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Abstract

In previous studies, we showed the possibility of expanding *in vitro* polyclonal CTL lines directed against patient leukemia cells using effector cells derived from both HLA-matched and HLA-mismatched hematopoietic stem cell donors. Some CTL lines, especially those derived from an HLA-disparate donor, displayed residual alloreactivity against patient nonmalignant cells. In this study, we evaluated the possibility of separating *in vitro* CTLs with selective graft-versus-leukemia (GVL) activity from those potentially involved in the development of graft-versus-host disease (GVHD) through single T-cell cloning of antileukemia polyclonal CTL lines. We showed that CTLs that were expanded from a single T-cell clone (TCC), able to selectively kill leukemia blasts and devoid of alloreactivity towards nonmalignant cells, can be obtained from antileukemia alloreactive polyclonal CTL lines. TCCs expressed a wide repertoire of different T-cell receptor (TCR)-Vβ families, mainly produced IFNγ and interleukin 2, irrespective of CD8 or CD4 phenotype, and could be extensively expanded *in vitro* without losing their peculiar functional features. The feasibility of our approach for *in vitro* separation of GVL from GVH reaction opens perspectives for using TCCs, which are selectively reactive towards leukemia blasts, for antileukemia adoptive immune therapy approaches after hematopoietic stem cell transplantation, in particular from HLA-mismatched donors. (Cancer Res 2006; 66(14): 7310-6)

Introduction

Allogeneic hematopoietic stem cell transplantation has been employed for many years as part of the treatment for hematologic malignancies, in particular of acute leukemia (1, 2). The therapeutic effect of allogeneic hematopoietic stem cell transplantation is largely dependent on the graft-versus-leukemia (GVL) effect exerted by donor-derived, immunologically active cells transferred with the graft, which importantly contribute to eradicate leukemia cells surviving the preparative regimen, thus preventing posttransplantation leukemia recurrence (3).

For many patients in need of allogeneic hematopoietic stem cell transplantation, one of the major limitations of this treatment is the lack of an immediately available HLA-matched donor. For this reason, T cell–depleted hematopoietic stem cell transplantation using an HLA-haploidentical family donor has been increasingly used to treat patients who cannot find an HLA-matched, either related or unrelated, donor (4). Although this type of transplantation has the advantage of being immediately applicable in almost all patients, it is associated with prolonged impairment of immune recovery, predisposing patients to development of life-threatening infections (4, 5). Moreover, the absence of the T cell–mediated GVL effect renders the recipients of a T cell–depleted allograft more susceptible to leukemia relapse (6), although a GVL effect displayed by donor natural killer (NK) cells can, at least partially, compensate this lack of specific alloreactivity when an HLA-disparate NK-alleicative relative is employed as a donor (7–9).

Adoptive immune therapy with donor leukocyte infusion represents one of the most effective approaches to treat leukemia relapse occurring after allogeneic hematopoietic stem cell transplantation from an HLA-matched donor. The greatest donor leukocyte infusion–induced GVL effect has been observed in patients with chronic myeloid leukemia (CML) whereas a relatively low benefit has been reported in patients relapsing with acute leukemia (7, 10, 11). Moreover, donor leukocyte infusion may be associated with life-threatening complications [i.e., myelosuppression and especially graft-versus-host disease (GVHD)], which occur with high frequency and particular severity after transplantation from donors other than an HLA-matched sibling (10, 11).

The development of new techniques for generating and expanding *in vitro* leukemia-specific T cells devoid of alloreactive capacity against nonmalignant cells could offer a useful adoptive immune therapy approach for preventing or treating leukemia relapse occurring after allogeneic hematopoietic stem cell transplantation, without increasing the risk of severe GVHD. However, one of the major limitations of *in vitro* generation of donor-derived, leukemia-reactive polyclonal CTL lines is the difficulty of obtaining T cells unable of inducing the development of GVHD, particularly when the donor and recipient are HLA disparate.

In previous studies, we showed the possibility of generating and expanding *in vitro* CTL lines directed towards different types of either acute leukemia blasts or myelodisplastic cells, derived from both HLA-matched and partially matched donors (12, 13). Most of the antileukemia CTL lines we generated displayed very low levels of residual alloreactivity directed against patient nonmalignant...
cells (i.e., phytohaemagglutinin-induced T-lymphoblastoid cell lines and fibroblasts; ref. 13). However, some CTL lines, especially those derived from an HLA-mismatched donor, displayed a sizeable cytotoxic capacity against patient nonmalignant cells and were defined as alloreactive CTL lines (13). Cytolytic activity of alloreactive, antileukemia CTL lines towards patient nonmalignant cells was, indeed, lower than that observed against patient leukemia blasts, suggesting that at least a fraction of CTLs could be selectively directed against leukemia blasts.

The aim of the present study was to test the possibility of separating in vitro T cells able to mediate a GVL effect from those potentially involved in the development of GVHD through single T-cell cloning of alloreactive antileukemia polyclonal CTL lines. We show that CTLs that were expanded from a single T-cell clone (TCC), able to selectively kill leukemia blasts and devoid of alloreactivity towards patient nonmalignant cells, can be recovered from hematopoietic stem cell transplantation donor-derived, antileukemia polyclonal CTL lines. Several CD8+ or CD4+ TCCs, expressing a wide array of different TCR-V\(\beta\) families, and mainly producing IFN\(\gamma\) and interleukin (IL)-2, could be extensively expanded in vitro without losing their peculiar functional features.

Materials and Methods

Patients. Five patients with acute leukemia [three with acute myeloid leukemia (AML) and two with acute lymphoblastic leukemia (ALL)], given an allograft from either an HLA-identical sibling (two children) or an HLA-partially matched relative (three children), were included in the study. Antileukemia CTL lines were derived from peripheral blood mononuclear cells (PBMC) obtained from their respective donors. The institutional review board of Pediatric Haematology-Oncology, Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo, Pavia approved the study. Written informed consent for use of PBMCs and leukemia blasts for this study was obtained from both donors and patients. Clinical characteristics of patients and donors are reported in Table 1.

Stimulator and target cells. Leukemia blasts, used as stimulators for ex vivo cultures and as targets for cytotoxicity assays, were prepared from heparinized bone marrow aspirates (containing >90% leukemia blasts) obtained from patients either at diagnosis or leukemia relapse. Mitogen-stimulated, patient-derived or donor-derived T-lymphoblastoid cell lines used as control targets for the GVH reaction in cytotoxicity assays were established by stimulation of cryopreserved pretransplant or posttransplant PBMC, respectively, using a method previously described (14). Fibroblasts, also used as control targets, were obtained from a punch skin biopsy and cultured as previously described (13). Patient-derived or donor-derived bone marrow remission cells were obtained from patients before or after transplantation, respectively, during routine controls and after demonstration of complete hematologic remission.

Induction of antileukemia polyclonal CTL lines. Dendritic cells and antileukemia CTL lines were generated as previously described (12, 13). Briefly, dendritic cells were generated using CD14+ PBMCs from hematopoietic stem cell transplantation donors; donor-derived, CD8-enriched cells, obtained from PBMC after negative depletion using anti-CD4 magnetically labeled microbeads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany), were cocultured with donor-derived dendritic cells, irradiated (20,000 rad) patient leukemia blasts, and irradiated (3,000 rad) donor-derived CD4+ lymphocytes. Recombinant human IL-7 (10 ng/mL; R&D Systems Europe) and 10 pg/mL IL-12 (R&D Systems Europe, Abington, United Kingdom) were added to the culture at day 0. After 7 to 10 days, cultures were restimulated with irradiated recipient leukemia blasts and irradiated adherent PBMCs as feeder cells. Adherent feeder cells included >80% CD14+ cells. Two days later, 25 units/mL recombinant human IL-2 (RIL-2; Chiron, Emeryville, CA) was added to the cultures. The same protocol was used for each successive round of stimulation.

Isolation and expansion of antileukemia T-cell clones. Effector cells derived from antileukemia polyclonal CTL lines recovered after three rounds of leukemia-specific stimulation were cloned at 0.3 per well in Terasaki plates (Nunc Brand Products, Roskilde, Denmark) in the presence of rIL-2 (100 units/mL), phytohemagglutinin-L (8 \(\mu\)g/mL; Boehringer, Mannheim, Germany), and allogeneic irradiated (3,000 rad) feeder cells as previously described (15). After 12 to 14 days of culture, all growing wells were harvested and expanded in the presence of IL-2, phytohemagglutinin, and allogeneic irradiated feeder cells. TCCs thus obtained were tested for their capacity to lyse patient leukemia blasts in a \(^{51}\text{Cr}\)-release assay. Thereafter, leukemia blast–directed TCCs were further characterized for specificity by evaluating their reactivity against patient T-lymphoblastoid cell lines and fibroblasts.

Antileukemia TCCs able to selectively lyse patients’ leukemia blasts were further expanded using a cycle of antigen-independent rapid expansion (13) through the use of irradiated allogeneic feeder cells (2 \(\times\) 10\(^6\)/mL), anti-CD3 antibody (30 ng/mL OKT3; Ortho, Raritan, NJ), and repeated addition of rIL-2 (50 units/mL every 3–4 days).

Table 1. Patient and donor characteristics

<table>
<thead>
<tr>
<th>Donor/recipient pair no.</th>
<th>CTL line number</th>
<th>Patient disease</th>
<th>Gender</th>
<th>Type of donor employed</th>
<th>Age at time of hematopoietic stem cell transplantation (patient/donor), y</th>
<th>HLA typing of the donor/recipient pair</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LES001.3</td>
<td>AML, FABM2</td>
<td>46, XX, t(6;9)</td>
<td>F/M</td>
<td>PMFD</td>
<td>7/35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R = A 24.2 B 40,14,1 C 03,03 DRB1 04,04</td>
</tr>
<tr>
<td>2</td>
<td>LTE002.3</td>
<td>ALL, CD10+</td>
<td>46, XX, t(9;22)</td>
<td>M/F</td>
<td>PMFD</td>
<td>5/30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R = A 32,03 B 35,18,1 C 03,03 DRB1 11,14</td>
</tr>
<tr>
<td>3</td>
<td>LGM004.3</td>
<td>AML, FABM5</td>
<td>46, XY</td>
<td>M/F</td>
<td>HLA-identical sibling</td>
<td>14/12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R = A 33,03 B 35,18,1 C 04,12 DRB1 01,15</td>
</tr>
<tr>
<td>4</td>
<td>LPE002.3</td>
<td>AML, FABM2</td>
<td>46, XX</td>
<td>F/M</td>
<td>PMFD</td>
<td>3/40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R = A 02,32 B 34,40,1 C 03,04 DRB1 08,11</td>
</tr>
<tr>
<td>5</td>
<td>LCL001.3</td>
<td>ALL CD5+, CD7−</td>
<td>46, XY</td>
<td>M/F</td>
<td>HLA-identical sibling</td>
<td>12/16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>R = A 3,32 B 17,35,1 C 04,06 DRB1 13,03</td>
</tr>
</tbody>
</table>

Abbreviations: F, female; M, male; PMFD, partially-matched family donor; D, donor; R, recipient.
Induction and expansion of antileukemia T-cell lines and clones according to good manufacturing practice criteria. In two donor/recipient pairs, we evaluated the possibility of selecting and expanding leukemia blast–specific TCC according to good manufacturing practice (GMP) criteria. In particular, we employed CellGro DC Medium (Cell Genix, Freiburg, Germany) for generation of dendritic cell whereas CellGro Stem Cell growth Medium (Cell Genix) was used for induction and expansion of antileukemia T-cell lines and clones. Granulocyte macrophage colony-stimulating factor was Leucomax (Novartis Farma, Basel, Switzerland). Dedicated lots of recombinant human IL-4, IL-7, and IL-12 (R&D Systems Europe) were employed. Instead of recombinant IL-2, we used Proleukin (Aldesleukin, Chiron) for clinical use. For antigen-independent expansion, we used Orthoclone OKT3 (muramab-CD3; Ortho Biotech, Bridgewater, MA). All reagents were employed at the recommended experimental conditions. CD14+, CD8-enriched, and CD4+ populations were obtained by using CliniMACS CD14 and CliniMACS CD4 microbeads (Miltenyi Biotec).

Flow cytometry analyses. The monoclonal antibodies (mAb) used in this study included FITC- or phycoerythrin-labeled anti-Leu-4 (CD3), anti-Leu-3a (CD4), anti-Leu-2a (CD8), anti-Hle-1 (CD45), anti-perforin, and anti-TCR-V\(h\) families (BD Bioscience, Mountain View, CA). Evaluation of T-lymphocyte subset phenotype, TCR-V\(h\) chains, and intracellular perforin expression was done by direct immunofluorescence according to previously reported methods (16, 17). Cell population flow cytometry analysis was done on a FACScalibur flow cytometer and data were calculated using CellQuest software (BD Bioscience).

For cytokine detection at the single-cell level, TCCs were stimulated with 25 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma, Milan, Italy) plus 1 µg/mL ionomycin (Sigma) for 4 hours in the presence of Brefeldin A (10 µg/mL; Sigma). PMA/ionomycin–stimulated and nonstimulated cells were fixed and permeabilized with Fix and Perm solution (Caltag Laboratories, Valter Occhiena, Turin, Italy), following the instructions of the manufacturer, and stained with FITC or phycoerythrin mAbs specific for IL-2, IFN-\(\gamma\), IL-4, and IL-10 (BD Bioscience; ref. 18).

\({}^{51}\)Cr-release cytotoxicity assay. Target cells included patient-derived leukemia blasts, T-lymphoblastoid cell lines, bone marrow remission cells and fibroblasts, and donor-derived T-lymphoblastoid cell lines and bone marrow remission cells. Antileukemia polyclonal CTL lines were tested in an 8-hour cytotoxicity assay and TCCs in a 4-hour cytotoxicity assay as previously described (12). Briefly, in all experiments, we tested cytotoxic activity at effector/target (E/T) ratios ranging from 40:1 to 0.1:1. Blocking experiments of antileukemia TCCs cytolytic activity were done with anti–HLA class I or anti–HLA class II mAbs (12).

Results

Induction and characterization of antileukemia polyclonal CTL lines. Five antileukemia polyclonal CTL lines were generated from CD8-enriched lymphocytes obtained from a family donor, either HLA mismatched (LES001.3, LTE002.3, and LPE002.3) or HLA identical (LGM004.3 and LCL001.3). Lines LPE002.3 and LCL001.3 were generated using reagents, medium, and standard operating procedures fulfilling GMP criteria. Antileukemia CTL lines cytolytic activity was evaluated after the third round of leukemia-specific stimulation. CTL lines were simultaneously tested against patient leukemia blasts and patient nonmalignant cells (T-lymphoblastoid cell lines and fibroblasts). All polyclonal CTL lines were able to efficiently kill patient leukemia blasts (Fig. 1). CTL lines LGM004.3 and LCL001.3 derived from an HLA-identical sibling showed very low reactivity (<15% lysis) at the highest E/T ratio against patient nonmalignant cells. By contrast, CTL lines LES001.3 and LTE002.3 derived from an HLA-mismatched family donor displayed sizeable levels of

Figure 1. Cytotoxic activity of antileukemia CTL lines towards recipient leukemia blasts and nonmalignant recipient cells. Results of the cytolytic activity of the five antileukemia CTL lines after the third round of stimulation. CTL lines were simultaneously tested against recipient leukemia blasts (●), recipient T-lymphoblastoid cell lines (●), and recipient fibroblasts (▲). Cultures were tested 7 to 10 days after each round of stimulation at different E/T ratios.

Figure 2. Selection of antileukemia TCCs. A to C, results obtained after cloning of antileukemia CTL lines LES001.3, LTE002.3, and LGM004.3, respectively. Antileukemia CTL lines, obtained after the third round of leukemia-specific stimulation, were cloned by limiting dilution, and among the total number of TCCs, TCCs able to lyse patients leukemia blasts were defined as leukemia blast–directed TCCs. TCC/T-LCL, TCCs killing both leukemia blasts and T-lymphoblastoid cell lines; TCC/FB, TCCs killing leukemia blasts and fibroblasts; TCC/T-LCL/FB, TCCs killing leukemia blasts, T-lymphoblastoid cell lines, and fibroblasts.

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cytotoxicity against patient nonmalignant cells; although the reactivity was lower than that observed against leukemia blasts, especially at the lowest E/T ratio, this suggests that at least a fraction of the cells could be selectively directed against leukemia blasts. Phenotypic analysis done after the third round of stimulation showed that in the polyclonal CTL lines LTE002.3, LPE002.3, and LCL001.3, there was a prevalence (>85%) of CD3+/CD8+ lymphocytes whereas CD3+/CD4+ cells were <20%. On the contrary, the CTL line LES001.3 showed the prevalent expansion of CD4+ T cells, which increased from 3% before stimulation to 60% after three rounds of stimulation. No significant differences were observed in the magnitude or in the kinetics of generation between antileukemia CTL lines generated according to the previously described experimental approach (LES001.3, LTE002.3, and LPE002.3), exhibited a wide specificity, capable of killing patient T-lymphoblastoid cell line (range, 65-215), fibroblasts (range: 90-120), or both target cells, T-lymphoblastoid cell line/fibroblast (range 70-120; lysis >20% at E/T ratio of 1:1). However, 64, 38, and 55 TCCs derived from LES001.3, LTE002.3, and LPE002.3 CTL lines, respectively, were selectively able to lyse only patient leukemia blasts and were operationally defined as leukemia blast–specific TCCs. The majority of TCCs (280 of 318 and 140 of 167), obtained from the non-alloreactive antileukemia polyclonal CTL lines LGM004.3 and LCL001.3, were leukemia blast–specific TCCs; they were selectively directed against leukemia blasts and were devoid of alloreactivity towards patient nonmalignant cells.

Also in this case, because the number of cells present in each clone was variable, we defined these as "leukemia blast–specific cytotoxicity against patient nonmalignant cells; although the reactivity was lower than that observed against leukemia blasts, especially at the lowest E/T ratio, this suggests that at least a fraction of the cells could be selectively directed against leukemia blasts. Phenotypic analysis done after the third round of stimulation showed that in the polyclonal CTL lines LTE002.3, LPE002.3, and LCL001.3, there was a prevalence (>85%) of CD3+/CD8+ lymphocytes whereas CD3+/CD4+ cells were <20%. On the contrary, the CTL line LES001.3 showed the prevalent expansion of CD4+ T cells, which increased from 3% before stimulation to 60% after three rounds of stimulation. No significant differences were observed in the magnitude or in the kinetics of generation between antileukemia CTL lines generated according to the previously described experimental approach (LES001.3, LTE002.3, and LPE002.3), exhibited a wide specificity, capable of killing patient T-lymphoblastoid cell line (range, 65-215), fibroblasts (range: 90-120), or both target cells, T-lymphoblastoid cell line/fibroblast (range 70-120; lysis >20% at E/T ratio of 1:1). However, 64, 38, and 55 TCCs derived from LES001.3, LTE002.3, and LPE002.3 CTL lines, respectively, were selectively able to lyse only patient leukemia blasts and were operationally defined as leukemia blast–specific TCCs. The majority of TCCs (280 of 318 and 140 of 167), obtained from the non-alloreactive antileukemia polyclonal CTL lines LGM004.3 and LCL001.3, were leukemia blast–specific TCCs; they were selectively directed against leukemia blasts and were devoid of alloreactivity towards patient nonmalignant cells.

Also in this case, because the number of cells present in each clone was variable, we defined these as "leukemia blast–specific...
TCC,” displaying >25% lysis against patient leukemia blast and <10% lysis against patient-derived nonmalignant cells. However, to confirm levels of specific lysis, leukemia blast–specific TCCs were further tested at various effector target ratios (5:1, 1:1, and 0.2:1) against patients leukemia blast, patient-derived nonmalignant cells, and donor-derived cells before further expansion. All TCCs selected displayed <10% lysis against donor-derived and patient-derived nonmalignant cells whereas the lysis against patient leukemia blasts ranged between 40% and 80% (mean of 57% at E/T ratio of 1:1). Figure 3 reports results of the three representative TCC.

**Rapid expansion of leukemia blast–specific TCCs.** Leukemia blast–specific TCCs were further expanded with a round of antigen-independent stimulation using OKT3, allogeneic feeder cells, and IL-2. After 15 to 20 days of culture, TCCs were expanded >100 times the number of cells seeded at the beginning of cultures. In particular, for each TCC, 10⁶ cells were seeded and the recovery at the end of the culture ranged between 1.1 x 10⁸ and 1.7 x 10⁸ cells. Evaluation of expanded TCCs cytotoxic activity showed that all of them maintained their capacity to selectively lyse leukemia blasts and were still devoid of alloreactivity towards patient nonmalignant cells. Fig. 4 shows the expansion magnitude (Fig. 4A) and cytotoxic activity against patient leukemia blasts and fibroblasts (Fig. 4B) displayed by representative TCCs derived from the two antileukemia TCC. It is noteworthy that, based on the expansion capacity displayed by antileukemia TCCs, we can assume that by expanding the total cell number available, we could obtain for each TCC a range of 2 x 10⁸ to 8 x 10⁸ cells at the end of the culture. No significant difference was observed in the magnitude of expansion between TCCs expanded under experimental conditions and those generated and maintained in compliance with GMP.

**Phenotypic and functional characterization of leukemia blast–specific TCCs.** Leukemia blast–specific TCCs deriving from antileukemia CTL lines LES001.3, LTE002.3, and LGM 004.3 were analyzed for surface phenotype, TCR-Vβ repertoire usage, and intracellular cytokine production. Thirty-seven of 64 TCCs derived from polyclonal CTL line LES001.3 were CD3+/CD8+ whereas 27 of 64 displayed a CD3+/CD4+ phenotype. The presence of a consistent percentage of CD4+ TCCs was in keeping with the considerable expansion of CD4+ T cells observed in the polyclonal CTL line. TCCs derived from the polyclonal CTL line LTE002.3 were mainly CD3+/CD8+ (30 of 38); the remaining TCCs were CD3+/CD4+. All TCCs derived from CTL line LGM 004.3 were CD3+/CD8+.

Evaluation of TCR-Vβ repertoire showed that leukemia blast–specific TCCs were heterogeneous in terms of different Vβ family expression (Fig. 5A-C).

<table>
<thead>
<tr>
<th>Antileukemia CTL lines*</th>
<th>CD8±</th>
<th>CD4⁺</th>
<th>CCR7⁻</th>
<th>CD45RA⁻</th>
<th>CCR7⁻</th>
<th>CD45RA⁺</th>
<th>IFNγ⁺IL-2⁺</th>
<th>IFNγ⁺IL-2⁺IL-4⁺</th>
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<tbody>
<tr>
<td>LES001.3</td>
<td>37 of 64</td>
<td>27 of 64</td>
<td>44 of 64</td>
<td>20 of 64</td>
<td>42 of 50</td>
<td>8 of 50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTE002.3</td>
<td>30 of 38</td>
<td>8 of 38</td>
<td>24 of 38</td>
<td>14 of 38</td>
<td>26 of 30</td>
<td>4 of 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LGM004.3</td>
<td>70 of 70</td>
<td>0 of 70</td>
<td>51 of 70</td>
<td>19 of 70</td>
<td>48 of 60</td>
<td>12 of 60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Antileukemia CTL lines from which TCCs were derived.
† Number of positive TCCs/total number of TCCs evaluated.
TCCs expressed variable levels of intracellular perforin, in keeping with their capacity to kill leukemia blasts. These data are in accordance with those obtained from polyclonal antileukemia CTL lines from which TCCs were derived as we documented in the lines a consistent presence of effector cells displaying an effector/memory, or terminally differentiated, phenotype. In these antileukemia CTLs, a small percentage (~5%) of central memory T cells was also identified (data not shown).

Evaluation of intracellular cytokine production showed that the majority of both CD8+ and CD4+ TCCs evaluated produced IFNγ and IL-2, irrespective of CD4 or CD8 phenotype. In addition, ~20% were able to produce IL-4 but did not produce IL-10 (Table 2).

Blocking experiments, done with anti–HLA class I mAbs, showed that the cytotoxic activity of all CD8+ TCC was strongly inhibited by anti–HLA class I mAbs. Twelve CD8+ TCCs, derived from different anti-leukemia CTL lines were tested and the mean inhibition was 82% (range, 77-90%). On the contrary, only 4 of 12 CD4+ TCCs showed a sizeable reduction of cytotoxic activity after incubation with anti–HLA class II mAbs (mean inhibition, 54%; range, 30-65%).

### Discussion

During the last few years, much effort has been directed at identifying strategies capable of obtaining and enhancing a GVL response not associated with the development of GVHD. One of the most intriguing approaches is the generation of T-cell lines or clones preferentially directed against leukemia cells. In particular, the possibility of infusing TCCs, rather than polyclonal T-cell lines, represents an attractive and more sophisticated way for the use of these cells in adoptive immune therapy, as specificity and effector functions of infused cells can be more precisely defined. In the present study, we first showed that single T-cell cloning of antileukemia polyclonal CTL lines is feasible, allowing the generation of a large number of donor-derived TCCs able to kill patient leukemia blasts. The high proportion of antileukemia TCCs that we were able to isolate among the total number of TCCs grown from each antileukemia CTL line indicates that polyclonal CTL lines contain a sizeable frequency of CTLs directed against leukemia blasts.

We also found that it is possible to select and expand in vitro TCCs with different specificities, separating T cells able to mediate a GVL effect from those potentially involved in the development of GVHD. In fact, we can distinguish TCCs capable of selectively killing patient leukemia blasts from those directed against cells derived from tissues that can be a target for GVHD, such as skin fibroblasts, or against cells deriving from the hematopoietic system, such as T-lymphoblastoid cell lines. Antileukemia CTLs also able to lyse T-lymphoblastoid cell lines, but not patient fibroblasts, represent an interesting approach in adoptive immune therapy for treating leukemia relapse after allogeneic hematopoietic stem cell transplantation (20). Although we cannot exclude that TCCs directed against T-lymphoblastoid cell lines but not against fibroblasts can also be safely used in adoptive immune therapy approaches, we decided to focus on the characterization of leukemia-specific TCCs selectively directed against patient leukemia blasts. A number of leukemia-specific TCCs, which interestingly displayed a wide repertoire of TCR-Vβ families, were isolated from the alloreactive antileukemia CTL lines, thus suggesting that leukemia-specific T cells are not derived from the expansion of a few clones but represent a polyclonal response elicited by in vitro priming of donor T-lymphocytes with patient leukemia blasts. This peculiar feature of leukemia-specific TCC to express a polyclonal TCR-Vβ repertoire is relevant because the infusion of cells with multiple specificities should, in principle, diminish the possibility of selecting for escape variants, likely induced by the poor immunogenicity of leukemia blasts in vivo. Moreover, the applicability of our approach does not depend on the presence of either a defined HLA specificity or a disparity for certain minor histocompatibility antigens between donor and recipient. It must be noted that, although target antigens recognized by antileukemia CTLs are not known, antileukemia TCCs here described could be also used for the identification and molecular cloning of leukemia-associated antigens.

CD8+ T cells likely play a major role in specific tumor killing. However, results obtained in patients affected by either AML or CML, as well as by various types of solid tumors, suggest that CD4+ T cells are also involved in antitumor effector activity (21, 22). Results of our study, documenting the possibility of expanding both CD8+ and CD4+ leukemia-specific TCCs, provide further evidence that both CD3+/CD8+ and CD3+/CD4+ T cells can cooperate in mediating antileukemia cytotoxic activity. Although CD4+ TCCs isolated with our approach displayed lower levels of antileukemia cytotoxic activity than CD8+ TCCs, our data further support the role played by CD4+ T cells not only in providing help for optimal priming and expansion of antitumor CD8+ CTL (23, 24) but also as active effectors of the immune response.

The rapid expansion of TCCs with relatively low doses of IL-2 and allogeneic feeder cells allows the generation of a large number of clinically suitable leukemia-specific TCCs, which maintain their specificities. After isolating these clones, they may be pooled to obtain a polyclonal, leukemia-specific CTL line devoid of alloreactivity.

The demonstration of the feasibility of generating and expanding a large number of antileukemia TCCs devoid of alloreactivity against patient nonmalignant cells from donors HLA-disparate with the recipient, in compliance with a GMP protocol, is of clinical relevance. In fact, the use of these cells in adoptive immune therapy protocols could mediate the elimination of residual leukemia blasts surviving the preparative regimen in patients given a T cell–depleted hematopoietic stem cell transplantation from an HLA-partially-matched donor. It is, in fact, known that patients with leukemia given T cell–depleted hematopoietic stem cell transplantation from an HLA-partially-matched family donor may be exposed to an increased risk of posttransplant relapse, especially when affected by ALL (4) and/or when the donor does not display NK alloreactivity towards recipient cells (8).

An important question in adoptive immune therapy is on the fate of infused cells and, in particular, the in vivo homing of extensively in vitro expanded CTLs and their capacity to proliferate in vivo in response to antigens. T cells can be divided into different populations of effector and memory cells that play distinct roles in the immune response and that are characterized by a particular pattern of surface markers, chemokine receptors, and cytokine secretion (19–23). Although a measurable percentage of cells, present in our antileukemia CTL lines, displayed a central memory phenotype (25), we could not isolate TCCs with this phenotype. However, most of the TCCs we isolated displayed an effector/memory phenotype despite periodic restimulation and long-term culture. We speculate that the infusion of antileukemia TCCs devoid of alloreactivity, displaying an effector/memory phenotype, characterized by rapid effector function, could be a useful tool to...
control the growth of malignant cells, without inducing GVHD, during the early posttransplant period.

Although the approach described here is quite labor-intensive and requires at least 2 months to obtain a sizeable number of leukemia-specific TCCs, it permits us to finely characterize the reactivity of the cells to be infused for clinical adoptive immunotherapy strategies, aimed at controlling minimal residual disease or, possibly, at treating initial leukemia relapse after hematopoietic stem cell transplantation also when the donor is HLA mismatched. Future clinical trials should allow the definition of the optimal number of TCCs to be infused and the most suitable time interval between each infusion.

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