PROPHYLACTIC TRANSFER OF BCR-ABL-, PR1-, AND WT1-REACTIVE DONOR T CELLS AFTER T-CELL-DEPLETED ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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Abstract
Donor lymphocyte infusions have been effective in patients with chronic myeloid leukemia (CML) relapsing after allogeneic stem-cell transplantation, but their use is associated with the risk of graft-versus-host disease. We investigated the effects of prophylactic infusion of in vitro-generated donor T cells reactive against peptides derived from CML-associated antigens. Fourteen CML patients received conditioning therapy followed by CD34+-selected peripheral blood stem cells from matched siblings (n = 7) or unrelated (n = 7) donors. Donor-derived mature dendritic cells generated in vitro from CD14+ monocytes were loaded with HLA-restricted peptides derived from PR1, WT1, and/or BCR-ABL and used to repetitively stimulate donor CD8+ T cells in the presence of IL-2 and IL-7. Stimulated T cells were infused 28, 56, and 112 days after transplantation. Thirteen patients are alive and 7 remain in molecular remission (median follow-up, 45 months). Interestingly, all 4 patients receiving CD8+ T cells displaying marked cytotoxic activity in vitro and had detectable peptide-reactive CD8+ T cells during follow-up have not experienced graft-versus-host disease or relapse. Our study reveals that prophylactic infusion of allogeneic CD8+ T cells reactive against peptides derived from CML-associated antigens is a safe and promising therapeutic strategy. This trial was registered at http://clinicaltrials.gov as NCT00460629

Key-words: Chronic myeloid leukemia; adoptive transfer; leukemia-reactive CD8+ T cells; BCR-ABL
Introduction

Despite the advent of tyrosine kinase inhibitors (TKIs) such as imatinib mesylate (IM), allogeneic stem-cell transplantation (SCT) remains the only therapy with definitive curative potential for patients with chronic myeloid leukemia (CML). For patients relapsing after allogeneic SCT, donor lymphocyte infusions (DLI) have been established as an effective therapy that may reinduce remission when employed at an earlier stage in cases of molecular or cytogenetic relapse. Nevertheless, the risk of graft-versus-host disease (GvHD) associated with DLI remains considerable even when it is administered in incremental doses.

Efforts to decrease the risk of GvHD by depleting T-cell numbers in the graft have been associated with an increased incidence of relapse and subsequent treatment failure. Newer protocols using grafts comprising CD34+-selected hematopoietic cells from matched sibling donors, and subsequent infusion of T cells in incremental doses to treat or avoid disease relapse, seem to be more promising. However, individual T-cell thresholds required to induce graft-versus-leukemia (GvL) effects without relevant GvHD are difficult to predict.

CD8+ T-cell responses against the HLA-A2-restricted nonamer peptide PR1 derived from proteinase 3, have been shown to be associated with long-term survival of CML patients after therapy with interferon alpha or allogeneic hematopoietic cell transplantation. Similarly, cytotoxic CD8+ T lymphocytes (CTL) reactive against HLA-A2- and -A24-restricted peptides derived from Wilms' tumor antigen 1 (WT1) expressed in subtypes of myeloid leukemia have been described. WT1-specific CD8+ T cells were detected in leukemic patients, and a correlation between GvL effects and detectable WT1-specific CTL was observed after allogeneic SCT for CML and acute lymphoblastic leukemia. Peptide-based vaccination trials with regard to both PR1 and WT1 have demonstrated clinical responses, and an association between therapeutic response and increased frequency of WT1-specific CTL was reported.
In contrast to the aforementioned leukemia-associated antigens, the BCR-ABL fusion protein represents a tumor-specific antigen expressed in CML stem and progenitor cells but not in healthy hematopoietic stem cells (HSC). Several studies have demonstrated that CD8+ T cells reactive against BCR-ABL-derived peptides can be generated in vitro and are able to lyse peptide-loaded target cells or CML cells\textsuperscript{13-15}. Furthermore, it has been reported that BCR-ABL peptide-reactive CD8+ T cells are detectable in CML patients\textsuperscript{10,16}. BCR-ABL-derived peptides can also be used to induce specific T-cell responses in CML patients\textsuperscript{17}.

Pivotal clinical studies have demonstrated that leukemia-specific CTL can be successfully generated to treat post-transplant relapse\textsuperscript{18}. In addition, HLA-restricted minor-histocompatibility antigen (mHAg)-specific CTL have been detected in vivo after DLI, which was followed by complete remission of leukemia\textsuperscript{19}. Recently, Warren and colleagues were able to demonstrate the safety and efficacy of CD8+ T-cell clones specific for tissue-specific mHAg when administered to patients with relapsed leukemia after allogeneic SCT\textsuperscript{20}.

In the present study, donor CD8+ cytotoxic T cells reactive against PR1-, WT-1-, and/or BCR-ABL-derived peptides were generated in vitro and prophylactically administered to CML patients after allogeneic T-cell-depleted SCT. Besides the feasibility and safety of this approach, we were able to demonstrate for the first time that the prophylactic infusion of these CD8+ T-cell preparations allogeneic SCT is associated with durable molecular remission in 50% of patients.

**Patients and Methods**

*Study design and procedures*

We conducted a clinical trial to determine the feasibility and efficacy of the adoptive transfer of in vitro-stimulated leukemia antigen-reactive donor T cells after allogeneic transplantation of CD34+-selected peripheral blood stem cells. The trial was approved by the institutional review board of the University Hospital Dresden and registered at http://clinicaltrials.gov as NCT00460629. Patients 18 years of age or older with (i) the presence of BCR-ABL-positive CML, (ii) chronic or accelerated phase, and (iii) identification
of an HLA-identical family donor or an unrelated volunteer donor matched for 9 out of 10 HLA alleles were eligible for enrolment. Written informed consent or assent was obtained from all patients and donors. Additional inclusion criteria were either the presence of a b3a2 BCR-ABL transcript in HLA-A3-, -A11-, and -B8-positive patients and/or HLA-A2 positivity. Patients with blast phase, major organ dysfunction or uncontrolled infection were excluded.

Primary outcome measures of this trial were the safety and feasibility of transferring leukemia-reactive donor T cells after T-depleted SCT. Secondary endpoints included the probability of surviving in molecular remission, the incidence of acute and chronic GvHD and the rate of opportunistic infectious complications.

To compare the kinetics of donor T cell chimerism with or without prophylactic T cell infusion, a historical cohort of ten patients comprising 2 CML, 4 acute myeloid leukemia and 4 acute lymphoblastic leukemia patients with a similar age distribution receiving the identical conditioning regimen and T-depleted SCT without prophylactic donor T-cell infusion was selected.

**Conditioning therapy and transplantation**

According to protocol, total body irradiation (TBI) divided into fractions of 200 cGy was performed with a total dose of 800 cGy (days −8 to −7) in patients >40 years of age (n = 9) and with 1200 cGy (days −9 to −7) in younger patients (n = 5). The regimen also included intravenous fludarabine (40 mg/m² over 5 days, days −6 to −2, total dose 200 mg/m²), thiotepa 2 × 5 mg/kg on day −6 (total dose 10 mg/kg), antithymocyte globulin (ATG) Fresenius 5 mg/kg over 5 days (days −5 to −1, total dose 25 mg/kg), and cyclosporine A at 1 mg/kg per day from days −10 to −3. CD34⁺-selected donor cells were infused on day 0 (Figure 1). No further post-transplant GvHD prophylaxis was performed.

**Cell processing and generation of activated CD8⁺ T cells**

Clinical-scale immunomagnetic CD34⁺ selection using the CliniMacs® device (Miltenyi Biotec, Bergisch Gladbach, Germany) was performed as previously described.
Immunomagnetic CD8⁺ T-cell isolation (Miltenyi Biotech) was subsequently performed using the leukapheresis product of the second day or the CD34⁻ fraction of the aforementioned positive selection procedure. CD14⁺ monocytes were also immunomagnetically isolated from an aliquot of the first or second leukapheresis product at a purity of >90% according to the manufacturer’s instructions (Miltenyi Biotech). To generate immature dendritic cells (DCs), the selected CD14⁺ cells were incubated at 37°C in 5% CO₂ at a density of 2-3 10⁶/ml in X-Vivo 15 medium (Bio-Whittaker, Walkersville, MD) supplemented with interleukin (IL)-4 (1000 IU/ml; R&D Systems, Minneapolis, MN), GM-CSF (2000 IU/ml; Novartis, Nuernberg, Germany), and 1% pretested AB serum (c-c pro, Neustadt/W., Germany). On day 8, DCs were incubated with TNF-\textit{alpha} (1100 IU/ml; R&D Systems, Minneapolis, MN), PGE2 (1 mg/ml; Pharmacia, Puurs, Belgium), IL-1\textit{beta} (1900 IU/ml; R&D, Minneapolis, MN), and IL-6 (1000 IU/ml; R&D Systems, Minneapolis, MN) to induce maturation. For the second and third infusion of activated CD8⁺ T cells, immature DCs were cryopreserved and thawed later before the final maturation step was induced.

HLA-restricted peptides derived from proteinase 3 (HLA-A2, VLQELNVTV²³), WT1 (HLA-A2, RMFPNAPYL²⁴; HLA-A24, RWPCQKKK²⁵) and BCR-ABL (HLA-A3, KQSSKALQR; HLA-A3/HLA-A11, HSATGFKQSSK; HLA-A3/HLA-A11, ATGFKQSSK; HLA-B8, GFKQSSKAL)¹³,²⁶,²⁷ synthesized to >95% purity were purchased from Jerini, Berlin, Germany. To generate peptide-specific CD8⁺ T cells, mature DCs were loaded with 20 µg/ml of leukemia antigen-derived peptides according to the patient HLA type. After washing, 2 × 10⁵ peptide-loaded DCs were co-cultured with 2 × 10⁶ immunomagnetically isolated CD8⁺ T cells per well of a 24-well tissue culture plate (Greiner, Frickenhausen, Germany). T cells were cultured in 2 ml RPMI 1640 medium/well supplemented with AB-serum, 50 U/ml IL-2 (Proleukin; Chiron, Munich, Germany), and 100 U/ml IL-7 (R&D Systems, Wiesbaden, Germany). T cells were subsequently restimulated weekly 2–4 times with peptide-loaded DCs and evaluated for peptide-specific cytotoxic activity. Prior to infusion, CD8⁺ T cells were washed twice in PBS and reconstituted in 0.9% physiologic NaCl solution for infusion into the recipient. The administration of in vitro-stimulated donor T cells was scheduled for days 28,
56, and 112 after transplantation (Figure 1). The target CD8+ T cell dose per protocol was 0.5 x 10^6/kg recipient on day 28 and 1 x 10^6 on days 56 and 112, respectively. Release criteria for infusion of the cells were vitality of ≥90% determined by trypan blue exclusion, sterility and negativity for mycoplasma by culture and PCR in an aliquot derived from the last medium change. Only fresh cells were infused on day 28. For infusions on day 56 and 112, previously cryopreserved CD8+ T cells were thawed and stimulated with peptide-loaded DCs as described above. After having demonstrated the safety of that dose level in the first three sequentially enrolled patients, the maximum CD8+ T-cell dose was amended to 1 x 10^7/kg. The protocol required the patient to have no signs of acute GvHD before the T cell infusion could be scheduled.

**Chromium release assay**

Cytotoxic activity of in vitro-stimulated T cells was tested against (i) the HLA-A2-positive mutant cell line T2 or (ii) autologous mature DCs loaded with peptides derived from leukemia antigens or an irrelevant HLA-A2-restricted peptide derived from HIV-1 reverse transcriptase as targets in a 4 h standard ^51^Cr release assay28. Briefly, T2 cells or DCs were incubated for 4 h with individual peptides at a concentration of 50 μg/ml, labeled with ^51^Cr for 1 h (sodium chromate; PerkinElmer Life Sciences, Rodgau-Jügesheim, Germany), and used as targets. Chromium-labeled target cells were washed 3 times and plated in round-bottomed 96-well plates at 5 x 10^3 cells/well. Effector cells were added as triplicates at different ratios. After 4 h of incubation, ^51^Cr release was determined with a beta counter (PerkinElmer Life Sciences).

**Fluorescence-activated cell sorting and tetramer/pentamer analyses**

Standard flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA) using fluorochrome-labeled antibodies was performed to measure reconstitution of CD3+/CD4+ T cells, CD3+/CD8+ T cells, CD3+/CD56+ NK cells, and CD19+ B cells. To confirm the quality of monocyte-derived DCs, the coexpression of CD83, HLA-DR as well as the costimulatory
molecules CD80 and CD86 was quantified for each DC preparation. HLA/peptide tetramer or pentamer complexes were acquired from ProImmune Limited (www.proimmune.com). In the second phase of the trial, frequencies of peptide-specific CD8\(^+\) T cells were determined in donor-derived peripheral blood mononuclear cells (PBMC) before stimulation with peptide-loaded DCs and in the T-cell preparations, which were used for infusion. In addition, the frequency of peptide-specific CD8\(^+\) T cells after adoptive transfer was analyzed. Cells were incubated with R-PE-labeled HLA/peptide tetramer or pentamer complex for 10 min at room temperature. Then, cells were washed and incubated with APC-labeled anti-CD3 and FITC-labeled anti-CD8 (Becton Dickinson) for 20 min at room temperature.

**Analysis of donor chimerism**

The methods used for DNA isolation and chimerism analysis have previously been described in detail\(^{29}\). The HumanType Chimera kit (Biotype, Dresden, Germany), which amplifies 12 STR loci and the amelogenin locus, was used. In order to assess the effects of CD8\(^+\) T-cell infusion on the development of T-cell chimerism, 30 - 40 ml heparinized blood was collected on days 56, 84, 112, and 180. As previously described, CD3\(^+\)/CD4\(^+\) and CD3\(^+\)/CD8\(^+\) T cells were sorted by FACS (FACS Vantage or from 2009 FACS Aria II, Becton Dickinson) and were subjected to DNA isolation with subsequent chimerism analysis\(^{30}\). Whenever possible, between 1,500 and 10,000 cells were sorted for each population. Samples were also collected to assess the purity of the sorted cells. The median purity, as measured by repeated FACS analysis, was 98% (range, 92% - 100%).

**BCR-ABL and WT1 mRNA analysis**

A quantitative BCR-ABL PCR assay (qPCR) was performed before conditioning, every 4 weeks during the first 12 months after transplantation, and every third month during the second to fifth year of follow-up. For all samples, real-time qPCR was performed using the published EAC quantitative polymerase chain reaction (qPCR) primers and probes\(^{31}\), with minor modifications and detection on an ABI7500 system (Applied Biosystems, Darmstadt,
Germany). In brief, white blood cells (WBC) were obtained after erythrocyte lysis from PB and BM samples, with approximately $5 \times 10^5$ to $1 \times 10^7$ cells being used for RNA extraction by the RNeasy Blood kit (Qiagen, Hilden, Germany). cDNA synthesis was performed as described and up to 5 µL of cDNA was used for real-time PCR. All reactions were performed in duplicate. Results were obtained using a second PCR reaction for a housekeeping gene (ABL) as an internal standard, which results in expression of BCR-ABL levels relative to ABL levels (ratio of copy number BCR-ABL : copy number ABL) as percentages. These results were adjusted to an international scale. Absolute quantification was performed using commercially available plasmid standards for BCR-ABL and ABL genes (Ipsogen, Marseille, France). The assay detected 1 BCR-ABL-positive cell in 100,000 cells, as determined by dilution experiments with CML cells in normal controls. In the case of qPCR negativity, nested PCR with a sensitivity of about $1-5 \times 10^6$ was performed as previously described. WT1 mRNA expression in the peripheral blood was assessed in four patients using real-time qRT-PCR based on commercially available primer and probe sets (Applied Biosystems, Darmstadt, Germany) and normalized to GAPDH using the \( \Delta C_t \)-method. In normal peripheral blood, WT1 is not detectable in our hands (\( \Delta C_t \gt 25 \) cycles), in the four patients (nos. 11-14) analyzed, the \( \Delta C_t \) values were 8.9, 15.6, 17.9 and 22.6. However, only the first patient could be analyzed at diagnosis, the others were investigated during follow-up.

**Statistical analysis**

Means (± SE) or median with ranges were provided as descriptive parameters whenever applicable. The probability of overall and molecular relapse-free survival, including 95% confidence intervals, was calculated by the method of Kaplan and Meier using the SPSS software package 12.0 (SPSS Software GmbH, Munich, Germany).

**Results**

**Patient characteristics**

Fourteen patients aged between 21 and 67 years (median, 45 years) were included between August 2003 and October 2008. Patient characteristics, the interval between diagnosis and transplantation, disease status at the time of transplantation, the European
Group for Blood and Marrow (EBMT) risk score\textsuperscript{34} and the details of pretreatment are summarized in Table 1. Given the lower frequency of referral of CML patients after approval of imatinib mesylate, these 14 patients represent 93\% of patients with chronic or accelerated phase CML who underwent SCT at our centre during the accrual period. Only 1 patient was not eligible due to an HLA type mismatch. The reasons for transplantation from HLA-A-, -B-, -C-, -DR-, and -DQ-matched related ($n = 7$) or unrelated ($n = 7$) donors were either (i) loss of hematologic response/acceleration ($n = 5$), loss of cytogenetic remission ($n = 2$), loss of major molecular remission as warning signal ($n = 3$) or detection of a T315I mutation ($n = 1$), (ii) intolerance to tyrosine kinase inhibitor therapy ($n = 1$, elevated liver enzymes), or (iii) the patient’s decision ($n = 2$). Only 7 patients (50\%) were in the first chronic phase (CP) at the time of transplantation, whereas 7 patients were in second CP ($n = 4$) or presented with signs of acceleration ($n = 3$). Two patients had previously achieved a major cytogenetic response, but preferred elective allogeneic SCT. Intensive chemotherapy with cytarabine and anthracyclines had become necessary to control blast transformation in 3 patients. In 1 patient (no. 10) with a disease history of more than 10 years, autologous transplantation with PB stem cells collected after combination therapy was performed 5 years prior to allogeneic SCT. A second-line TKI (dasatinib) was prescribed in 3 patients after the occurrence of IM-resistant disease. A T315I mutation within the tyrosine kinase domain of BCR-ABL was identified as the reason for resistance against IM in 1 case. The high-risk characteristics of the included population is supported by the fact that the median EBMT risk score of the cohort was 3 (range 2 to 6) with eleven patients having a score of $> 2$ predicting a transplant-related mortality of 40 to 70\%.

\textit{Engraftment- and transplant-related toxicity}

After immunomagnetic CD34$^+$ selection, the grafts infused on day 0 contained a median of $6.7 \times 10^6$/kg CD34$^+$ cells (range, $4.0 - 10.8 \times 10^6$/kg) and only $0.5 \times 10^4$/kg CD3$^+$ T cells (range, $0 - 9.8 \times 10^4$/kg). Prompt neutrophil (absolute neutrophil count [ANC] >0.5 × $10^9$/L) and platelet engraftment (>50 × $10^9$/L) was achieved without growth factor
administration after 13.5 (range, 10 - 20) and 20 (range, 12 - 28) days, respectively. Only patients receiving 1,200 cGy TBI \( (n=5) \) suffered from grade 3 mucositis. Grade 3–4 gastrointestinal toxicity (according to National Cancer Institute common toxicity criteria [CTCAE 3.0]) was observed in 7 patients (50%). In 4 patients this was associated with neutropenic fever and positive blood cultures; the occurrence of neutropenic enterocolitis was most likely related to the combination of TBI and thiotepa and was reversible in all cases. Of 5 CMV-seropositive patients, 2 experienced CMV reactivation as measured by quantitative PCR, which resolved after preemptive therapy with ganciclovir. HHV-6 viremia associated with exanthema occurred in 1 patient on day 40 after transplantation and remitted after 10 days of oral valganciclovir. Patient no. 10 with a 10-year disease history experienced pulmonary aspergillosis with central nervous manifestations, from which death occurred 4 months after transplantation despite escalation of antifungal therapy. Pulmonary tuberculosis was reactivated in patient no. 1 four months after transplantation, and the patient required tuberculostatic therapy for 24 months. In 1 patient, toxoplasmosis of the retina occurred 6 months after transplantation; specific therapy was instituted and all symptoms resolved without any functional impairment. No patient experienced acute GvHD and 4 out of 13 patients at risk (31%) had to be treated for mild or moderate chronic GvHD. Immunosuppressive therapy had been stopped in all these 4 cases at the time of reporting.

Donor chimerism and immune reconstitution

The number of total nucleated cells (TNC) and CD8\(^+\) T cells per kilogram recipient infused on each occasion as well as the percentage of CD8\(^+\) T cells and the vitality of each cell preparation is provided for every patient in Table 2. A median dose of 1.85 (range, 0.06 - 4.1), 1.7 (range, 0.06 - 5.7) and 2.6 (range, 1.1 - 9.5) \( \times 10^6 \)/kg CD8\(^+\) T cells could be administered on day 28, 56 and 112, respectively. In patient no. 2, only 2 infusions were possible due to the limited quantity and viability of cells obtained after culture at day 112. Patient 10 could only receive the first infusion of CD8\(^+\) T cells on day 28 because his clinical condition did not allow further participation in the trial. All other CTL infusions could be
performed within three days of the scheduled dates. The infused preparations contained a median of 80% CD3+/CD8+ T cells (range, 50 - 98%), 4.7% CD3+/CD4+ cells (range, 0 - 15.2%), and 0% CD3−/CD56+ natural killer cells (range, 0 - 7.2%). The median vitality was 98% (range 90 - 100%). There was a considerable variability in the amplification of CD8+ T cells with a faster cell growth in the initial phase of stimulation and lower expansion factors during subsequent restimulation. The median expansion factor of CD8+ T cells after 3-4 weeks of cell culture was 1.6 (range, 0.3 - 10).

As shown in Figure 2A, the infusion of activated donor CD8+ T cells on day 28 was associated with a prompt increase in the percentage of donor CD4+ and CD8+ T cells in the patient’s peripheral blood. At the time of the third infusion (day 112), complete T-cell chimerism could be documented in most patients. Figure 2B summarizes the course of CD4+ and CD8+ donor chimerism in a historical control cohort that was transplanted with T-cell depleted grafts from matched sibling donors without receiving prophylactic DLI. In these patients, the mean percentage of donor chimerism in T-cell subsets remained below 90% until 6 months after transplantation. The absolute count of lymphocyte subsets is depicted in Figure 2C. CD4+ T cells were the only subset not to return to normal levels, remaining below 200/µL within the first 12 months after transplantation. CD8+ T cells dominated during that period, CD19+ B cells reached normal levels as soon as 3 months after transplantation, while CD3−/CD56+ NK cells peaked 2 months after transplantation and slowly declined for further 10 months.

*Induction of peptide-reactive CD8+ T cells in vitro and detection of peptide-specific CD8+ T cells in vivo after adoptive transfer*

Immunomagnetic selection of CD14+ monocytes from an aliquot of the leukapheresis product resulted in a median purity of 98% (range, 94 - 100%) with a median recovery rate of 95% (range, 60 - 100%). FACS analysis of HLA-DR, CD80, CD83, and CD86 was used to evaluate DC maturation. The median percentage of mature DCs coexpressing CD83/HLA-DR and CD80/CD86 after maturation was 70% (range, 22 - 93%) and 80% (range, 24 -
89%), respectively. To obtain sufficient amounts of peptide-specific CD8+ T cells for adoptive transfer, DCs were loaded with peptides derived from proteinase 3, WT1, and/or BCR-ABL and co-cultured with CD8+ T cells.

In the second phase of the clinical trial, we systemically analyzed the frequency of peptide-specific CD8+ T cells in donor-derived PBMC before stimulation with peptide-loaded DCs and in the T-cell preparations, which were adoptively transferred. As demonstrated in Table 3, the percentage of pre-existing peptide-specific CD8+ T cells in the blood of 8 evaluated donors for patients no. 7-14 was low or not detectable. After stimulation with peptide-loaded DCs, the frequency of peptide-reactive CD8+ T cells was markedly increased in cell preparations for 3 patients (no. 11, 12 and 14).

In further experiments, we evaluated the cytotoxic potential of the generated CD8+ T cells. As shown in Table 4, peptide-specific CD8+ T cells exhibiting marked cytotoxic activity could be induced in 7 out of 14 donors. The frequency of peptide-specific CD8+ T cells in the peripheral blood was monitored for at least one year after transplantation. Maximum values related to the day of analysis are included in Table 4. Prior to the first infusion on day 28, the level of CD3+ T cells in the PB was, in general, too low for subset chimerism and multimer FACS analyses, as is expected after T-depleted SCT.

Table 5 categorizes patients according to the infusion of CD8+ T cells with cytotoxic activity in vitro and the occurrence of peptide-specific CD8+ T cells in vivo, as determined by tetramer or pentamer staining. Interestingly, 4 out of 5 patients in whom the infusion of donor T cells with peptide-specific cytotoxicity was followed by measurable pentamer+/CD8+ T cells in vivo (patient no. 3, 5, 11, and 14, marked in yellow) have remained BCR-ABL negative between 31 and 55 months after transplantation. None of these patients have showed clinical signs of acute or chronic GvHD during follow-up. Additionally, table 5 provides BCR-ABL mRNA levels measured by PCR four weeks after conditioning therapy/transplantation before the first infusion of activated CD8+ T cells and by the time of last follow-up. Figure 3 shows the cytotoxic potential of peptide-reactive CD8+ T cells and the course of tetramer-positive CD8+ T cells after adoptive transfer in 2 patients (no. 11 and no. 14) from this group in
conjunction with the level of minimal residual disease after transplantation until final follow-up. Only patient no. 1, who had undergone transplantation in advanced phase of CML and had received a low dose of donor T cells, experienced cytogenetic relapse and was successfully treated with IM.

In vitro-generated CD8+ T cells displaying marked cytotoxic potential without detectable increase in the frequency of CD8+ T-cell responses in vivo occurred in patients nos. 1 and 6, who experienced cytogenetic and molecular relapse, respectively. In both cases a major molecular response could be achieved by salvage therapy with IM and unmodified DLI. In the case of patient no. 2, neither in vitro cytotoxicity of the T cell preparation nor pentamer-positive CD8+ T cells in the recipient were detectable. Molecular relapse in this patient occurred 11 months after transplantation and was successfully treated with IM and DLI.

Pentamer-positive CD8+ T cells have been identified after transplantation in 6 recipients in whom donor CD8+ T cells with either no detectable in vitro cytotoxicity (nos. 4, 7, 8, 9, and 13) or a cytotoxic response against another peptide (no. 12) were infused. Three of these patients (nos. 4, 7, and 8) experienced molecular or hematologic relapse and had to be treated with IM and/or DLI. Patient no. 12, who had received PR1-reactive CD8+ cells but showed WT1/pentamer-positive cells during follow-up, has not achieved complete molecular remission. Sustained negativity for BCR-ABL has been maintained in patient nos. 9 and 13 for 42 and 39 months, respectively. Figure 4 summarizes the course of patient no. 9, in whom a considerable proportion of BCR-ABL-specific CD8+ T cells could be detected during follow-up. Panel A shows the representative heat map of KQSSKALQR/HLA-A3 pentamer+ CD8+ T cells detectable in PB of patient no. 9 three weeks after the third infusion of the in vitro-generated CD8+ donor T cell preparation. As depicted in panel 4B, this was accompanied by the detection of 1.5% ATGFKQSSK/HLA-A11/-A3 pentamer+ CD8+ T cells. The patient has remained in molecular remission more than 3 years after transplantation (panel 4C).
Survival

With a median follow-up of 45 months, the probability of overall and molecular relapse-free survival is 93% (CI, 79 - 100%) and 49% (CI, 22 - 76%), respectively (Figure 5A, B). Although the lack of a control arm precludes drawing any conclusions on the association between the specific intervention and clinical outcome, the favourable survival and the limited rate of molecular relapse are encouraging, in particular in the light of the older patient cohort included with high EBMT pre-transplantation risk scores. As mentioned above, only 1 patient succumbed to cerebral aspergillosis 3 months after transplantation. Six patients experienced molecular ($n = 4$), cytogenetic ($n = 1$), or hematologic relapse ($n = 1$). Five of them were successfully treated with IM, which was combined with unmodified donor lymphocyte infusions in 4 cases. Two of these patients have subsequently achieved molecular remission. A major molecular response has been reached in 3 cases, whereas 1 patient with molecular relapse has maintained a stable Bcr-Abl:Abl ratio of 0.12 without further intervention until 3 years after transplantation.

Discussion

Establishing GvL effects without inducing GvHD remains a major challenge after allogeneic SCT. This is especially true for patients with CML who are less frequently referred for transplantation due to the success of TKI therapy with IM or second-line compounds. Although scoring systems have been developed to estimate the individual risk of therapy-related death, most referring physicians are hesitant to recommend a procedure associated with considerable morbidity and mortality. Obviously, the aforementioned scoring systems were developed before the era of IM in a retrospective fashion and are therefore difficult to extrapolate into current therapeutic algorithms. All efforts to avoid these risks by employing in vitro or in vivo T-cell depletion have been offset by a significant increase in the incidence of relapse. Infusion of unselected donor T cells to treat relapse in leukemia is effective in a significant proportion of CML patients, but also causes significant morbidity due to GvHD even when incremental doses are infused. The encouraging clinical results of our study are
in line with the data reported by Elmaagacli and coworkers. They transplanted CD34+ purified
granulocyte colony-stimulating factor-mobilized allogeneic stem cells from matched sibling
donors, and infused unmodified donor T cells in increasing doses starting on day 120, to
preemptively encounter molecular or cytogenetic relapse. The high probability of survival
and limited incidence of acute and chronic GvHD in this series prompted us to use this
platform to prophylactically infuse ex vivo-activated donor T cells. Most importantly, this
strategy allowed avoidance of the use of pharmacologic immunosuppression, which would
suppress the activity of adoptively transferred T cells and reduce the antileukemic efficacy of
the overall approach. Stable hematopoietic engraftment could be achieved even in recipients
of transplants from unrelated donors. This of course requires an immunosuppressive
conditioning regimen. Besides TBI, thiotepa, and ATG, the use of cyclosporine before
transplantation may have ensured rapid and stable hematopoietic engraftment in all patients.
The omission of post-grafting cyclosporine and methotrexate contributed to a major reduction
in extramedullary toxicity compared to transplantation with T-cell-replete grafts. Further
reduction in the intensity of our protocol will have to be developed with caution, because less
intensive conditioning therapy followed by the infusion of T-cell-depleted donor cells has
been associated with a high incidence of relapse in CML patients.

The successful infusion of leukemia-reactive T-cell lines generated against leukemic
targets was described over 10 years ago in a patient with CML relapsing after allogeneic
SCT. Protocols to generate CD8+ T cells specifically targeted against hematopoiesis-
restricted mHAg ex vivo have subsequently been developed. Although considered to be
leukemia specific, a recent report has demonstrated that the adoptive transfer of ex vivo-
expanded mHAg-specific CTL can lead to significant pulmonary toxicity due to cross-
reactivity with antigens expressed in lung tissue. Again, clinical responses were observed,
but only a minority of T-cell lines or clones that had been prospectively isolated could be
infused at the time of hematologic relapse. Interestingly, peptide-based vaccination therapy
inducing T-cell responses against potential autoantigens like proteinase 3 or WT1 has so far
not been associated with clinically relevant toxicity or autoimmunity.
Our protocol allowed the induction of peptide-specific CD8+ T cells exhibiting marked cytotoxic activity in 50% (n = 7) of healthy donors. The frequency of detectable peptide-specific CD8+ T-cell responses against various epitopes may vary significantly. Whereas BCR-ABL-specific CD8+ T cells have rarely been detected or induced in healthy volunteers, this may be possible for up to 90% with regard to WT1. Because we did not infuse T-cell clones or purified antigen-specific T-cell populations, we cannot exclude the possibility that a proportion of the antileukemic efficacy may be linked to alloreactivity of T cells within bulk populations.

To decrease the risk of immune escape by target deletion we combined several antigens, whenever possible. In HLA-A2-positive donors, PR1 and WT1 were used concomitantly, but the responses observed were always stronger for WT1. This also correlated with a higher frequency of CD8+ T cells binding with the WT1/A2 pentamer during follow-up, whereas no significant increase in PR1-reactive CD8+ T cells was detectable even after adoptive transfer of in vitro-activated CTL. PR1-specific T-cell frequencies have been correlated with clinical response to interferon or after allogeneic SCT. Results of a recent vaccination trial using both WT1 and PR1 peptides together with adjuvant suggest that robust cellular immune responses can be induced against both epitopes in vivo, and that these responses correlate with eradication of residual disease. This may be different when CD8+ T cells with a minimal precursor frequency are primed with peptide-loaded antigen-presenting cells in vitro. In addition, the avidity of PR1-specific T cells induced in vitro may be closely related to the peptide concentration chosen, thereby suggesting that activation-induced cell death may contribute to the rapid deletion of these cells after infusion. Another reason could be the low level of target antigen in the first 6-8 weeks after conditioning therapy, which may hamper proliferation of infused CTL.

Besides leukemia-associated antigens, BCR-ABL represents a disease-specific target, which has been established as the ideal candidate for cellular immune therapies. Both MHC class I-restricted and 17-mer peptides have been described which are able to induce a CD8+ and CD4+ T-cell response after vaccination. In our series, the highest frequency of
BCR-ABL-specific CD8⁺ T cells was observed in response to treatment with HLA-A3/-A11-specific peptides. The HLA-B8-restricted peptide was used in 4 patients, and peptide-specific CD8⁺ T cells could be found in 2 of these patients.

Our data confirm a recent report in which BCR-ABL-specific CD8⁺ T cells could be detected in CML patients as well as in healthy donors, albeit in much lower frequencies. Interestingly, in this report an in vitro induction of higher frequencies of peptide-specific CD8⁺ responses was barely possible in CML patients but could be achieved in healthy donors, thus arguing for the presence of a tolerance mechanism in CML patients. This finding argues in favor of our approach using HLA-matched CD8⁺ T cells from healthy donors and allogeneic SCT as a platform for effective adoptive immunotherapy.

A recent report from Warren and coworkers suggests that the half-life of adoptively transferred CTL clones is about 7 days, and homing to the bone marrow may occur. Of course, the dynamics may be different in patients with minimal levels of residual disease at the time of infusion of polyclonal T-cell preparations. Although we can only speculate on the fate of infused T-cell preparations, we have indirect evidence of their biologic activity. Lineage-specific chimerism analyses revealed a steep increase in donor signals for CD8⁺ and CD4⁺ T cells following the first and second infusion of ex vivo-generated CTL. This increase in donor T-cell chimerism occurred 3 - 4 months earlier compared to a historical cohort of patients receiving T-cell-depleted SCT with no planned adoptive T-cell transfer. Therefore, we hypothesize that lymphodepletion induced by the conditioning regimen and the infusion of CD34⁺ donor cells allowed for a rapid peripheral proliferation of transferred T-cell preparations without any competition against existing T-cell populations and the lack of calcineurin inhibitors as pharmacologic immunosuppression did not jeopardize the proliferation of donor lymphocytes infused early after transplantation. Although the dose of ATG used in this trial is at the lower end of current dosing schedules, there might have been residual levels of ATG in the plasma of our patients potentially suppressing the activity of the first CTL dose on day 28. Pharmacokinetic data obtained in recipients of T-depleted transplants suggest that the levels of active ATG decrease below therapeutic levels between
8 and 38 days after transplantation\textsuperscript{42}. Therefore, no relevant interference with the second and third CTL infusion can be assumed.

The current approach can be improved in order to obtain increased numbers of T cells reactive against leukemia antigen-derived peptides and to include a higher proportion of patients with CML requiring transplantation after failure of conventional pharmacologic therapy. Thus, loading of DCs with synthetic overlapping long peptides comprising various epitopes for CD8\textsuperscript{+} T cells as well as CD4\textsuperscript{+} T cells can induce and expand both T cell subsets simultaneously, which can subsequently be administered to patients. This immunotherapeutic strategy combines the antitumor effects mediated by both T cell subsets and may induce more effective tumor-directed responses\textsuperscript{43}. Furthermore, peptides derived from additional leukemia antigens such as elastase, prame and hyaluronic acid receptor\textsuperscript{44-46} can be used to generate specific T cells. Purifying antigen-specific T cells by their secretion of interferon gamma or their binding to peptide/HLA complexes may increase the specificity of adoptive cellular therapy and thereby decrease the risk for acute and chronic GvHD\textsuperscript{47,48}. Another attractive approach would be the combination of vaccination and adoptive cellular therapy in order to maximize efficacy in the lymphopenic host after T-depleted allogeneic SCT\textsuperscript{49}.

In conclusion, this is the first study to demonstrate that the prophylactic infusion of BCR-ABL-, WT1-, and PR1-reactive CD8\textsuperscript{+} donor T cells after allogeneic SCT from matched sibling and unrelated donors is safe and feasible. In cases with detectable in vitro cytotoxicity and measureable peptide-specific CD8\textsuperscript{+} T cells in the recipient, molecular remission of Ph\textsuperscript{*} CML could be maintained for more than 4 years after transplantation, with no occurrence of acute or chronic GvHD. Given the minimal treatment-related morbidity and mortality observed so far in this cohort with predominantly high pre-transplantation risk scores, this strategy may represent a promising therapeutic option for CML patients with IM resistance or intolerance to TKI therapy.
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Contributions: M.B., M.S., E.P.R., and G.E. designed the study protocol; C.T., U.O., J.B., R.W., H.J., M.B. and M.S. performed the monitoring of immune monitoring and of minimal residual disease; A.K., U.P., S.T., J.R., M.W., and J.S. were responsible for clinical care and out-patient follow-up; M.B., M.P.B., and M.S. analyzed data; M.B. and M.S. wrote the paper; and all authors have read and approved the final version of the manuscript.

Conflict-of-interest disclosure: the authors declare no competing financial interests.

References


41. Rusakiewicz S, Madrigal A, Travers P, Dodi AI. BCR/ABL-specific CD8+ T cells can be detected from CML patients, but are only expanded from healthy donors. *Cancer Immunol Immunother.* 2009;58(9):1449-1457.


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M, male; F, female; Dx–Tx, interval between diagnosis of CML and transplantation (months); EBMT, European Group for Blood and Marrow Transplantation; CP, chronic phase; AP, accelerated phase; T315I, P-loop mutation of Bcr-Abl; HU, hydroxyurea; IF, interferon; IM, imatinib mesylate; DA, dasatinib; PCT, polychemotherapy; Tx, autologous transplantation.
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* 10<sup>6</sup>/kg; Pent, Pentamer.; NA, not administered; NT, not tested.
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WT1, wilms-tumor antigen 1; PR1, proteinase 3-derived peptide sequence; NT, not tested; * 10⁶/kg; Pent, Pentamer.; d, day, ND, not detectable; NA, not administered.
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CTL, cytotoxic T lymphocytes; E:T, effector:target ratio; WT1, Wilms’ tumor antigen 1; PR1, proteinase 3-derived peptide sequence; *, specificity of cytotoxic response or HLA+/peptide* CD8* T cells detectable by flow cytometry is provided in brackets whenever CTL with specificities for more than one peptide were infused;
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<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
<td>PCR neg.</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>4†</td>
</tr>
</tbody>
</table>

MMR, major molecular response (<0.1); cyt, cytogenetic relapse; Mol, molecular relapse; DLI, infusion of unmodified donor lymphocytes; IM, imatinib mesylate; cGvHD, chronic graft-versus-host disease; CNS, central nervous system; N.A., not applicable; †, death; pos., positive; neg. negative; ND, below the detection limit of the nested PCR assay (<1-2 x 10⁻⁶).
Figure legends

Figure 1: Conditioning regimen and schedule for CTL infusion

Panel A shows the conditioning regimen used before allogeneic stem cell transplantation (SCT). The regimen for patients aged 18-40 years consisted of hyperfractionated total body irradiation (TBI) with $6 \times 200$ cGy from days −9 to day −7, with lung shielding (maximum lung dose of 800 cGy). Patients older than 40 years received only $4 \times 200$ cGy. Fludarabine was infused at a daily dose of 40 mg/m$^2$ from days −6 to day −2. On the same days, 5 mg/kg antithymocyte globulin (ATG) Fresenius was infused over 4 h. Two doses of thiotepa (5 mg/kg) were infused over 4 h on day −5. Treatment with cyclosporine A was initiated on day −10 at a daily dose of 1 mg/kg until day −3. CD34$^+$-selected peripheral blood stem cells were infused on day 0 (SCT). Panel B shows the planned schedule of infusion of in vitro-generated CD8$^+$ T-cell preparations. Whenever possible, in vitro-cultured donor CD8$^+$ T cells were infused on days 28, 56, and 112. No post-grafting immunosuppressive therapy was administered.

Figure 2: T-cell chimerism and immune reconstitution

As shown in panel A, infusion of ex vivo-activated donor T cells on days 28, 56, and 112 was associated with a prompt increase in the percentage of donor CD4$^+$ and CD8$^+$ T cells in the peripheral blood of recipients. Six months after transplantation, all 13 patients who could be evaluated showed complete donor T-cell chimerism. When compared to a historical cohort of patients who did not receive prophylactic donor T-cell infusion after T-cell-depleted SCT (panel B), the increase in donor T-cell chimerism to levels >50% had occurred more than 3 months earlier. The absolute count of lymphocyte subsets is summarized in panel C. CD4$^+$ T cell numbers remained <200/µL within the first 12 months after transplantation, while CD8$^+$ T cells dominated during that period. CD19$^+$ B cells reached normal levels as soon as 3 months after transplantation, while CD3$^−$/CD56$^+$ NK cells peaked 2 months after transplantation and slowly declined for further 10 months.
Figure 3: In vitro cytotoxicity of CD8+ T cells, the frequency of peptide-specific CD8+ T cells in vivo, and BCR-ABL levels

In vitro cytotoxicity of infused cytotoxic T cells (CTL) was tested with peptide-loaded antigen-presenting cells (DCs or T2 cells) as targets. Panels A and D confirm antigen-specific cytotoxicity against an HLA-A3-restricted BCR-ABL-derived peptide (A) and an HLA-A2-binding WT1 peptide (D) in patient no. 11 and 14, respectively. The corresponding frequency of HLA/peptide tetramer-positive CD8+ T cells after transplantation and CD8+ T-cell infusion in each case is depicted in panels B and E. As shown in Figure 3C and F, BCR-ABL mRNA became undetectable in both patients and PCR negativity was maintained until the last follow-up.

Figure 4: Peptide+/HLA+ CD8+ T cells and BCR-ABL levels

Panel A shows the FACS heat map of KQSSKALQR/HLA-A3 pentamer+ CD8+ T cells detectable in the peripheral blood of patient no. 9 three weeks following the third infusion of the in vitro-generated CTL preparation. As demonstrated in panel B, this was accompanied by the detection of 1.5% ATGFKQSSK/HLA-A11/-A3 pentamer+ CD8+ T cells. The patient has remained in molecular remission throughout follow-up, with no signs of acute or chronic GvHD (panel 3C).

Figure 5: Overall and molecular relapse-free survival

The graphs depict Kaplan–Meier estimates of overall (A) and molecular relapse-free survival (B) for all 14 patients. The dashed line represents the respective 95% confidence interval.
Figure 1

A

Day

-10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0

Cyclosporine A 1 mg/kg

TBI 6 x 200cGy

Fludarabine 5 x 40 mg/m²

Thiotepa 2 x 5 mg/kg

ATG Fresenius 5 x 5 mg/kg

CD34+

SCT

B

CD34+ SCT

CTL

CTL

CTL

Conditioning

No immunosuppression

Day

0 +28 +56 +112
**Figure 2**

**A**  
CML patients with CTL  

Graph showing percent donor chimerism over days after transplantation.  
- CD4⁺  
- CD8⁺  
- CTL (down arrow)

**B**  
Historic control w/o CTL  

Graph showing percent donor chimerism over days after transplantation.  
- CD4⁺  
- CD8⁺

**C**  
Graph showing cell counts (10⁶/L) over days after transplantation.  
- CD4⁺  
- CD8⁺  
- CD3⁺/CD56⁺  
- CD19⁺
Figure 3

A. Patient no. 11

- MoDC
- BCR-ABL (ATGFKQSSK)
- BCR-ABL (KQSSKALOR)
- BCR-ABL (HGATFKQSSK)

B. Patient no. 14

- T2
- HIV-Peptide
- WT1 (RMFPNAPYL)

C. CTL

- PR1
- WT1
- BCR-ABL (A3)

D. CTL

- PR1
- WT1

E. CTL

- PR1
- WT1

F. CTL

- PR1
- WT1

- Ratio BCR-ABL to ABL

Day after transplantation

- 0.0001
- 0.001
- 0.1
- 1
- 10

Day after transplantation
Figure 5

A

Overall Survival (N=14)

B

Molecular Relapse-Free Survival (N=14)
Prophylactic transfer of BCR-ABL−, PR1−, and WT1-reactive donor T cells after T-cell–depleted allogeneic hematopoietic cell transplantation in patients with chronic myeloid leukemia

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