Depletion of Alloreactive T Cells by a Specific Anti–Interleukin-2 Receptor p55 Chain Immunotoxin Does Not Impair In Vitro Antileukemia and Antiviral Activity

By Daniela Montagna, Eric Yvon, Valeria Calcaterra, Patrizia Comoli, Franco Locatelli, Rita Maccario, Alain Fisher, and Marina Cavazzana-Calvo

The success of bone marrow transplantation (BMT) from HLA-disparate donors depends on the development of new strategies able, on one hand, to efficiently prevent graft-versus-host disease (GVHD) and, on the other hand, to protect leukemic patients from relapse and infections. Using an immunotoxin (IT) directed against the α chain (p55) of the human interleukin-2 receptor (RFT5-SMPT-dgA), we previously showed that it is possible to kill mature T cells activated against a specific HLA complex by a one-way mixed lymphocyte culture (MLC). The present study was performed to investigate whether this protocol of allodepletion affects the capacity of residual T cells to display antileukemia and antiviral activity evaluated by limiting dilution assays (LDA), measuring the frequency of cytotoxic T-lymphocyte precursors (CTLp) directed against autologous leukemic blasts (LB) and cytomegalovirus (CMV)- and Epstein-Barr virus (EBV)-infected target cells. Antileukemia activity was evaluated in peripheral blood mononuclear cells (PBMC) of 3 patients treated for acute myeloid leukemia who had developed a high frequency of LB-reactive CTLp after either autologous or allogeneic BMT. Results demonstrate that (1) depletion with RFT5-SMPT-dgA efficiently inhibited MLC; (2) fresh PBMC of patients yielded a high frequency of LB-reactive CTLp comparable to that of the mock-treated PBMC; and (3) effector cells obtained after alloreduction fully retained the capacity to lyse pretransplant LB. By contrast, the frequency of CTLp directed against patient’s pretransplant BM remission cells was always undetectable. Data obtained in 4 healthy donors showed that specifically alloreduced T cells recognized and killed autologous CMV-infected fibroblasts and autologous EBV-B lymphoblastoid cell lines. In conclusion, our data indicate that allodepletion using RFT5-SMPT-dgA efficiently removed alloreactive cells, while sparing in vitro antileukemic and antiviral cytotoxic responses.

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able to proliferate against third-party cells, Candida, and cytomegalovirus (CMV) antigens. Moreover, in vivo studies in a murine animal model showed that this particular T-cell depletion was efficient, at least partially, in preventing both graft rejection and GVHD in a complete haplotype-mismatched combination. However, so far, few data are available regarding the possibility to maintain antileukemia reactivity after elimination of alloreactive T cells.

The clinical and experimental data reported in the literature on the possibility of separating GVHD and graft-versus-leukemia reaction (GVLR) still remain controversial. We showed that cytotoxic T-cell clones reactive to autologous leukemic blasts (LB) but not to autologous bone marrow remission cells (BMRC) can be obtained from peripheral blood mononuclear cells (PBMC) of leukemic children by in vitro lymphocyte stimulation with autologous tumor cells and recombinant IL-2 (rIL-2). Moreover, we showed that cytotoxic T-lymphocyte precursors (CTLp), specifically reactive towards recipient LB, were undetectable or low in donor peripheral blood, although their frequency reached high values in the recipients after transplantation. This provided evidence for an in vivo expansion of donor T cells able to recognize leukemic recipient cells.

The aim of this study was to assess whether in vitro alloreactive T-cell depletion could affect the capacity of spared T cells to kill LB or virus-infected cells. To address these issues, we established a limiting dilution assay (LDA) to measure the frequency of CTLp directed against autologous LB, CMV-infected fibroblasts, and Epstein-Barr virus lymphoblastoid cell lines (EBV-LCL) before and after depletion of alloreactive T cells. Antileukemia activity was assessed in PBMC of 3 patients with acute myeloid leukemia (AML) who had developed a high frequency of LB-reactive CTLp after either autologous or allogeneic HSCT. PBMC of patients were activated in vitro in a one-way mixed lymphocyte culture (MLC) against PBMC of their father, depleted of activated T cells by an anti-CD25-ricin chain IT (RFT5-SMPT-dgA), and then tested for antileukemia activity. This new IT has been previously reported that cytotoxic T-cell clones reactive to alpha chain (IT-treated) were calculated as the percentage of the mock-treated sample.

Materials and Methods

Isolation of PBMC and BM cells from patients and healthy subjects. Three children with AML were studied to evaluate antileukemia activity after elimination of alloreactive T cells activated against paternal HLA-antigens. One patient (BP) had been treated with autologous BM transplantation (BMT), whereas patients ER and GA had received allogeneic BMT from HLA-identical sibling donors. Patients were analyzed 6 months after BMT, when the presence of sizeable values of LB-directed CTLp was documented. Persistence of antiviral activity was evaluated in PBMC of 4 healthy donors.

Cell preparation and cell line establishment. Heparinized BM aspirate containing greater than 90% LB was obtained from patients at the time of diagnosis and BMRC were collected after demonstration of complete hematological remission. PBMC of patients were collected 6 months after either allogeneic or autologous BMT, while patients were in remission. BMRC and PBMC were isolated by Ficoll-hypaque density gradient of anticoagulated whole blood, cryopreserved in fetal calf serum (FCS; Gibco Ltd., Paisley, UK) supplemented with 10% dimethyl sulfoxide (DMSO), and stored in liquid nitrogen.

In vitro antiviral and antileukemia activity after TCD of alloreactive cells. Immunocytotoxicity was evaluated in PBMC of healthy controls after allodepleted MLC. We showed that allodepletion using RFT5-SMPT-dgA was efficient, at least partially, in preventing both alloreactive T cells but did not significantly affect the cytotoxicity of RFT5-SMPT-dgA. After two washes, cells were resuspended at 10^6 cells/mL and used immediately for proliferation and LDAs.

Study of MLC inhibition. Two hundred microliters of treated and mock-treated cell mixtures (AB* and AA*) were plated in duplicate in 96-well, round-bottomed microtiter plates (Limbro; Flow Lab, Scotland) and incubated at 37°C in 5% CO2 until day 6.

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supplemented with 2 mmol/L L-glutamine, 50 µg/mL gentamicin, and 10% FCS (RPMI-FCS). Cells were continuously incubated at 37°C, 5% CO₂ for 3 to 4 weeks. Each week, 2 mL of culture medium was removed and 2 mL of fresh medium was added until growth of B-LCL was established.

LDA for evaluation of CMV and EBV-specific CTLp. To evaluate CTLp frequency to CMV-infected fibroblasts, fresh PBMC or cultured IT-treated and mock-treated cells obtained from healthy controls were seeded in a final volume of 200 µL in 96-well round-bottom microplates. Autologous fibroblast were infected with AD169 strain CMV following a previously described method. A decreasing number of responder cells (10⁵, 5 × 10⁴, 2.5 × 10³, 1.25 × 10³, and 0.6 × 10³) were stimulated with 2 × 10⁵ autologous infected fibroblasts. When the IT- or mock-treated cells were used as responders, 4 × 10⁴ irradiated (3,000 rads) were added as feeder cells. On days 4 and 9, 40 U/mL rIL-2 was added to the cultures. On day 12, the plates were accurately split as described above and tested against CMV-infected and mock-infected fibroblasts. For evaluation of EBV-specific CTLp, responder cells were stimulated with autologous irradiated B-LCL following a previously described method.

Cytotoxicity assay. At the end of the cultures, effector cells were assayed for cytolytic activity against ⁵¹Cr-labeled targets. Target cells included autologous LB, autologous BMRC, CMV-infected or mock-infected fibroblasts, and EBV B-LCL. Briefly, 2 to 3 × 10⁶ LB, autologous BMRC, and EBV B-LCL or 5 × 10⁵ fibroblasts were labeled with 100 to 200 µCi ⁵¹Cr for 2 hours, washed four times, and added to the wells. Plates were incubated for 5 hours at 37°C and then centrifuged at 200 g for 10 minutes. Finally, 100 µL of supernatant was collected from each well and counted for 1 minute in a gamma-counter. To provide necessary controls, spontaneous and total ⁵¹Cr release from target cells were also determined. Spontaneous release from all types of target cells was consistently less than 20%.

Calculation of CTLp frequency. Assay wells were defined as positive when ⁵¹Cr release exceeded the average plus 3 SD of control wells. The frequency of responding cells was determined by maximum likelihood estimation using a statistical program and the variance by the use of 95% confidence limits.

Generation of leukemia-reactive T-cell clones. LB-reactive effector cells of patient ER were recovered from pooled positive wells obtained from 10-day LDA of both untreated and allodepleted cultures and seeded into Terasaki trays at 0.3 cells/well in the presence of rIL-2 (200 U/mL), phytohemagglutinin (PHA; GIBCO; M-form 1:100), 2 × 10⁵ irradiated (7,000 rads) autologous LB, and 5 × 10⁵ autologous infected fibroblasts. After allospecific T-cell depletion, an apparent increase in LB-reactive CTLp frequency was observed in all patients analyzed, with frequencies being 1/2,480, 1/5,141, and 1/24,374, respectively (Fig 1). Undetectable frequencies of CTLp were found against autologous BMRC both before and after treatment with IT (data not shown).

Surface phenotype and cytolytic activity of LB-reactive TCC. Effector subsets of cytotoxic activity against recipient LB before and after T-cell–specific alloredepletion were characterized by cloning in limiting dilution pooled positive wells from patient ER. The growing clones were expanded and then screened and selected for their capacity to lyse recipient LB but not BMRC in a cytotoxicity assay. Different subsets of LB-reactive TCC could be defined on the basis of the surface phenotype. In untreated cultures (when effector cells were fresh PBMC), we were able to isolate 5 LB-reactive TCC. Two TCC were CD3+ /TCRβ+/CD8+ cells, 1 TCC was CD3+/TCRγδ+/CD8+, 1 TCC was CD3+/TCRβ+/CD4/CD8 double negative (DN), and the last was CD3+/TCRγδ+/CD4/CD8DN cells. Six TCC were obtained from cultures after depletion of alloreactive T cells: 4 of them were CD3+/TCRβ+/CD8+ lymphocytes, 1 TCC was CD3+/TCRγδ+/CD8+, and 1 displayed CD3+/TCRγδ+/CD4/CD8DN phenotype.

Some LB-reactive TCC derived from either untreated and IT-treated cultures could be expanded sufficiently to be tested in blocking experiments with anti-CD3 and anti-HLA class I

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Untreated (cpm x 10⁻⁹)</th>
<th>Treated (cpm x 10⁻⁹) (%)</th>
<th>Untreated (cpm x 10⁻⁹)</th>
<th>Treated (cpm x 10⁻⁹)%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (pt BP)</td>
<td>17.4</td>
<td>0.3 (1.7)</td>
<td>13.6</td>
<td>12.2 (70.5)</td>
</tr>
<tr>
<td>2 (pt ER)</td>
<td>47.4</td>
<td>0.9 (1.9)</td>
<td>43.6</td>
<td>51.6 (108)</td>
</tr>
<tr>
<td>3 (pt GA)</td>
<td>31.5</td>
<td>0.6 (1.9)</td>
<td>27.8</td>
<td>30.8 (96.7)</td>
</tr>
<tr>
<td>4 (donor 1)</td>
<td>16.4</td>
<td>0.3 (1.8)</td>
<td>25.2</td>
<td>24.1 (150)</td>
</tr>
<tr>
<td>5 (donor 2)</td>
<td>51.7</td>
<td>0.5 (0.9)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6 (donor 3)</td>
<td>21.3</td>
<td>0.4 (1.9)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7 (donor 4)</td>
<td>13.1</td>
<td>0.2 (1.5)</td>
<td>7.2</td>
<td>19.1 (146)</td>
</tr>
</tbody>
</table>

Data reported represent the mean of duplicate cultures for each independent experiment. Standard deviation was less than 10% of mean values.
MoAbs (Table 2). The killing capacity of the αβ⁺/DN TCC, γδ⁺/DN TCC, and γδ⁺/CD8⁺ TCC was not affected by the addition of the two MoAbs. In contrast, the addition of these MoAbs strongly reduced cytolytic activity of αβ⁺/CD8⁺ TCC.

**Evaluation of virus-specific CTLp frequency.** The capacity to kill autologous CMV-infected fibroblasts was evaluated in PBMC from 2 healthy controls. As shown in Fig 2, we observed that allodepletion did not affect the frequency of CMV-specific precursors, because in the first subject it was 1/19.834 in untreated cells versus 1/15.212 after allodepletion and in the second subject frequencies were 1/5.052 and 1/4.566, respectively. Reactivity against mock-infected fibroblasts was always undetectable. Similar results were obtained against EBV-LCL (Fig 3). In particular, CTLp frequencies were 1/25.899 and 1/17.719 in PBMC of the 2 donors tested, and they reached values of 1/3.799 and 1/26.442 after T-cell allodepletion, respectively.

**DISCUSSION**

The present study demonstrates that allospecific T-cell depletion obtained by using RFT5-SMPT-dgA does not affect the frequency of CTLp directed against autologous LB in transplanted patients who had developed a sizeable frequency of LB-directed CTLp after transplantation. Moreover, the spared T cells maintained a high frequency of CTLp directed against EBV- and CMV-infected cells. Treatment with RFT5-SMPT-dgA results in strong inhibition of primary MLC response from PBMC of subjects used to evaluate antileukemia and antiviral activities, whereas residual T cells maintain the capacity to proliferate in the presence of third-party stimulating cells.

In a previous study, we demonstrated that specific depletion of donor T cells reactive against the host led to a reduction in the incidence and severity of GVHD after haploidentical HSCT in mice. The persistence of antileukemia activity after removing host-specific reactive T cells is crucial for successful allogeneic HSCT in patients with malignancy. A number of clinical and experimental data indicate that mature adoptively transferred donor lymphocytes are involved in the generation of both GVHD and GVLR after HSCT. In particular, clinical evidence suggests the existence of a strong relationship between GVHD and GVLR. After allogeneic HSCT from HLA-matched sibling donors, it is believed that non–MHC-encoded minor histocompatibility antigens (mHAg) are involved in both GVHD and GVLR activities. Although widely distributed mHAg account for the GVLR associated to GVHD, tissue-restricted or leukemia-specific antigens can elicit a specific GVLR, and

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**Table 2. Effect of the Addition of Anti-CD3 and Anti-HLA Class I MoAbs on the Cytolytic Activity of LB-Reactive TCC Against Autologous Pretransplant LB**

<table>
<thead>
<tr>
<th>TCC No.</th>
<th>Phenotype</th>
<th>% of Specific Lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>+Anti-CD3</td>
</tr>
<tr>
<td>6⁺</td>
<td>TCR αβ⁺/CD8⁺</td>
<td>35</td>
</tr>
<tr>
<td>8⁺</td>
<td>TCR αβ⁺/CD8⁺</td>
<td>68</td>
</tr>
<tr>
<td>13⁺</td>
<td>TCR αβ⁺/CD8⁺/CD4⁺</td>
<td>26</td>
</tr>
<tr>
<td>15⁺</td>
<td>TCR γδ⁺/CD8⁺</td>
<td>26</td>
</tr>
<tr>
<td>2⁺</td>
<td>TCR αβ⁺/CD8⁺</td>
<td>58</td>
</tr>
<tr>
<td>7⁺</td>
<td>TCR αβ⁺/CD8⁺</td>
<td>36</td>
</tr>
<tr>
<td>15⁺</td>
<td>TCR γδ⁺/CD8⁺</td>
<td>15</td>
</tr>
<tr>
<td>18⁺</td>
<td>TCR γδ⁺/CD8⁺/CD4⁺</td>
<td>35</td>
</tr>
</tbody>
</table>

Results are expressed as the percentage of specific lysis at an E:T ratio of 5:1.

* TCC isolated from untreated cultures.
† TCC isolated from IT-treated cultures.
it has been demonstrated that both CD4+ and CD8+ CTL recognizing mHAg in a classical MHC-restricted fashion can be generated in vitro. In particular, mHAg-specific CD8+ CTL can display strong lysis of mature leukemia cells as well as suppress, together with CD4+ mHAg-specific CTL, the growth of clonogenic leukemia precursor cells.

We have previously described how CTLp reactive towards recipient LB can be detected in PBMC of children receiving allogeneic HSCT. LB-reactive CTLp developed in the first months after transplant and persisted for at least up to 18 months in patients in hematological remission. The GVLR we observed in vitro seems not to be strictly correlated with GVHD, given that the emergence of a high frequency of LB-reactive CTLp was not related to the occurrence and severity of clinical GVHD. Results of the present work, obtained in patients who have developed in vivo antileukemia activity, demonstrate that, notwithstanding depletion of alloreactive T cells, a high frequency of precursor cells able to kill LB is still present among spared lymphocytes. Our data confirm results recently reported by Mavroudis et al, who demonstrated the maintenance of antileukemia reactivity after depletion of alloreactive T cells through an IT targeting the α-chain of IL-2 receptor. Moreover, evaluation of LB-reactive TCC documents that clones derived from allodepleted cultures exhibit surface phenotype and functional characteristics similar to clones derived from untreated cultures. In particular, we demonstrate that LB-reactive, CD3+/CD8+, or CD3+/CD4-CD8 DN TCC expressing either TCR αβ or γδ are involved in the generation of in vitro antileukemia activity obtained from untreated and T-cell allodepleted cultures. These results confirm the data on LB-directed TCC previously obtained in children who have received allogeneic HSCT. They demonstrate that several types of CTL clones can contribute to antileukemia activity in vitro, because we also isolated, together with CD3+/CD8+ MHC-restricted TCC, TCC expressing TCR αβ or γδ, whose cytolytic activity is not affected by the addition of MoAb directed against HLA class I antigens and the CD3 complex.

The isolation of a number of HLA-unrestricted TCC that use a TCR/CD3-independent recognition pathway suggests that some T-lymphocyte subsets may use alternative mechanisms to kill malignant cells. These nonconventional recognition and activation pathways could be due to the variable expression of surface molecules on LB used as stimulator and target cells. In particular, LB of patients used in this study express neither CD80 nor CD86, whereas LB-directed TCC displayed a CD28+ phenotype (data not shown). It is indeed well known that CD80-CD86 costimulation pathway plays a critical role in HLA-restricted, TCR-mediated activation of T cells. The inefficient delivery of crucial costimulatory signals may allow the development of alternative pathways of target cell recognition. We can, therefore, hypothesize that both HLA-restricted CTL directed towards mHAg and other CTL subsets, such as those expressing γδTCR and using a TCR/CD3-independent activation pathway, are involved in mediating GVLR in vitro and in sustaining in vivo anticancer immunosurveillance.
Persistence of a high frequency of LB-directed CTLp after removal of alloreactive T cells in patients receiving HSCT provides further support to the possibility that GVLR and GVHD can be at least partially separated and that some lymphocyte subsets are able to selectively recognize leukemia-specific antigens, heat-shock proteins, or overexpressed tissue-restricted molecules on tumor cells. Whether these lymphocytes can contribute in vivo to the eradication or immune control of clonogenic malignant cells, as well as the capacity of this approach to prevent GVHD occurrence in human beings, remains to be proven in a clinical study.

In a previous work, we have shown that the proliferative response of IT-treated cells toward candidin and CMV, as well as cytotoxic activity against EBV, was conserved. In the present study, we demonstrate that the number of cytotoxic precursor cells evaluated by LDA and directed against both CMV-infected fibroblasts and EBV-LCL are not affected by such T-allospecific depletion. It has been previously reported that (1) among the population of T cells specific for allogeneic MHC, there is a mix of virgin and experienced cells; (2) TCRs are broadly cross-reactive; and (3) many T cells specific for environmental antigens also cross-react on foreign MHC. On the basis of these data, it could be theoretically hypothesized that T-cell allodepletion be associated with reduction of the number of T cells specific for viral antigens. On the contrary, present data demonstrate that virus-specific precursor cells are not impaired by the strategy of T-cell depletion here described.

In conclusion, our data indicate that treatment with RFT5-SMPT-dgA is able to delete alloantigen specific T cells while sparing in vitro antiviral and antileukemia activity. Use of this IT is now under evaluation in a phase I clinical trial aimed at testing the feasibility of this approach for extending the applicability of allogeneic HSCT from HLA-disparate donors.

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