HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse

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Key Points
• Haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents high-risk acute leukemia relapse.
• The GVL effect is separated from GVHD even across major HLA barriers.

Introduction

In patients with acute leukemia (AL) at high risk of relapse because of unfavorable cytogenetics, molecular markers and disease status (complete remission [CR] ≥2), the most powerful postremission therapy is hematopoietic stem cell transplantation (HSCT) from a matched sibling (MSD) or unrelated donor (MUD).12-15 When patients do not have a MSD or MUD, unrelated cord blood (UCB)3 and haploidential-related donor (haplo)3 HSCTs are emerging as alternatives.

In eradicating malignancy, ie, the so-called graft-versus-leukemia (GVL) effect, clinical observations2,6 and experimental models3-9,16-18 established that the allogeneic immune system played a crucial role. Donor T cells recognize host alloantigens on leukemic cells, although hematopoietic-specific and leukemia-specific responses may also occur.10 They also mediate graft-versus-host disease (GVHD), a major cause of morbidity and mortality after HSCT.

In T-cell–replete HSCT, pharmacological immunosuppression for GVHD prophylaxis and treatment is nonspecific and only partially successful. More importantly, it may compromise the T-cell–induced GVL effect. Indeed, relapse is still the major cause of treatment failure in high-risk AL patients.11-14

To date, attempts to manipulate alloreactive T cells to spare normal cells while killing leukemic cells have been unsuccessful. In HLA-haploidentical transplantation, we reported that donor-derived T regulatory cells (Tregs), coinfused with conventional T cells (Tcons), protected recipients against graft-versus-host disease (GVHD). The present phase 2 study investigated whether Treg–Tcon adoptive immunotherapy prevents post-transplant leukemia relapse. Forty-three adults with high-risk acute leukemia (acute myeloid leukemia 33; acute lymphoblastic leukemia 10) were conditioned with a total body irradiation–based regimen. Grafts included CD34+ cells (mean 9.7 × 10^6/kg), Tregs (mean 2.5 × 10^6/kg), and Tcons (mean 1.1 × 10^6/kg). No posttransplant immunosuppression was given. Ninety-five percent of patients achieved full-donor type engraftment and 15% developed ≥grade 2 acute GVHD. The probability of disease-free survival was 0.56 at a median follow-up of 46 months. The very low cumulative incidence of relapse (0.05) was significantly better than in historical controls. These results demonstrate the immunosuppressive potential of Tregs can be used to suppress GVHD without loss of the benefits of graft-versus-leukemia (GVL) activity. Humanized murine models provided insights into the mechanisms underlying separation of GVL from GVHD, suggesting the GVL effect is due to largely unopposed Tcon alloantigen recognition in bone marrow. (Blood. 2014;124(4):638-644)
acute and chronic GVHD and favored posttransplant immunological reconstitution.24

To address the issue of leukemia relapse, the present study investigated whether Treg-Tcon adoptive immunotherapy provided a powerful Tcon-mediated GVL effect in the absence of GVHD.

**Patients and methods**

**Patient inclusion criteria**

The protocol was approved by the Umbria Regional Hospital Ethical Committee and registered as 0108. Adults (18-65 years) with high-risk AL were eligible for haplo-HSCT with Treg-Tcon adoptive immunotherapy if they did not have an MSD or MUD. Before enrollment, all patients provided written informed consent in accordance with the Declaration of Helsinki.

**Donors**

Donors included healthy family members with 1 HLA haplotype identical to the patient’s who were able to donate hematopoietic stem cells after treatment with granulocyte colony-stimulating factor (G-CSF) and undergo leukapheresis sessions for collecting hematopoietic stem cells, Tregs, and Tcons. A natural killer (NK)-alloreactive donor was preferentially selected when available (Table 1).

**Transplantation procedure**

Figure 1 illustrates the transplant timeline schema. Briefly, the conditioning regimen included total body irradiation (TBI), thiotepa, and fludarabine. The first 25 patients also received cyclophosphamide. To reduce extrahematological toxicity and the risk of veno-occlusive disease, instead of cyclophosphamide the other 18 patients were given alemtuzumab (8 patients) or thymoglobulin (12 patients) when alemtuzumab was withdrawn from patients with hematological diseases. Anti-T antibodies were administered 21 days before transplant to prevent interference with Treg-Tcon adoptive immunotherapy. All patients received freshly isolated donor Tregs on day −4, followed by a megadose of purified CD34⁺ cells and Tcons on day 0. The 4-day interval between Treg and Tcon infusions was in accordance with animal data indicating that early Treg administration provided greatest protection against GVHD.18 No pharmacological posttransplant GVHD prophylaxis was given. Patients were managed according to our standard haplo-HSCT protocol.25

**Graft processing**

Tregs and Tcons were collected from donors before they underwent G-CSF treatment of CD34⁺ cell collection. Two total blood volumes from a single leukapheresis procedure were processed with COBE Spectra (Terumo BCT, Table 1. Patient demographics

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Treg-Tcon</th>
<th>Historical controls</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Number of patients</td>
<td>43</td>
<td>114</td>
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<tr>
<td>M/F</td>
<td>20/23</td>
<td>63/51</td>
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</tr>
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<td>Age, y (median and range)</td>
<td>40 (range 18-65)</td>
<td>37 (range 8-66)</td>
<td>.8</td>
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<tr>
<td>Median time and range from diagnosis to transplant, CR1</td>
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<tr>
<td>Median length of remission and range, CR2</td>
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<td>7 mo (2-55)</td>
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<tr>
<td>Disease and status</td>
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<tr>
<td>ALL</td>
<td>10 (23%)</td>
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</tr>
<tr>
<td>1 CR</td>
<td>7</td>
<td>27</td>
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</tr>
<tr>
<td>≥II CR</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>WBC &gt;50 000/mmc at diagnosis for ALL</td>
<td>4</td>
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</table>

F, female; M, male; NA, not applicable; WBC, white blood cell.

Figure 1. Conditioning regimen and inoculum. (A) Twenty-five patients (September 2008-October 2009): 8 Gy in a single fraction at a fast dose rate with lung shielding on day −10, thiotepa (4 mg/kg per day) on days −10 and −9, fludarabine (40 mg/m² per day) from days −10 to −6, and cyclophosphamide (35 mg/kg per day) on days −7 and −6. (B) Eighteen patients (May 2010-December 2012): alemtuzumab lot #8403D 20 mg/m² and thymoglobulin 9-7 mg/kg.

BLOOD, 24 JULY 2014 • VOLUME 124, NUMBER 4 T REGULATORY CELLS IN HAPLOIDENTICAL TRANSPLANT 639
Lakewood, CO). Tcons were separated from peripheral blood mononuclear cells by negative selection using CliniMACS CD19 reagent (Miltenyi Biotec) and cryopreserved. Naturally occurring CD4\(^+\)CD25\(^-\)FoxP3\(^+\) Tregs were selected by means of a fully automated immunomagnetic procedure (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) by depleting the leukapheresis product of CD8\(^+\)CD19\(^-\) cells and then positively immunoselecting CD25\(^+\) cells. The initial leukapheresis products contained a median of 12.1 \(\times\) 10\(^6\) (range 5.4-18 \(\times\) 10\(^6\)) nucleated cells. After magnetic cell separation, a median of 272 \(\times\) 10\(^6\) (range 142-412 \(\times\) 10\(^6\)) Tregs was recovered.

Immediately after Treg and Tcon collection, donors were treated with G-CSF to mobilize peripheral blood progenitor cells. After collection, CD34\(^+\) cells were positively immunoselected using the CliniMACS device (Miltenyi Biotec).

The target numbers of Tregs, Tcons, and CD34\(^+\) cells were achieved using direct immunofluorescence with a panel of monoclonal antibodies as described previously. Overall, 3 days of apheresis were needed for each donor (1 for Treg and Tcon collection; 2 for CD34\(^+\) cells).

Suppression assays

Treg suppressive capacity was established as follows. Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled CD4\(^+\)CD25\(^-\) T lymphocytes were cultured in 96-well plates at 2 \(\times\) 10\(^4\) cells/well with phytohemagglutinin (PHA; 4 \(\mu\)g/mL; Biochrom) in the presence of varying numbers of CD4\(^+\)CD25\(^-\) (Tregs). For a CFSE-based measurement of proliferation, the suppressive capacity of Tregs toward responder cells in coculture (a Tcon:Treg ratio of 1:1 or 1:2) was expressed as the relative inhibition of the percentage of CFSE\(^-\) cells as follows: 100 \(\times\) (1 - %CFSE\(^-\)/CD4\(^+\)CD25\(^-\) T cells in coculture/ %CFSE\(^-\)/CD4\(^+\)CD25\(^-\) T cells alone).

Chimerism analysis and immunological studies

Chimerism was assessed on DNA extracted from peripheral blood samples by multiplex fluorescent short-tandem repeat analysis. Peripheral blood was collected weekly for the first 2 months and then monthly for cytometric lymphocyte subset immunophenotyping. Alloreactive NK cell subsets were phenotyped in donors and monthly in recipients after transplant. Function was analyzed in cytotoxicity assays against KIR ligand mismatched recipient target cells to determine the frequency of alloreactive NK clones.

End points and definitions

The primary end point was to assess the impact of Treg/Tcon immunotherapy in haploidentical transplantation on the cumulative incidence of posttransplant leukemia relapse. Disease status was assessed in bone marrow in all transplant recipients on days 30, 60, 90, 120, 180, 240, and 360, and then annually. Relapse was defined as disease recurrence according to the following: marrow morphology, flow cytometry, cytogenticities including fluorescence in situ hybridization and polymersome chain reaction for disease markers. The secondary end points were full donor type engraftment; probability of disease-free survival (DFS) based on patients who were alive without evidence of disease. Incidence of grades 2-4 and 3-4 acute GVHD on day +100; chronic GVHD at 1 year; cumulative incidence of nonrelapse mortality (NRM) (ie, death by any cause in the absence of disease relapse); GVHD mortality included patients with a history of GVHD who died from infections or organ failure during immunosuppressive therapy.

Historical controls

We compared outcomes of the present Treg-Tcon transplants with 114 high-risk CR1, \(\geq\)CR2 AL patients (50 acute lymphoblastic leukemia [ALL]; 64 acute myeloid leukemia [AML]; median age 37 years) (Table 1) from previous haploidentical-HSCT trials. All had received a similar conditioning regimen with TBI, thiotapec, cyclophosphamide/fluorouracil, and thymoglobulin that, unlike the present series, was administered during conditioning and not 21 days earlier. The inoculum included a megadose (mean CD34\(^+\) cells 10 \(\times\) 10\(^6\)kg \pm 2.1) of hematopoietic progenitor cells. The contaminating T lymphocytes ranged in number from 1 \(\times\) 10\(^6\) to 1 \(\times\) 10\(^8\)kg body weight.

Statistical analyses

Cumulative incidence estimates (evaluated by the Gray test) were used for relapse and nonleukemic mortality because they are competing risks. The Kaplan-Meier method evaluated event-free survival. The log-rank test assessed impact of disease (AML vs ALL), disease status, gender, age, pretransplant donor-recipient pair cytomegalovirus (CMV), and donor-recipient NK cells on event-free survival. Multivariate analysis (Cox regression model) also investigated the impact of these factors on nonleukemic mortality, relapse, and event-free survival. Two-sided \(P\) values <.05 were considered significant.

Mouse models

Colonies of nonobese diabetic-scidII2gnot (NSG) mice were bred at the Perugia University Animal House. All experiments were performed in accordance with the National Ethics Approval Document for animal experimentation.

NSG mice were irradiated with 3.5 Gy and infused IV with human primary AML cells (7 \(\times\) 10\(^6\)/mouse), human normal Ph1\(^+\) ALL cell line (SUP-B15, 2 \(\times\) 10\(^4\)/mouse), or human Burkitt-like cell line (Namalwa, 2 \(\times\) 10\(^4\)/mouse). After primary AML leukemia engraftment, mice were coinoculated with human Tcons (3 \(\times\) 10\(^6\)/mouse) and Tregs (3 \(\times\) 10\(^6\)/mouse). In mice treated with lymphoblastic cell lines, Tregs and Tcons were infused with leukemic cells. Controls were either untreated or infused with human Tcons (3 \(\times\) 10\(^6\)/mouse) or Tregs (3 \(\times\) 10\(^6\)/mouse). Tregs and Tcons came from the same donor and were HLA-mismatched with the primary leukemia and leukemia cell lines. At different time points, mice were evaluated for GVHD (by observation score and histopathology), leukemia infiltration, and T-cell homing (by flow cytometry with a combination of anti-human monoclonal antibodies: CD45, CD25, FoxP3, CD34, CD13 [eBioscience, CA]; CD3, CD56, CD8, CD4, CD14 [Miltenyi, Köln, Germany]; CD33, CD19, CD20, CD10 [Beckman Coulter, Marseille, France]; and CD117, CD7, kappa, lambda, and immunoglobulin M [Invitrogen, CA]). Each group comprised 5 mice. All experiments were repeated in duplicate. Results of duplicated experiments were combined.

Human CD3 T cells were purified from murine bone marrow by anti-human CD3-conjugated microbeads (Miltenyi) and assessed in a chromium release cytotoxicity assay with a 5:1 ratio as an effector against the targets (leukemia and autologous to leukemia PHA blasts).

Results

Patients and graft characteristics

Forty-three consecutive AL patients (median age 40 years; range 18-65) were treated between September 2008 and December 2012. Thirty-three had AML (18 CR1, 15 \(\geq\)CR2), 10 had ALL (7 CR1; 3 in \(\geq\)CR2). Twenty-four of these patients have already been reported in a previous article. All patients who were transplanted in CR1 were at high risk of relapse (8 FLT-3/ITD, 8 complex karyotypes, 4 with t(9:22), 2 primary induction failures, 1 secondary AML, 1 CR after second-line induction, and 1 with central nervous system and skin involvement at diagnosis).

All patients received a full haplotype mismatched graft that included CD34\(^+\) cells (mean 9.7 \(\times\) 10\(^6\)/kg \pm 3.1; mean contaminating T cells: 0.8 \(\times\) 10\(^6\)/kg \pm 0.4), Tregs (mean 2.5 \(\times\) 10\(^6\)/kg \pm 1), and Tcons (mean 1.1 \(\times\) 10\(^6\)/kg \pm 0.6). The Treg phenotype was FoxP3\(^+\), 81.01% \pm 16.47, Helios/FoxP3\(^+\) 54% \pm 8.4, and CD127\(^+\) 11.72% \pm 7.653. CD45RO\(^+\) cells were 90% and CD45RA\(^+\) cells 10%. Treg suppressive capacity in vitro was 67% \pm 22 (\pm standard deviation) (ratio Tcons/Tregs 1:2). Infused Tcons were 90.72% \pm 9.6 CD3\(^+\), 57.77% \pm 8.85 CD4\(^+\), and 31.21% \pm 8.59 CD8\(^+\).

Table 1 shows the demographic characteristics and disease status of the present series of patients vs the historical control group. Median age, the proportion of AML vs ALL cases, and remission
status were similar in the 2 cohorts. All patients in both cohorts who underwent transplantation in CR1 had high-risk features. Median time from diagnosis to transplantation was 6 months (range, 3-48 months in the present cohort) vs 5 months (range, 2-60 months in the historical controls); \( P = .4 \). For patients who underwent transplantation in CR2, duration of remission was 6.5 months (range, 1-60 months present series) vs 7 months (range, 2-55 months historical controls); \( P = .6 \).

**Engraftment and posttransplant immune recovery**

Forty-one of 43 patients achieved primary, sustained full-donor type engraftment. Neutrophils reached \( 1 \times 10^9/L \) (median 16 days; range 10-39). Platelets reached \( 20 \times 10^9/L \) and \( 50 \times 10^9/L \) (medians 13 and 15 days, respectively; ranges 8-48 and 13-60). The incidence of sustained engraftment was similar for Treg/Tcon vs historical control patients (95% vs 90%; \( P = .8 \)). Likewise, time to neutrophil and platelet recovery was not different between Treg/Tcon and historical controls (\( P = .11 \)).

There was a rapid, sustained increase in peripheral blood T-cell subpopulation recovery: CD4⁺ and CD8⁺ cells reached 50/µL at a median of, respectively, 30 days (range 16-65) and 25 (range, 12-90); 100/µL at a median of 40 (range 25-150) and 45 days (range 18-100); and 200/µL at a median of 55 (range 45-160) and 60 days (range 50-140). Compared with standard haploidentical transplantation, specific CD4⁺ and CD8⁺ for opportunistic pathogens such as *Aspergillus fumigatus, Candida albicans*, CMV, adenovirus, herpes simplex virus, and varicella zoster virus toxoplasma emerged significantly earlier (at each time point \( P < .0001 \)).²⁴

NK cells reached 200/µL at a median of 25 days (range 19-35) and 400/µL at a median of 45 days (range 22-70). Posttransplant alloreactive NK clones against KIR ligand–mismatched targets had a higher frequency than in historical controls (15 ± 6 vs 7 ± 5 at 1 month posttransplant).

**GVHD and NRM**

Only 6/41 patients (15%) developed ≥grade 2 acute GVHD. In 2, GVHD responded rapidly to a short course of immunosuppression, and only 1 of these patients developed chronic GVHD. Even though \( 1.1 \times 10^5/kg \) ± 0.6 Tcons were infused, the incidence of ≥grade 2 acute GVHD was similar (\( P = .2 \)) to the 11% in historical controls. They had received extensively T-cell–depleted grafts and antithymocyte globulin, which exerted additional in vivo T-cell depletion. Three patients (7.5%) died of GVHD (1 grade 2 acute GVHD, 2 grade 3-4 acute GVHD) compared with 10% (9%) in the historic cohort.

Overall, in the present series, the cumulative incidence of NRM was 0.40, which fell to 0.21 in 18 patients who received anti-T antibodies during conditioning. Causes of nonrelapse death were: veno-occlusive disease in 3 patients who were heavily pretreated until a few days before transplantation, GVHD (3), adenoviral infection (2), bacterial sepsis (2), HHV6 infection (2), multiorgan failure (1), and systemic toxoplasmosis (1). Lung (3) and central nervous system (1) aspergillosis occurred in patients with invasive fungal disease before transplantation.

**Relapse and DFS**

At a median follow-up of 46 months (range 18-65 months), only 2/41 evaluable patients have relapsed. Both had NPM1 FLT3⁺ AML in molecular relapse at time of HSCT and had received a transplant from non-NK-alloreactive donors. Risk of relapse was not related to modification of the treatment plan over time. In 114 historical controls, relapse occurred in 11/50 patients with ALL, in 10/32 patients with AML transplanted from non–NK-alloreactive donors, and in 4/32 AML patients who were transplanted from NK-alloreactive donors. Figure 2 reports relapse rates in the Treg-Tcon–treated patients and historical controls who underwent haplo-HSCT from NK-alloreactive and non–NK-alloreactive donors.

In the Treg-Tcon cohort, the cumulative incidence of relapse was significantly lower than in historical controls (0.05 vs 0.21; \( P = .03 \)). Multivariate analysis identified Treg-Tcon adoptive immunotherapy as the only predictive factor associated with a reduced risk of relapse (relative risk 0.06; 95% confidence interval, 0.02-0.35; \( P = .02 \)).

Twenty-three of 41 patients are alive and well with DFS probability of 0.56 at 18 months’ minimum follow-up. DFS in the control cohort was 0.39 (\( P = .07 \)), indicating a trend toward better survival under the Treg/Tcon protocol.

**Murine studies**

All mice that received myeloid or lymphoblastic leukemias, Tregs, and Tcons were rescued from leukemia and survived without GVHD. Cytolourimetric analysis of all organs and tissues showed disease eradication. All mice that received leukemic cells with or without Tregs died of leukemia within 60 to 75 days. Those that received leukemia cells plus Tcons developed severe GVHD and died within 60 days (\( P < .002 \) mice with Treg/Tcon infusions vs all others).

Human T cells from bone marrow in mice that were rescued from leukemia by Treg-Tcon adoptive immunotherapy exhibited similar cytotoxicity against both leukemic and PHA blast cells that were autologous to leukemia (Figure 3). This experiment was repeated twice.

**Discussion**

The present study is the first to analyze the impact of Treg-Tcon adoptive immunotherapy on AL disease eradication. Infusion of freshly isolated FoxP3⁺ Tregs in this large series of high-risk patients...
undergoing full-HLA haplotype mismatched transplantation controlled the allogeneity of up to 10^6/kg T lymphocytes, which is about 2 log more than the threshold dose for GVHD. Indeed, nearly 90% of patients were protected against GVHD, thus confirming previous results.24 Brunstein et al, who employed ex vivo expanded polyclonal Tregs as supplemental GVHD prophylaxis in double-unit UCB transplantation, also reported a significantly reduced incidence of grade 2 to 4 GVHD.28

Present results and those of Brunstein et al suggest that adoptive immunotherapy with Tregs does not compromise general immunity or blunted responses to infectious agents. Immunological reconstitution in our transplant recipients was stronger and faster than in the historical controls. Prevention of CMV disease improved markedly, with no CMV-related deaths, which had been one of the major causes of mortality in the historical control group.

Overall, NRM is still unsatisfactory in the present series but it is interesting to note it dropped sharply in patients who received anti-T antibides in the conditioning regimen. Better outcomes may have been linked to cyclophosphamide suspension because less extramedullary toxicity was associated with lack of severe veno-occlusive disease and multigang failure. In the future, to reduce the risk of posttransplant infection–related morbidity and mortality, it might be worth increasing the number of Tcons in the graft by means of CD3+CD45RO− cells,29 thus approaching what is termed a “designed” graft for haploidentical transplantation.30

One major concern about the use of Tregs in HSCT is their potential to suppress antineoplastic immune responses, as suggested by some studies in solid tumors31,32 and hematological malignancies.33,34 For example, Tregs accumulated in leukemic sites in mice, preventing adoptively transferred anti-AML T cells from proliferation and cytolyis.35 Conversely, interleukin-2 diphertheria toxin, which depleted Tregs expressing CD25, increased the number of cytotoxic T lymphocytes at tumor sites and resulted in tumor regression.36

On the other hand, in mismatched transplant mouse models, adoptive immunotherapy with Tregs and Tcons protected mice from GVHD without impairing Tcon control of neoplastic cell line expansion, such as A20 leukemic cells of BALB/c origin,22,23 BCL1 lymphoma,22 and P815 mastocytoma.23 The present study shows for the first time in humanized mouse models that human Treg-Tcon immunotherapy eradicated human primary myeloid leukemia and lymphoblastic leukemia cell lines without triggering GVHD.

In the present series of AL patients, infusion of Tcons under the Treg protective umbrella, in the absence of posttransplant pharmacological immunosuppression, guaranteed almost total control of AML and ALL relapse. The cumulative incidence of posttransplant leukemia relapse was 0.05 at a medium follow-up of 46 months, which is extremely low considering these patients were at high risk of relapse according to cytogenetics, molecular markers, and disease stage at transplant. In our historical controls, relapse rates were >30% in high-risk ALL patients and in AML patients who were not transplanted from NK alloreceptive donors. Similarly, relapse rates ranged from 28% to 40% in recent clinical trials of T-cell–replete HSCT, independently of whether the donor was a MSD, MUD, UCB, or haplo.11-14 In the present series, the T-cell–dependent GVL effect was so powerful that it masked the anti-AML activity exerted by posttransplant generation of alloreceptive donor-versus-recipient NK cell repertoires.35-39

The mechanisms underlying Treg suppression of GVHD with no loss of GVL activity are still obscure. In animal models, Edinger et al observed that Tregs inhibited early expansion of allogeneic donor T cells and their capacity to induce GVHD but did not inhibit cotransplanted Tcon activation and cytotoxicity against leukemia and lymphoma cells in vitro and in vivo. Thus GVL activity appears to rely mainly on Tcon activation rather than expansion and consequently requires transplantation of sufficient Tcons.22

In our murine models, human T cells that were harvested from bone marrow killed human leukemia cells and autologous PHA blasts in vitro, confirming their cytotoxicity activity was preserved and suggesting the T-cell–dependent GVL effect is mainly due to allogeneic recognition.

The GVL effect in the absence of GVHD could also be related to Treg migratory properties that are linked to expression of homing molecules for diverse sites. Tracking Treg in vivo dynamics in animal models of incompatible transplantation showed that upon infusion of polyclonal Tregs, alloantigen-specific Treg were activated and expanded in lymph nodes and then migrated to peripheral tissues (skin, gut, liver, lung).18 Thus alloantigen-specific Tregs controlled Tcon alloreactivity not only in lymph nodes but in nonlymphoid tissues, which are GVHD targets.

In the clinical transplant setting, one might hypothesize the GVL effect is due to largely unopposed Tcon alloreactivity in bone marrow. In fact, few infused donor Tregs are likely to migrate there because they mask the anti-AML activity exerted by posttransplant generation of alloreceptive donor-versus-recipient NK cell repertoires.22
without the risk of Tcon activity being inhibited, $1 \times 10^{9}$kg T-effector cells are sufficient to eradicate minimal residual disease. Consequently, the 30% to 35% incidence of relapse, which is generally reported by conventional T-cell-replete HSCT in high-risk AL patients, is practically eliminated.

Present results also demonstrate that adoptive immunotherapy with Tregs does not require ex vivo Treg expansion systems,\textsuperscript{42,43} which are cumbersome, expensive, and require good manufacturing practices facilities.

In conclusion, we are confident the innovative use of Tregs and Tcons is a major advance over conventional HSCT that, as leukemia immunotherapy, is associated with severe side effects and is only partially efficacious. In the future, the present strategy might be applied to exploit the GVL effect of Tcons that recognize minor histocompatibility antigens in matched-donor HSCT.

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Authorship

Contribution: M.F.M., M.D.I., L.R., and A.V. designed the study, oversaw the results, and drafted the paper; Y.R., F.A., and B.F. contributed to the design and interpretation of the study; A.C., A.T., A.P., M.S.M., and L.A. provided clinical data; and L.R., F.F., E.U., B.D.P., T.Z., R.I.O., D.C., and R.T. performed the in vitro studies and interpreted the results.

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