC are reviewed by Gyger et al. [28]. However, Rondelli et al. also showed that the T-cell proliferative response to alloantigens was maintained in most of the cases after G-CSF treatment [24], indicating that G-CSF-PBMC can be used as responder cells in vitro. However, for a predictive test system PBMC should be preincubated with G-CSF, and no data are available on this in vitro-generated cell population.

Despite the shift to TH2 cells by G-CSF mobilization, acute and chronic GVHD are more common after PBSCT than after BMT [29]. This was not expressed in significantly higher CTLp frequencies in our patient cohort, although observed in most publications concerning BMT [4–12, 30]. The majority of patients (4/5) developed acute GVHD grade II. Therefore, it was not possible to correlate host-specific CTLp frequencies with GVHD grades.

The predictive value of host-specific CTLp frequencies has so far been shown predominantly for patients receiving grafts from HLA-matched unrelated volunteer donors. However, the inclusion of unrelated grafts was avoided for several reasons. (1) Host-specific CTLp frequencies may be predictive for complications after HLA-identical SCT when analyzed in the respective stem cell graft. (2) Usually, unrelated stem cell grafts are transported by aircraft and over various periods of time. Therefore, a constant quality for all stem cell grafts was not guaranteed for the experiments.

A trend to lower rates of relapse as observed after PBSCT [29, 31] was expressed in lower CTLp frequencies in G-CSF-PBMC compared to BMMC, although statistically not significant. The association between a low CTLp frequency in PBMC and relapse, although analyzed in a

small patient number, was striking and confirmed the results of several groups [9, 10, 32], but was inconsistent with the data of Keever-Taylor et al. [6].

Besides the analysis of CTLp and HTLp frequencies, which is also rather contradictory, no established test system has been available to date for the prediction of outcome after SCT. Granzyme A, determined by enzyme-linked immunoassay in supernatants of mixed lymphocyte cultures, may have a predictive value for the development of GVHD, as we have shown in a preliminary cohort of patients [33]. At least in renal transplant recipients, the ELISPOT assay measuring the frequency of interferon-gamma-producing cells reflected the alloreactivity against minor histocompatibility antigens and might also play a role in identifying patients at risk for developing GVHD [34].

On the other hand, GVHD is often associated with graft-versus-leukemia (GVL) activity. Therefore, methods predicting the risk of developing GVHD should also include the analysis of cells exerting GVL activity but having no effect on GVHD such as T helper 2 and T cytotoxic 2 lymphocytes or natural killer cells. Several ways to manage the prevention of GVHD while preserving a GVL effect were reviewed by Barrett et al. [35].

In conclusion, this study is the first to investigate the predictive value of cytotoxic and helper LDA when CTLp and HTLp frequencies are determined in the respective stem cell graft. CTLp frequencies were not predictive for the risk of acute or chronic GVHD or relapse when analyzed in donor BMMC and G-CSF-PBMC. CTLp frequencies measured in PBMC, however, were significantly lower in patients who relapsed after HLA-identical BMT or PBSCT.

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