Advances in Brief

Infusion of CD4+ Donor Lymphocytes Induces the Expansion of CD8+ Donor T Cells with Cytolytic Activity Directed against Recipient Hematopoietic Cells

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Abstract

Purpose: Donor lymphocyte infusions (DLIs) provide effective therapy for patients with relapsed chronic myeloid leukemia after allogeneic bone marrow transplantation. Previous studies have suggested that depletion of CD8+ T cells from the infused donor lymphocytes can reduce the incidence of graft-versus-host disease associated with DLI without reducing antileukemia activity. In this situation however, the immune effector cells responsible for tumor rejection have not been identified. The goal of this study was to characterize these effector populations.

Experimental Design: We studied three representative patients with relapsed chronic myeloid leukemia who achieved complete molecular remission after receiving CD8+ T-cell-depleted DLI from HLA-identical sibling donors. Effector T cells were characterized in patient samples after in vitro stimulation and functional assessment. T-cell clones relevant to the immune response were then isolated and further characterized.

Results: Analysis of peripheral blood samples collected after DLI indicated the presence of a high frequency of circulating host-reactive cytolytic CD8+ T cells secreting IFN-γ. These HLA class I-restricted CTLs were specific for recipient minor histocompatibility antigens (mHAs) because they did not recognize target cells of donor origin. One CTL clone was further expanded in vitro and shown to recognize a broadly expressed mHA presented by HLA-B5701. Using a molecular approach, we demonstrated that this clone was expanded in peripheral blood and marrow after DLI. It was not detected before DLI.

Conclusions: These results indicate that CD4+ DLI elicits a potent allogeneic response mediated by mHA-specific CD8+ T cells.

Introduction

After administration of myeloablative therapy, infusion of allogeneic bone marrow or cytokine mobilized peripheral blood cells leads to the engraftment of pluripotent donor stem cells and the subsequent reconstitution of all hematopoietic functions. The presence of large numbers of mature donor T cells in the bone marrow or peripheral stem cell graft contributes to the reconstitution of cellular immune function and also enhances the elimination of residual leukemia cells (1-3). However, in addition to these beneficial outcomes, donor T cells are also the primary mediators of GVHD and thus contribute to substantial toxicity after allogeneic BMT (4-6). Depletion of T cells from the donor marrow markedly reduces the incidence and severity of GVHD after allogeneic BMT and obviates the need for additional immunosuppressive therapy in most patients. Nevertheless, removal of donor T cells also adversely affects the elimination of residual leukemia cells and results in increased risk of relapse after BMT (2, 7, 8).

The critical role of donor T cells in eliminating tumor cells is also evident in the effectiveness of DLI for patients with relapsed leukemia after allogeneic BMT. These patients frequently respond to single infusions of mature donor lymphocytes without additional therapy (9, 10). The effectiveness of DLI was first demonstrated after infusion of relatively large numbers of polyclonal T cells including both CD3+CD4+ and CD3+CD8+ cells. As expected, GVHD has been the primary toxicity associated with infusion of normal donor lymphocytes (9-11). In efforts to reduce this toxicity, previous clinical studies have demonstrated that clinical responses are maintained after depletion of CD3+CD8+ cells and selective infusion of relatively low numbers of CD3+CD4+ donor T cells (12). Although polyclonal CD3+CD4+ donor T cells maintain GVL activity, the depletion of CD3+CD8+ donor cells significantly reduces the incidence and severity of GVHD (12-14). This observation suggests that GVHD and GVL may represent distinct immune responses. This may occur through activation of the donor lymphocytes which elicits a potent allogeneic response mediated by mHA-specific CD8+ T cells.
different effector T-cell mechanisms or, alternatively, by targeting distinct sets of antigens expressed by different recipient cells.

In conjunction with the elimination of relapsed leukemia, several previous studies have pointed out that complete remissions after DLI are invariably associated with the elimination of mixed hematopoietic chimeraism (13, 15, 16). This conversion to complete donor hematopoiesis often becomes evident at the time of antitumor response, but the development of clinical GVHD is not required. These consistent observations suggest that the DLI-induced immune response targets recipient mHAs expressed on both tumor cells and normal recipient hematopoietic cells. Although the mHAs targeted in both GVL and GVHD reactions are not fully characterized, these data support the hypothesis that the distinction between GVL and GVHD responses may primarily be due to the varying levels of expression of these antigens in different tissues (17, 18).

The present studies were conducted to better characterize the effector cells that mediate tumor rejection after DLI. Experiments were performed in a series of three representative patients with relapsed CML who received CD3⁺CD4⁺ DLI. Because recipient mHAs were known to be involved in the DLI response, we focused our investigation on T-cell subsets that would have targeted these antigens. Samples of PBMCs collected at the time of clinical response were examined to determine whether host-reactive CD3⁺CD8⁺ effector T cells were expanded in vivo. Although the DLI had been depleted of CD8⁺ T cells before infusion, we found evidence that the tumor rejection response was nevertheless mediated by the expansion of CD3⁺CD8⁺ cytotoxic T cells of donor origin. This observation suggests that the infusion of donor CD4⁺ T cells initiates a coordinated immune response that includes the generation of conventional CTLs directed against recipient mHAs.

Patients and Methods

Patient Characteristics and DLIs. Clinical characteristics of the three patients studied in this report are summarized in Table 1. All three patients underwent allogeneic BMT for the treatment of CML in chronic phase. Patients received myeloablative conditioning and marrow cells from HLA-identical sibling donors that were depleted of CD6⁺ lymphocytes. This CD8⁺-depleted donor lymphocytes (3 × 10⁷ CD4⁺ T cells/kg) from the same sibling that had donated marrow for the initial transplant. Results of the clinical protocol for DLI and the method for CD8⁺ T-cell depletion have been described previously (13). No additional treatment for leukemia was given after DLI. Clinical protocols for stem cell transplantation and DLI were approved by the Human Subjects Protection Committee of the Dana-Farber Cancer Institute. Blood and bone marrow samples were collected from donors and patients before and after DLI for in vitro experiments.

Hematopoietic Chimerism Assays. Molecular assays used to determine hematopoietic chimeraism for patients 1 and 2 have been described previously (16). Briefly, PCR amplification using sets of primers for polymorphic microsatellites was carried out with genomic DNA samples to identify a locus capable of distinguishing donor and recipient cells. Once an informative locus was identified, genomic DNA extracted from pre- and post-DLI samples was amplified using specific primers (one of which was conjugated to a fluorescent dye), electrophoresed on an automated 377 DNA sequencer (Applied Biosystems, Foster City, CA), and analyzed using GeneScan software (Perkin-Elmer Cetus Instruments, Emeryville, CA). The proportion of donor DNA in the sample was determined by comparing the results to those obtained with standard mixtures of donor and recipient DNA (ranging from 10:90 to 90:10). For patient 3, chimeraism was monitored by assessing the presence of a SNP found to be divergent between donor and recipient. PCR primers (Biotin-TTCAAGGCTCTGTCAGTG, forward; AAGCAAA-AACAGAAGAAACAA, reverse; and ACAACAACAAACCCACACAG, probe) flanking a SNP in the lipoprotein lipase gene (National Center for Biotechnology Information dbSNP ID 285) were used to amplify genomic DNA extracted from patient samples obtained before and after treatment. The biotinylated PCR product was purified, and single-strand DNA was obtained using streptavidin-coated magnetic beads. The sequence of the purified single-strand DNA was determined using the pyrosequencing method according to the manufacturers protocol (Pyrosequencing, Uppsala, Sweden; Ref. 20). This quantitative method allowed us to determine the percentage of DNA containing each divergent nucleotide in samples obtained after transplant.

Cell Lines and Culture Conditions. Donor and recipient EBV-transformed B-cell lines were obtained by incubating PBMCs from donor or pre-BMT recipient samples with superantigen from EBV-producing B95-8 Marmoset cells. EBV-B cell

Table 1. Patient characteristics and responses to DLI

<table>
<thead>
<tr>
<th>Patient</th>
<th>Stage of disease at the time of DLI</th>
<th>Time from BMT to DLI (months)</th>
<th>Age at DLI (yrs)</th>
<th>Amount of CD3⁺CD4⁺ T cells infused</th>
<th>Pt/Dnr sex</th>
<th>Time to cytogenetic response (wks)</th>
<th>Time to molecular response (wks)</th>
<th>Time to GVHD (wks)</th>
<th>GVHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cytogenetic relapse</td>
<td>27</td>
<td>43</td>
<td>3 × 10⁷ cells/kg</td>
<td>F/M</td>
<td>14</td>
<td>54</td>
<td>12</td>
<td>Acute GVHD</td>
</tr>
<tr>
<td>2</td>
<td>Hematologic relapse</td>
<td>42</td>
<td>28</td>
<td>3 × 10⁷ cells/kg</td>
<td>M/M</td>
<td>18</td>
<td>23</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>Hematologic relapse</td>
<td>37</td>
<td>50</td>
<td>3 × 10⁷ cells/kg</td>
<td>M/M</td>
<td>10</td>
<td>13</td>
<td>No</td>
<td>Limited chronic skin</td>
</tr>
</tbody>
</table>

*Pt/Dnr, patient/donor.*
lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, glutamine (4 mm), sodium pyruvate (1 mm), HEPES (10 mm), and antibiotics. HEL92.1.7, U937, HL-60, K562, and KG-1 cell lines (American Type Culture Collection, Manassas, VA) were cultured in the same medium. Normal T-cell lines and clones were cultured in RPMI 1640 supplemented with 10% heat-inactivated human AB serum, glutamine, sodium pyruvate, HEPES, and antibiotics (referred to as T-cell medium) with additional IL-2 (100 units/ml). They were re-expanded as needed on feeder cells consisting of irradiated recipient EBV-B cells (1.5 x 10^6 cells/well) and allogeneic PBMCs (7 x 10^4 cells/well) in the presence of IL-2.

**Stimulation and Generation of T-cell Lines and Clones.**

T-cell stimulation was performed by incubating 5 x 10^5 PBMCs from a patient sample collected after DLI with 1 x 10^6 irradiated recipient EBV-B cells (60 Gy) in 1.5 ml of T-cell media supplemented with IL-2 (100 units/ml). One-third of the media was replaced with fresh media containing IL-2 every 3 days, and the culture was split to maintain a cell concentration of approximately 0.5-1 x 10^6 cells/ml. After 1 week of stimulation, cells were harvested, labeled with anti-CD3-FITC and anti-CD8-PE Mab (Beckman Coulter, Brea, CA), and sorted with an Epics ELITE ESP cell sorter (Beckman Coulter). Purified CD3+CD8+ T cells (94–98% in different experiments) were cultured for an additional 4–7 days in T-cell medium and IL-2 before being tested in cytotoxicity assays. To generate T-cell clones, CD3+CD8+ T cells obtained after a 2-week stimulation were cloned by limiting dilution on feeder cells consisting of irradiated recipient EBV-B cells (1.5 x 10^6 cells/well; 60 Gy) and allogeneic PBMCs (7 x 10^4 cells/well; 35 Gy) plus IL-2 (100 units/ml). All T-cell clones described in this report were harvested after limiting dilution cloning from 96-well plates with <30 growing wells. Clones were re-expanded as described above.

**Cytotoxicity Assays.** Target cells were labeled with 51Cr for 1 h at 37°C, washed three times, and plated at 2000 cells/well in conical bottomed, 96-well microplates. CTLs were added at varying E:T ratios, and 51Cr release was measured in the supernatant after 4 h of incubation at 37°C. In blocking experiments, target cells were incubated with W6/32 Mab (anti-HLA class I; American Type Culture Collection) or B1.23.2 (anti-HLA-B and -C alleles) 1 h before adding the effector cells.

**IFN-γ Secretion Assays.** IFN-γ secretion by T-cell clone 6E4 was assessed after 48 h of effector cells were cultured in 96-well U-bottomed microplates with 5 x 10^4 stimulator cells in 150 μl of T-cell medium with IL-2 at 100 units/ml. After a 24-h incubation, supernatant (50 μl/well) was harvested, and the release of IFN-γ was determined using an ELISA kit (Endogen, Woburn, MA).

**Transduction of Cell Lines with HLA Class I cDNA.** HLA-B*1501 and HLA-B*5701 cDNAs were amplified by RT-PCR using RNA extracted from recipient EBV-B cells, cloned into pMSCVpuro retroviral expression vector (Clontech, Palo Alto, CA), and sequenced to ensure that no mutation was introduced. Vector DNA was used to transfect either the dual-tropic PT67 (Clontech) or the pantropic 293-GPG (VSV-G) packaging cell lines. Supernatants containing high titer retrovirus were collected and used to infect various cell lines in the presence of Polybrene. When the 293-GPG packaging cell line was used, retrovirus contained in the supernatant was concentrated by ultracentrifugation. After three rounds of infection, transduced cells were selected with puromycin and further cultured in the presence of this antibiotic.

**Quantitative Analysis of 6E4 in Vivo.** Amplification of TCR Vβ6 transcripts was performed by RT-PCR using RNA extracted from 6E4 cells and different patient samples. The PCR reaction was carried out using a Vβ6-specific forward primer (5'-AGGGCTTAGGAGATCCGTCCTC-3') and a Cβ-specific reverse primer (5'-CGGGCTGCTCCTTGAGGGCGCTGCG-3') as described previously (21). The amplified products were purified and cloned into pCR4 plasmid vector using the TOPO TA cloning kit (Invitrogen, Carlsbad CA). Ligated products were used to transform TOP10 Escherichia coli cells (Invitrogen). The plasmid insert obtained from clone 6E4 was sequenced, and the following rearrangement was found: 5'-ATGTATCGCTGT-GCCAGCACGC (TCRBV63A1N2T)-TTAGGAGGTCAGCTC (NDNregion)-ACTGAACTCTTTTGG (TCRB151S1)-3’. Subsequently, individual colonies obtained from blood and bone marrow samples were screened by hybridization for the presence of a plasmid containing a TCRβ chain insert and the 6E4-specific CDR3 sequence. Briefly, nylon membranes of 96 plasmid DNA dots, each corresponding to a single colony, were prepared by the dot blot technique. These membranes were sequentially hybridized with a 32P-labeled 6E4 clonotypic probe (5'-AGCT-TAGGGAGGTCAGCTC-3’) or a Cβ-specific probe (5’-CCGACACGTTGAGCTGCTTG-3’). All plasmid DNA spots showing a positive hybridization signal with the clonotypic probe were further sequenced to confirm the presence of the unique 6E4 CDR3 sequence. Results are reported as the ratio of confirmed 6E4 CDR3-specific dots compared to the total number of TCRβ chain-positive dots.

**Results**

**Patient Characteristics and Response to DLI.** The three CML patients chosen for this study were representative of a larger series of patients who received CD4+ DLI as treatment for relapsed leukemia after allogeneic BMT (13). Clinical characteristics of these patients are summarized in Table 1. At the time of DLI, all patients had cytogenetic evidence of relapse, and patient 3 also had evidence of hematological relapse. Each patient received 3 x 10^7 CD4+ T cells after ex vivo depletion of CD8+ cells. The number of residual CD3+ CD8+ T cells infused ranged from 2.9 x 10^6 to 4.6 x 10^5 cells/kg. All three patients achieved a complete cytogenetic response 14–18 weeks post-DLI and became PCR negative for BCR/ABL transcripts at day 100. Patient 1 developed grade 3 hepatic GVHD 3 months after DLI and responded to immunosuppressive therapy with corticosteroids. This was followed by limited chronic skin GVHD. Patients 2 and 3 were noted to have increased serum aspartate aminotransferase (SGOT) and LDH but no increase in serum bilirubin 11 and 12 weeks, respectively, after DLI. Abnormal liver function tests resolved spontaneously without immunosuppressive therapy, and these patients did not develop clinical evidence of acute or chronic GVHD.
Table 2  Hematopoietic chimerism before and after DLI.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pre-DLI</th>
<th>2 months</th>
<th>3 months</th>
<th>4 months</th>
<th>8 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20%(a)</td>
<td>75%</td>
<td>100%</td>
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</tr>
<tr>
<td>2</td>
<td>25%</td>
<td>50%</td>
<td>75%</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>85%</td>
<td>90%</td>
<td></td>
<td></td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) Results are expressed as a percentage of donor hematopoiesis in patient blood samples.

**Conversion to Donor Hematopoiesis after DLI.** PBMCs from each patient were examined for hematopoietic chimerism before and after DLI. As reported in Table 2, each patient showed evidence of mixed hematopoietic chimerism in PBMCs before DLI. All three patients gradually converted to complete donor hematopoiesis after DLI. By 12 months post-DLI, none of the patients had any evidence of residual recipient hematopoiesis.

**Presence of Host-reactive CD8+ T Cells at the Time of the DLI Response.** To determine whether host-reactive CD8+ T cells were present in vivo during the DLI response, PBMCs from samples obtained 1–3 months after the infusion were stimulated once in vitro with irradiated recipient EBV-B cells in the presence of IL-2. One week after stimulation, CD3+ CD8+ T cells were purified by cell sorting and further expanded in vitro for 4–7 days without any additional stimulus. In cytotoxicity assays, these CD3+ CD8+ T-cell lines displayed substantially higher levels of killing of EBV-B cells derived from the recipient compared with similar target cells derived from the donor (Fig. 1). Such selectivity for recipient cells indicated that within the polyclonal T cells, cytolytic effectors were targeting mHAs divergent between donor and recipient. As shown in Fig. 1, these ex vivo-activated polyclonal T-cell lines displayed significant cytolytic activity at low E:T ratios. For example, T cells from patient 1 displayed 30% killing of recipient cells at an E:T ratio of 16:1 compared with only 5% lysis of donor cells. The observation that this level of specific cytolytic activity was achieved after only one stimulation and 11–14 days in culture suggests that host-reactive T cells were represented with relatively high frequency in vivo after DLI.

To examine this allogeneic response at the single cell level, individual CD3+ CD8+ T-cell clones were derived from a blood sample from patient 1 obtained 3 months after DLI. After initial expansion of T cells on feeder cells composed of irradiated recipient EBV-B cells and allogeneic PBMCs, CD3+ CD8+ T cells were purified by cell sorting and cloned by limiting dilution on the same feeder cells. Thirty-two T-cell clones were expanded and screened for specificity against recipient and donor EBV-B cells using an IFN-γ release assay. Three distinct patterns of reactivity were observed: (a) no reactivity with either target cell (n = 2); (b) reactivity against both donor and recipient cells (n = 16); and (c) selective reactivity against recipient but not donor cells (n = 14). No T-cell clones reactive only to donor EBV-B cells were isolated. Several clones with selective reactivity against recipient cells were further expanded in vitro, and all were confirmed to be CD3+ CD8+. As shown in Fig. 2, six representative CTL clones displayed substantially higher levels of cytolytic activity against recipient EBV-B cells compared with donor EBV-B cells. In each case, cytotoxicity was blocked by a Mab (W6/32) specific for a monomorphic determinant on MHC class I molecules.

**Specificity of CTL Clone 6E4.** One of the CD3+ CD8+ clones, designated 6E4, isolated from patient 1 PBMCs 3 months after DLI was expanded for further analysis of CTL specificity. The clonality of 6E4 was confirmed by the detection of a single TCRβ chain transcript with a unique CDR3 (data not shown). As reported in Fig. 3A, clone 6E4 displayed selective cytolytic activity against patient but not donor EBV-B cells. Clone 6E4 also demonstrated selective killing of recipient-derived but not donor-derived PHA-activated T-cell blasts. Cytolytic activity against recipient EBV-B cells was blocked by the MHC class I Mab W6/32 as well as 8.1.23.2, a Mab that selectively binds to all HLA-A and -C alleles (Fig. 3B). These experiments indicated that the recipient mHA recognized by this
clone was presented by MHC class I molecules and was not restricted to EBV-B cells.

In additional studies, we proceeded to identify the MHC class I allele involved in antigenic recognition by clone 6E4. The previous blocking experiments suggested that clone 6E4 was restricted by one of the four HLA-B and -C alleles expressed on patient cells, namely, HLA-B*1501, HLA-B*5701, HLA-C*0304, or HLA-C*0602. We first focused on HLA-B alleles as candidates for the restriction element. HLA-B*1501 and HLA-B*5701 cDNA were amplified by RT-PCR from patient EBV-B cells, fully sequenced to confirm that no mutations were introduced, and then cloned into a mammalian expression retroviral vector. High titer vectors were prepared using dual-tropic or pantropic packaging cell lines and used to transduce several tumor cell lines. Transduced cell lines were expanded in vitro, and cells expressing high levels of HLA-B*1501 or HLA-B*5701 were further purified by cell sorting. The recognition of stable target cell lines expressing HLA-B*1501 or HLA-B*5701 by clone 6E4 was examined using an IFN-γ release assay. As shown in Fig. 4, HEL92.1.7 cells (erythroleukemia), U937 cells (histiocytic lymphoma), and K562 cells (BCR/ABL+ myeloid leukemia) were recognized by clone 6E4 when transduced with HLA-B*5701 but not when transduced with HLA-B*1501.

Fig. 2 Cytolytic activity of host-reactive CD3+CD8+ T cells. T cells shown to be host reactive in IFN-γ release assay were tested for their cytotoxicity against donor and recipient EBV-B cells. Results are expressed as the mean of triplicate assays and were obtained for an E:T ratio of 3:1. Blocking was performed using the pan-MHC class I Mab W6/32.

Fig. 3 Clone 6E4 is a class I-restricted host-reactive cytotoxic T lymphocyte. A, CD3+CD8+ clone 6E4 was used in cytotoxicity experiments against donor (○) or recipient (■) EBV-B cells as well as PHA-activated T-cell blasts of donor (□) and recipient (■) origin. B, cytolytic activity against recipient EBV-B cells was blocked by the addition of the pan-MHC class I-specific Mab W6/32 (▲) or the HLA-B and -C allele-specific Mab B1.23.2 (△). Results are expressed as the mean of triplicate assays.
to estimate its frequency. As summarized in Table 3, 6 of 467 complete 6E4 NDN junctional region. This method, previously using a specific clonotypic oligonucleotide probe spanning the plasmid clones containing V6 inserts were analyzed by hybridization after DLI and cloned into a plasmid vector. Random sample as well as from blood and bone marrow samples obtained after DLI and cloned into a plasmid vector. Random plasmid clones containing V66 inserts were analyzed by hybridization for their identity to the 6E4 TCRβ chain transcript using a specific clonotypic oligonucleotide probe spanning the complete 6E4 NDN junctional region. This method, previously developed by McHeyzer-Williams and Davis (22), enabled us to track the presence of 6E4 clone in different patient samples and to estimate its frequency. As summarized in Table 3, 6 of 467 clones obtained from the post-DLI blood sample corresponded to the 6E4 transcript. Thus, 6E4 represented approximately 1.3% of all V6+ T cells in peripheral blood 3 months after DLI. This frequency reached 3.1% in the bone marrow at the same time point. Over 600 independent clones from a blood sample before DLI were examined using the same procedure, but the 6E4 CDR3 sequence was not detected. Nevertheless, we cannot exclude the possibility that these cells were present at a lower frequency at that time (<1.0%). Remarkably, clone 6E4 was also detected in a blood sample collected 24 weeks after DLI. At this later time point, the frequency of these cells in peripheral blood was reduced compared with that seen 3 months after DLI (Table 3). Taken together, these data show that 6E4 cells underwent amplification in vivo after DLI and that these cells persisted in peripheral blood for at least 6 months.

**Discussion**

DLI represents a remarkably effective treatment for relapsed CML after allogeneic BMT. Clinical responses occur in >70% of patients with relapsed chronic phase CML, and the majority of these patients achieve long-lasting cytogenetic and molecular remissions without additional treatment (9, 10, 23). Infusion of polyclonal donor CD3+ T cells also frequently causes GVHD, which is the major toxicity associated with DLI. Previous studies have suggested that GVHD can be reduced by depleting CD8+ cells and infusing defined numbers of CD3+CD4+ donor T cells (12–14). Although depletion of CD8+ cells reduces the incidence of GVHD, it does not appear to reduce the GVL effect of DLI. Previous clinical trials performed at our institution have demonstrated that 71% of patients with cytogenic or stable phase CML relapse achieved complete cytogenetic responses after a single infusion of 3 × 10^7 CD4+ T cells/kg (13). With infusion of CD8-depleted donor cells, 57% of responding patients did not develop evidence of clinical GVHD (grade 2–4 GVHD) (13). The ability to examine the GVL response in patients with and without GVHD who received selected CD4+ DLI provides a unique opportunity to characterize the immunological effects induced by DLI. In particular, the experiments described in this report were undertaken in three representative patients to better define the cellular mechanisms that mediate GVHD and GVL in the responder population.

Although there is little doubt that donor T cells play a central role in both GVHD and GVL, the target antigens of these responses have not been well defined. Three distinct categories of cellular antigens have been postulated to be important targets for GVL, GVHD, or both effects. The first category includes tumor-specific antigens. For example, junctional peptides derived from the p210 protein product of the BCR/ABL oncogene represent potential tumor-specific epitopes that are only expressed by CML cells (24). Junctional p210 peptides are immunogenic in vitro in normal individuals, and both CD4+ and CD8+ T-cell responses toward these antigens have been documented (25–27). In previous studies, we were able to generate a p210-specific CD4+ CD3+ T-cell clone of donor origin by stimulating PBMCs obtained post-DLI with BCR/ABL junctional peptides in vitro (28). However, we found no evidence that patient CML cells naturally processed this target epitope for GVL.
that a response directed toward this antigen had occurred in vivo after DLI.

Nonmutated proteins that are preferentially expressed in tumor cells represent another category of potential target antigens. Proteinase-3 and its corresponding epitope, PR-1, are an example of such an antigen with a high level of expression in CML cells (29, 30). In a previous study, PR-1-specific T cells were found to be expanded in the peripheral blood of patients with CML after allogeneic BMT (31). Antigens such as PR-1 may also be targets of the DLI response, but T cells specific to these antigens have not yet been described in patients who have responded to the infusion.

mHAs represent a third category of potential targets after DLI. Antigens such as HA-1, HA-2, and HA-8 were initially characterized using T cells from patients who developed GVHD (32–34). Disparity in HA-1 genotype between donors and recipients of BMT has been associated with an increased risk of acute GVHD (35–37). Importantly, mHAs are also likely to play an important role as targets of GVL (18, 38). Leukemia cells have been shown to express mHAs that are expressed by normal hematopoietic cells, and immune responses that target such antigens are able to kill leukemia cells in vitro and in vivo (17, 39, 40). The striking observation that elimination of leukemia cells after DLI is frequently associated with the conversion of mixed hematopoietic chimerism to complete donor hematopoiesis supports the view that immune responses to mHAs expressed by all recipient hematopoietic cells represent common targets of the DLI response (13, 15).

In the present study, we sought to identify and characterize mHA-specific effector cells in selected patients with CML who responded to DLI. Because most human mHAs identified thus far are HLA class I-restricted epitopes recognized by conventional CD8+ cytotoxic T cells, we focused on the initial experiments on this T-cell subset. We studied three CML patients who received CD8-depleted DLI and achieved complete cytogenetic and molecular remission without additional therapy. Each patient also demonstrated conversion of mixed hematopoietic chimerism to complete donor hematopoiesis after DLI, indicating the induction of an immune response directed at normal recipient hematopoietic cells as well as leukemia cells. The presence of this allogeneic response was clinically more pronounced in patient 1, who also developed acute hepatic GVHD. Remarkably, a single in vitro stimulation of PBMCs obtained after DLI with recipient EBV-B cells was sufficient to generate host-reactive T-cell lines in all three patients, clearly suggesting that CD8+ T cells directed at recipient antigens were relatively abundant in peripheral blood at the time of clinical response. This response was further characterized at the single-cell level in patient 1 by isolating CD8+ CD3+ T-cell clones by limiting dilution. Consistent with the previous analysis of polyclonal cell lines, a significant proportion of emerging T-cell clones were found to be selectively reactive with host antigens, and this specificity was detected in cytokine secretion (data not shown) as well as in cytotoxicity assays.

To confirm that T-cell clones were specific for mHA, we analyzed one of these clones (6E4) in greater detail. Clone 6E4 was reactive with recipient PHA-activated T-cell blasts as well as recipient EBV-B cells but was not reactive with similar cells derived from the donor. Although the level of killing against recipient PHA blasts was low, it was significantly higher than that against donor T-cell blasts and was reproduced in four different experiments. Moreover, this killing was blocked by the addition of anti-MHC class I Mab. There are several possible explanations for this low level of killing. The recognized antigen might only be expressed by a small fraction of activated T cells, or, alternately, the level of expression of this antigen in activated T cells may be relatively low, resulting in low levels of lysis. The identification of the antigen recognized by the 6E4 clone would help clarify this issue. 6E4 cells also recognized several allogeneic hematopoietic cell lines transduced with HLA-B*5701 including the Ph+ CML cell line K562. No HLA-B57-restricted mHAs have previously been identified, and these results therefore indicate that 6E4 cells recognize a new mHA presented by HLA-B*5701. This mHA is expressed by a variety of hematopoietic cells, but the gene encoding this new mHA has not yet been identified, and additional studies will be necessary to determine whether expression of this mHA is restricted to hematopoietic cells. Once the molecular characteristics of the new mHA have been defined, additional studies will also be able to establish the allele frequency of this target antigen and determine whether this mHA is frequently recognized after allogeneic BMT.

Although the peptide specificity of clone 6E4 has not yet been determined, we could assess the presence of clone 6E4 in blood and bone marrow samples by the detection of its unique rearranged TCRβ chain transcript. Despite testing 628 clones in a blood sample collected before DLI, we could not detect the 6E4-specific CDR3 sequence. In contrast, 6E4 cells were found to represent more than 1% of all Vβ6+ cells in peripheral blood and more than 3% of Vβ6+ cells in the bone marrow 3 months after DLI. These results therefore provide evidence that clone 6E4 had undergone a vigorous antigen-driven expansion in vivo after DLI. Moreover, finding these cells in the blood as well as in the bone marrow showed that they were circulating between these two compartments and that the total number of 6E4 cells had reached relatively high levels at the time of CML response and conversion to complete donor hematopoiesis. The detection of clone 6E4 in a sample collected 6 months after the infusion provided further evidence that these cells participated in the sustained DLI response.

### Table 3  In vivo representation of clone 6E4 within the Vβ6+ T-cell subpopulation

<table>
<thead>
<tr>
<th></th>
<th>PBMCs pre-DLI</th>
<th>PBMCs 15 weeks post-DLI</th>
<th>BM 15 weeks post-DLI</th>
<th>PBMC 24 weeks post-DLI</th>
</tr>
</thead>
<tbody>
<tr>
<td>6E4 CDR3 frequency</td>
<td>0/628 (&lt;0.1%)</td>
<td>6/467 (1.3%)</td>
<td>12/384 (3.1%)</td>
<td>1/549 (0.2%)</td>
</tr>
</tbody>
</table>

* BM, bone marrow.

* Results are expressed as the number of 6E4-specific transcripts detected on total Vβ6 TCRβ chain transcripts screened. Corresponding percentages are indicated.
Knowing that the patients we studied received donor lymphocytes that had been depleted of CD8+ T cells before infusion, it was interesting to find expansion of recipient reactive CD8+ CD3+ T cells in each patient after DLI. Because the infused donor lymphocytes contained <1% CD8+ CD3+ T cells, the total CD8+ cell dose infused was calculated to be approximately 3 × 10^5 cells/kg. Although we have not excluded the possibility that the CD8+ CD3+ cells that expanded after DLI were derived from residual CD8+ cells in the DLI, this seems an unlikely explanation, given the relatively small number of CD8+ cells infused and the relatively low frequency of naïve mHA-specific T cells present in normal unstimulated peripheral blood. Thus, although mHA-specific cells such as 6E4 could not be detected before DLI, it seems more likely that these cells were present in the recipients at relatively low levels before the infusion. Additional experiments will be required to address this point and to assess whether such host-reactive effector cells could be obtained from pre-DLI patient samples as well as from the infused lymphocyte product. Previous studies have demonstrated the recovery of thymic function and gradual reconstitution of a diverse T-cell repertoire after T-cell-depleted BMT (41, 42). The mHA-specific CD8+ T cells reported here could originate from this repopulation of the T-cell compartment. Subsequently, infusion of additional donor CD4+ T cells capable of recognizing HLA class II-restricted mHAs on recipient dendritic cells may be sufficient to initiate a productive response to other recipient mHAs and the activation of naïve CD8+ effector cells. To investigate whether clone 6E4 was present before DLI, we attempted to develop a more sensitive assay to detect these cells. Unfortunately, clonotypic RT-PCR assays based on the 6E4 CDR3 sequence to detect this clone in pre-DLI samples were unsuccessful because of a high level of redundancy in the VDJ junction. Additional studies performed on additional clones displaying similar functional patterns will be required to resolve this issue and determine whether CD8+ CD3+ mHA-specific clones were derived from residual cells in the DLI or whether these donor cells were present in the patient before DLI.

In summary, the present studies demonstrate the expansion of host-reactive CD8+ CD3+ T cells in association with the conversion of mixed chimerism toward full donor hematopoiesis and the complete response observed in three patients undergoing DLI for relapsed CML. Although other cell populations are also likely involved in the overall CD4+ DLI response, the present findings support the view that CD8+ T cells are prominent effector cells in this response. These CTLs appear to be primarily reactive with recipient mHA and therefore target recipient hematopoietic cells as well as leukemia cells. Additional studies will be directed to define the new mHA we have identified and the mechanism whereby these CD8+ T cells are activated by infusion of CD4+ donor lymphocytes.

References


Infusion of CD4+ Donor Lymphocytes Induces the Expansion of CD8+ Donor T Cells with Cytolytic Activity Directed against Recipient Hematopoietic Cells

Emmanuel Zorn, Kathy S. Wang, Ephraim P. Hochberg, et al.


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