Expansion of Tumor-specific CD8+ T Cell Clones in Patients with Relapsed Myeloma after Donor Lymphocyte Infusion

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ABSTRACT

Donor lymphocyte infusions (DLI) provide effective therapy for patients with multiple myeloma who have relapsed after allogeneic bone marrow transplantation. However, the immunological mechanisms of the graft-versus-myeloma (GVM) effect have not been defined, and the target antigens of this response have not been identified. Molecular analysis of CD3/CD8 repertoire after CD4+ DLI demonstrated previously that the development of GVM and graft-versus-host-disease (GVHD) were associated with the clonal expansion of distinct T-cell populations in patient peripheral blood. In the current study, we undertook a molecular and functional characterization of GVM- and GVHD-associated T-cell clones. T-cell clones associated with GVM were detectable by clone-specific PCR at a low level in peripheral blood before DLI and expanded ~10-fold after DLI. In contrast, T-cell clones associated with GVHD were not detectable before DLI or before the development of clinical GVHD. Two T-cell clones associated with GVM were isolated and expanded in vitro, allowing their phenotypic and functional characterization. Both GVM clones were derived from donor cells and had a CD3+/CD8+/CD4− phenotype. One GVM clone specifically recognized patient myeloma cells in an HLA class I-restricted manner, but was not reactive with patient normal bone marrow cells or patient EBV transformed B cells. Taken together, these findings suggest that the GVM response is mediated by donor-derived CD8+ T-cell clones with antmyeloma specificity that may be present before DLI. In contrast, T-cell clones associated with GVHD are expanded de novo after DLI.

INTRODUCTION

DLIs provide effective therapy for patients with various hematologic malignancies who have relapsed after allogeneic stem cell transplantation. In patients with relapsed CML, long-lasting hematologic and cytogenetic remissions can be obtained in association with clinical onset of tumor response or GVHD. However, despite the temporal association of clonal T-cell expansion with clinical events, the phenotype and functional specificity of these cells have not been established. It has been postulated that these donor T cells can be directed at either tumor-associated antigens (5) or minor histocompatibility antigens (6–8), but the specific antigens that are targets of effective DLI responses in vivo have not been identified.

GVHD represents the major toxicity associated with DLI. GVHD is mediated primarily by donor T cells directed against multiple antigens expressed in different recipient tissues. To the extent that GVHD targets antigens that are also expressed by recipient tumor cells, GVHD can also be responsible for significant GVL activity. Despite this evident overlap between GVHD and GVL, different experimental models have demonstrated the feasibility of distinguishing these two effects of donor T cells (9–11). Different clinical strategies have also been developed to reduce the incidence of GVHD after DLI. These have included infusion of limited numbers of donor T cells, sequential infusion of increasing numbers of donor T cells, and infusion of selected subsets of donor T cells (12–15). The results of these clinical trials, in conjunction with studies in animal models, suggest that GVL activity of DLI can be maintained in the absence of clinical GVHD.

Depletion of CD8+ T cells before infusion appears to be one method for effectively reducing the incidence and severity of GVHD after DLI. Previous clinical trials have shown that infusion of CD4+ donor T cells, obtained after depletion of CD8+ cells without further in vitro manipulation or activation, induces substantial GVL activity despite the reduced incidence of GVHD (12, 13, 16). The immunological mechanisms responsible for GVL, distinct from GVHD, may involve the activation of different effector populations or, alternatively, may depend on the recognition of distinct sets of target antigens. To identify those T cells that mediate GVL and GVHD in vivo, we previously examined the TCR Vβ repertoire of peripheral blood lymphocytes at various times after CD8-depleted DLI in patients with CML and MM (17–19). CD8-depleted DLI frequently induces clinical responses in both of these patient populations, and serial analysis of TCR Vβ repertoire has provided a sensitive method for detecting the emergence of distinct clonal T-cell populations in peripheral blood and bone marrow at different times after DLI. Importantly, these studies demonstrated that different clonal T-cell populations expand in association with clinical onset of tumor response or GVHD. However, despite the temporal association of clonal T-cell expansion with clinical events, the phenotype and functional specificity of these cells were not established.

In this report, we describe the molecular characterization of T-cell clones that expand in vivo in patients with multiple myeloma at the time of clinical response after DLI. These results suggest that the antmyeloma effect of DLI is mediated by CD8+ T-cell clones of donor origin, detectable in the patient peripheral blood before DLI. These cells expand in vivo in the months after DLI and, when isolated in vitro, are capable of specifically recognizing antigens expressed by recipient myeloma cells and not by normal recipient myeloid cells or EBV transformed B cells. These results, therefore, support the hypothesis that tumor rejection after DLI is, at least in part, directed against tumor-associated antigens.

MATERIALS AND METHODS

CD4+ Donor Lymphocyte Infusions and Patient Histories. The clinical research protocol and the results of treatment of the two patients analyzed in

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The abbreviations used are: DLI, donor lymphocyte infusion; CML, chronic myeloid leukemia; MM, multiple myeloma; GVHD, graft-versus-host disease; GVL, graft-versus-leukemia; TCR, T-cell receptor; BMT, bone marrow transplantation; PBMC, peripheral blood mononuclear cell; IL, interleukin; TNF, tumor necrosis factor; GVM, graft-versus-myeloma.

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this report have been described previously (12, 17, 20). Both patients with MM relapsed after allogeneic BMT and received a single infusion of CD8-depleted lymphocytes from the same HLA identical sibling that had previously donated marrow for BMT. Donor lymphocyte infusions contained <1% CD6+ cells. All other therapy was discontinued before DLI and patients received no other immune-modulating therapy after DLI. The clinical characteristics of the 2 patients examined in this study are summarized in Table 1. Patient 1 relapsed 6 months after allogeneic BMT and received DLI 3 months after relapse. Serum monoclonal protein began to decrease 4 weeks after DLI but remained detectable for a prolonged period and he did not achieve a complete remission until 2 years after DLI. Patient 2 relapsed 4 years after BMT. A progressive decline in serum monoclonal protein began 2 months after DLI but a complete remission was never achieved. Progressive disease was again documented 2 years after DLI. Both patients developed acute and chronic GVHD after lymphocyte infusions.

**Table 1 Patient characteristics**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/sex</th>
<th>Myeloma paraprotein</th>
<th>Status at BMT</th>
<th>GVHD post-DLI</th>
<th>Response after DLI</th>
<th>DLI to response (wks)</th>
<th>DLI to GVHD (wks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42/M</td>
<td>IgA</td>
<td>Minimal disease</td>
<td>Grade 2 acute + Chronic</td>
<td>CR*</td>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>42/M</td>
<td>IgA</td>
<td>Minimal disease</td>
<td>Grade 1 acute + Chronic</td>
<td>IgA</td>
<td>9</td>
<td>17</td>
</tr>
</tbody>
</table>

* CR, complete remission; PR, partial remission.

**TCR Vβ Repertoire Analysis.** The PCR-based method for analysis of TCR Vβ repertoire has previously been described (17, 18, 21). Briefly, RNA was extracted from T-cell enriched preparations and cDNA was prepared. TCR Vβ gene segments were amplified from the cDNA with one of 26 Vβ subfamily-specific primers and a fluorescent-conjugated Cβ primer recognizing both Cβ1 and Cβ2 regions. PCR amplification of Vβ5 and Vβ13 each required the use of 2 primers to identify the entire Vβ subfamily. The size distribution of each fluorescent PCR product was determined by electrophoresis on a 5.5% polyacrylamide gel. Because the position of the 5’ and 3’ primers are fixed, fragment size differences within each Vβ subfamily are due entirely to different CDR3 region lengths, reflecting junctional diversity and N-random nucleotide insertions in the V-D-J region. Peaks corresponding to in-frame transcripts are detected at three nucleotide intervals. A normal transcript size distribution, reflecting polyclonal T cells, contains 8 to 10 peaks for each subfamily with a Gaussian size distribution (22). The appearance of dominant peaks corresponding to in-frame transcripts indicates the presence of exogenous cDNA of identical size, suggesting the presence of oligoclonal or clonal T cells.

**Determination of TCR Vβ and CDR3 Gene Sequences.** TCR Vβ gene transcripts were amplified as described above and the PCR product was purified using Wizard PCR prep kit (Promega, Madison, WI) and ligated into the PCR2.1 TA cloning vector (Invitrogen, San Diego, CA). The ligation product was transfected into INVaF competent cells (Invitrogen, San Diego, CA). White colonies growing in x-gal medium were randomly picked, checked for insert, and sequenced using standard methods.

**Clone-specific PCR.** PCR forward primers specific for the CDR3 region of individual clones were designed and used to amplify cDNA prepared as above in association with the Cβ antisense primer. Sequences of specific primers are listed in Table 2. PCR conditions were as follows: denaturing at 94°C for 1 min, annealing at 54–62°C (depending on each specific primer) for 1 min, and extension at 72°C for 1 min for a total of 35–40 cycles. To confirm reaction specificity, PCR products were purified using Wizard PCR prep kit (Promega, Madison, WI) and sequenced using standard methods. In each case, the amplified sequence matched the expected sequence.

**Dot Blot Assay with CDR3 Clone-specific Probes.** The oligonucleotides listed in Table 2 were synthesized to be complementary to the CDR3 sequences of the expanded clones and conjugated with [32P]y-ATP. cDNAs from different samples were amplified by PCR using the corresponding Vβ subfamily primer and aliquots of the amplified products were blotted onto nylon membranes, prehybridized, and hybridized overnight with the labeled probes at 42 or 55°C. Blots were washed twice at 4°C for 30 min in 6x SSC and twice at 65°C for 20 min in tetramethylammonium chloride. Signals were scanned and quantified using ImageQuant software (Amersham Biosciences, Piscataway, NJ).

For relative quantitation of identified clones, the same blots were stripped and hybridized with the internal Cβ probe described above, and the incorporated radioactivity was quantified using the same system. For each sample, the ratio between the two signals was calculated and considered indicative of the relative presence of the individual clone within the corresponding Vβ subfamily. Because hybridization efficiency is known to vary from one probe to another, these values do not reflect an absolute representation of the specific clone. However, because intersample comparison could be performed within each blot, results were expressed as relative variation over time and normalized to the maximal level of expansion.

**Phenotypic Analysis and Sorting by Flow Cytometry.** Expression of cell surface molecules was determined by either direct or indirect immunofluorescence using standard methods. The mouse monoclonal antibodies used were FITC- or phycoerythrin conjugated antibodies specific for CD3, CD4, CD8, CD138, TCR Vβ3, and TCR Vβ13.1 (Beckman Coulter, Fullerton, CA). Irrelevant isotype-matched antibodies were used as negative controls. Samples were analyzed using a Coulter Elite flow cytometer (Beckman Coulter, Fullerton, CA) and data were acquired in list-mode files.

To purify Vβ3- and Vβ3.1-positive cells, patient PBMCs were cryopreserved 3 months after DLI and thawed and stained with either purified Vβ3 or Vβ3.1 and CD3-FITC. After incubation, samples were washed and incubated with phycoerythrin-goat antimouse IgG2a or IgG2b, respectively. A FACStarflow cytometer (Becton Dickinson, San Jose, CA) was used to directly sort double-positive cells into 96-well round-bottomed plates at a concentration of 1 cell/well. To purify autologous myeloma cells, patient BM samples cryopreserved before DLI were thawed and stained with anti-CD138 monoclonal antibody recognizing the plasma cell-specific syndecan-1 antigen. Positive cells were sorted using a FACStarflow cytometer and cultivated at 37°C in complete medium (RPMI supplemented with 10% human AB serum, l-glutamine, Pen-Strep, HEPES buffer, and sodium pyruvate).

**Vβ3- and Vβ3.1-positive T-cell Cloning.** Single Vβ3/CD3 and Vβ3.1/CD3 double-positive cells were directly sorted into microwells containing the following reagents and feeder cells: 75,000 allogeneic irradiated PBL plus 15,000 irradiated donor EBV cells in 200 µL of complete medium with 50 ng/ml OKT3 and 50 units/ml IL-2. Growing wells were expanded and restimulated weekly using the same mixture. The clone-specific PCR primers p2–3 and p2–13.1 were used for the molecular analysis of growing clones. CDR3 fragment-size analysis, described previously, was used to confirm monoclonality of in vitro expanded clones and PCR product sequencing confirmed that these clones were identical to the clones that had been identified in vivo.

PCR amplification of the sY14-SRY (testis determining factor) gene on the Y chromosome was used to discriminate between donor (female) and recipient (male) origin of the in vitro expanded clones. Genomic DNA was amplified using the following primers: 5’-GAATATTTCCGCTTCCGGA-3’ and 3’-GCTGGTGCCCTATCTTGGAG-5’ Thirty-five cycles of 30 s at 94°C, 2 min at 58°C, and 2 min at 72°C were used, with a final extension of 7 min at 72°C.

**Table 2 Clone-specific PCR primers and CDR3 probes**

<table>
<thead>
<tr>
<th>Clone</th>
<th>PCR primer</th>
<th>CDR3 Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ TGT GCC AGC AAT GGG GGA A 3’</td>
<td>5’ AAT GGG GGA ACC TCC TAC GAG CAG 3’</td>
<td></td>
</tr>
<tr>
<td>5’ GCC ACC AGT AAT AGG GGA CGG GA 3’</td>
<td>5’ AAT AGG GGA CCG GAG AAC ACT GAA GCT 3’</td>
<td></td>
</tr>
<tr>
<td>5’ CCT TCT ATC TCT GGG CCT GGA TAA T 3’</td>
<td>5’ GTA ATG AAC ACT GAA GCT TTC TTT 3’</td>
<td></td>
</tr>
<tr>
<td>5’ TAT GTA CCT CTG TGC CAG CAG CTG G 3’</td>
<td>5’ AGC TGC ACA GGG TAC CAG CAG TAC 3’</td>
<td></td>
</tr>
<tr>
<td>5’ GCC AGC AGC TAC TCG GAG GGA TAT 3’</td>
<td>5’ TCG GAG GGA TAT CAG CAG TCT TCC 3’</td>
<td></td>
</tr>
</tbody>
</table>

* Pt., patient.
MYELOMA-SPECIFIC T CELLS AFTER DLI

Cytokine Production Assays. For functional analysis of the p2–3 and p2–13.1 clones, 1500 T cells/well were plated in 96-well V-bottomed plates, together with 2000 autologous plasma cells/well in complete medium. After 6 h of coculture, supernatants were harvested and tested for TNF production using a standard bioassay (23). Briefly, TNF-sensitive WHEI cells were cultivated overnight in flat-bottomed microwells in the presence of the testing supernatant, actinomycin B and LiCl. MTT was then added for 2 h, and after cell lysis, WHEI cell response to TNF was read using a colorimeter. TNF concentration was calculated using a standard curve carried out in the same experiment. All samples were tested in triplicate and results confirmed in at least two independent experiments. For anti-HLA class I and anti-HLA class II blocking experiments, purified W6/32 (American Type Culture Collection, Manassas, VA) and 9.49 antibodies (American Type Culture Collection) were incubated with the target cells for 30 min before adding responder T cells.

IFNγ production was tested in supernatants harvested after 48 h of coculture of 10,000/well patient plasma cells and 20,000/well p2–3 and p2–13.1 clones in complete medium with 20 units/ml IL-2, using a standard ELISA assay kit (Promega, Madison WI).

RESULTS

Identification of T-cell Clones Associated with GVM or GVHD Activity. In a previous report, we described the results of serial analysis of TCR Vβ repertoire in four patients with myeloma who underwent autologeneic BMT from HLA identical siblings and received infusions of CD4+ donor lymphocytes for treatment of relapse after BMT (17). Three of these patients responded to DLI and also developed GVHD weeks to months after the onset of tumor response. Because the onset of antmyeloma response and GVHD occurred at different times, the serial analysis of TCR Vβ repertoire allowed us to identify different sets of T-cell clones as being associated with either GVM or GVHD. To begin the functional characterization of T-cell clones associated with either GVM or GVHD activity, we undertook a more detailed analysis of five clones that were identified in two of these patients. Three clones shown in Fig. 1 appeared in peripheral blood 1–3 months after DLI and were, therefore, temporally associated with the GVM response, and two distinct clones appeared at later time points when patients developed GVHD.

To determine whether the TCR Vβ profiles shown in Fig. 1 represented true expansions of clonal T cells, we ligated PCR amplified products into a cloning vector and transfected into Escherichia coli. Ten to 30 growing colonies were randomly selected, and the inserted DNA sequence was obtained from each colony. In each case, the expansion of a unique T-cell clone was confirmed by demonstrating the frequent recurrence of the same sequence in the colonies that were analyzed. As summarized in Table 3, the frequency of clonal sequences varied from 30 to 100%. For each individual clone, the identified DNA sequence matched the predicted Vβ gene segment sequence and predicted length of the CDR3 segment. In addition, the specific J region and C region used in each of these clones could be identified. Table 3 also summarizes the complete CDR3 region sequence for five GVM-associated clones (designated p2–13.2, p2–3, and p2–13.1) and two GVHD-associated clones (designated p1–15 and p1–20).

Detection of Individual T-cell Clones by PCR. To further characterize the putative GVM and GVHD clones identified in Fig. 1 and Table 3, we used the CDR3 region sequences to design clone-specific PCR primers (Table 2). cDNA obtained from PBMCs before and after lymphocyte infusion were amplified with these clone-specific primers. As shown in Fig. 2, each target clone could be detected by clone-specific PCR in blood samples obtained from patients after DLI, but these clones were not detectable in blood samples from normal donors. Although pre-DLI blood samples demonstrated normal polyclonal CDR3 patterns, and the GVM-associated clones did not become evident as expanded populations until ~3 months post-DLI, clone-specific PCR shown in Fig. 2 demonstrated that the three GVM clones (p1–13.2, p2–3 and p2–13.1) were each detectable in patient blood samples before DLI. In contrast, the two GVHD-associated clones (p1–15 and p1–20) were not detectable by clone-specific PCR in patient samples before DLI.

Relative Expansion of T-cell Clones in Vivo after Donor Lymphocyte Infusion. To evaluate the relative expansion or regression of individual T-cell clones over time, we developed a semiquantitative hybridization assay using synthetic oligonucleotide probes specific for the junctional region of each clone. Aliquots of cDNA obtained from serial samples of PBMCs were amplified by PCR using TCR Vβ subfamily primers, blotted on nylon membranes, and hybridized with both clone specific probes indicated in Table 2 and an internal TCR CBβ probe. The results of the relative hybridization assay for the three candidate GVM T-cell clones over a period of 18–21 months after

<table>
<thead>
<tr>
<th>Clones</th>
<th>Patient no. (Vβ gene)</th>
<th>Frequency</th>
<th>V region</th>
<th>N-Dβ-N</th>
<th>J region</th>
<th>CBβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>p1-13.2</td>
<td>p1, Pt. 1 (Vβ13.2)</td>
<td>66%</td>
<td>AAT GGG GGA ACC</td>
<td>TCC-Jβ2.7</td>
<td>CB2</td>
<td></td>
</tr>
<tr>
<td>p2-3</td>
<td>Pt. 2 (Vβ3)</td>
<td>100%</td>
<td>AGC</td>
<td>AGC TGG ACA GGG</td>
<td>TAC-Jβ2.7</td>
<td>CB2</td>
</tr>
<tr>
<td>p2-13.1</td>
<td>Pt. 2 (Vβ13.1)</td>
<td>75%</td>
<td>TAC</td>
<td>TCC GAG GGA TAT</td>
<td>GAG-Jβ2.1</td>
<td>CB2</td>
</tr>
<tr>
<td>p1-15</td>
<td>Pt. 1 (Vβ15)</td>
<td>30%</td>
<td>AGT</td>
<td>AAT AGG CCG GA</td>
<td>GAA-Jβ1.1</td>
<td>CB1</td>
</tr>
<tr>
<td>p1-20</td>
<td>Pt. 1 (Vβ20)</td>
<td>53%</td>
<td>TGG</td>
<td>GTA AT</td>
<td>GAA-Jβ1.1</td>
<td>CB1</td>
</tr>
</tbody>
</table>

Table 3. CDR3 region sequence of expanded T cell clones

\* Pt., patient.
lymphocyte infusion are summarized in Fig. 3. These assays confirmed the presence of three clones (p1–13.2, p2–3, and p2–13.1) before DLI, but they were only detectable at relatively low levels at that time, consistent with the observation that no clonal peaks were evident in the CDR3 profiles. These clones appeared to expand at least 10-fold after DLI and reached maximal levels of expression 5–6 months post-DLI. Although the relative levels of expression subsequently declined, these clones persisted for prolonged periods and remained detectable in peripheral blood 18–21 months post-DLI. In patient 2, the increase in serum monoclonal protein levels at the time of tumor response, 6 months after DLI, was temporally associated with a decrease in the relative expansion of the GVM p2–3 and p2–13.1 T-cell clones.

A similar analysis of two T-cell clones (p1–15 and p1–20) that appeared to be associated with GVHD is also shown in Fig. 3. Neither clone was detectable in blood samples 1 or 3 months post-DLI, and both were first noted when GVHD became evident 5–6 months after lymphocyte infusion. Clone p1–15 persisted at relatively high levels 21 months post-DLI, which was consistent with the presence of this clone in the CDR3 profile at this time. In contrast, clone p1–20 was only detectable at low levels 21 months post-DLI, which was consistent with the return of a normal polyclonal CDR3 pattern at this time.

In Vitro Expansion and Characterization of p2–3 and p2–13.1 GVM Clones. To further characterize the phenotype and function of the T-cell clones that expanded in vivo at the time of tumor response, we developed methods to isolate and expand these cells in vitro. The availability of monoclonal antibodies specific for the Vβ3 and Vβ3.1 subfamilies allowed us to isolate and expand Vβ3+CD3+ and Vβ3.1+CD3+ T cells from patient 2. Flow cytometric analysis of PBMCs cryopreserved 3 months after DLI contained 4.8% Vβ3+ and 2.1% Vβ3.1+ T cells (Fig. 4). Double-positive cells were directly sorted into microwell plates and expanded in vitro. Samples from expanding clones were subsequently tested using clonotypic PCR primers. In this screening process, 24 Vβ3+ clones were tested, and 19 were positive for the clone-specific CDR3 sequence. Similarly, 86 Vβ3.1+ clones were tested, and 27 clones were positive for the clone-specific CDR3 sequence. Selected clones were further expanded, and CDR3 size analysis of PCR products shown in Fig. 5A confirmed that these were monoclonal populations expressing a single Vβ gene rearrangement. Nine identical Vβ3 clones and eight identical Vβ3.1 clones were selected for further in vitro expansion and functional analysis.

The cell surface phenotype of the p2–3 and p2–13.1 clones was also examined by flow cytometry. Both clones were CD3+CD8+CD4− T cells (data not shown). Because the patient and donor were sex mismatched (female donor/male recipient) we used PCR for a Y-encoded gene to determine the origin of each clone. The results of PCR amplification of a Y-associated gene (male determining factor) on the p2–3 and p2–13.1 clones are shown in Fig. 5B. Both clones were PCR negative, confirming their derivation from the female donor.

Antigenic Specificity of p2–3 and p2–13.1 GVM Clones. To examine the antigenic specificity of putative GVM clones, p2–3 and p2–13.1, we determined whether these clones were capable of recognizing patient myeloma cells using cytokine release assays. Bone marrow samples obtained before DLI contained a mixture of normal myeloid cells and myeloma cells. Myeloma cells were purified by flow cytometric sorting of CD138− (syndecan) cells and clonal T cells were tested for reactivity against both the CD138+ and CD138− fractions. As shown in Fig. 6, A and B, clone p2–13.1 demonstrated reactivity with patient CD138+ myeloma cells that was ~5-fold higher than stimulation with CD138 negative marrow cells and recipient EBV-transformed B cells. Similar results were seen using assays for IFNγ release (Fig. 6B). In contrast, stimulation of clone p2–3 with CD138+ myeloma cells resulted in relatively little cytokine production compared with PHA stimulation. Clone p2–3 had no detectable response to CD138− cells, and the low-level response to CD138+ cells was similar to the response to patient-derived EBV-transformed B cells.

The HLA restriction of the reaction was investigated using anti-HLA class I and anti-HLA class II monoclonal antibodies. As shown...
DISCUSSION

In this report, we describe the molecular and functional characterization of T-cell clones identified in peripheral blood from patients with relapsed myeloma responding to DLI. These clones were initially identified through analysis of TCR Vβ repertoire (spectratyping), a technique that provides a comprehensive characterization of the circulating T-cell compartment (21, 24, 25). Serial analysis of T-cell repertoire in patient samples has also been used to detect the emergence of oligoclonal and clonal T cells at different times in vivo (18, 26, 27). By combining analysis of TCR repertoire with clinical events, we observed the expansion of individual T-cell clones in peripheral blood that were temporally associated with the initiation of either a GVM or GVHD response (17). However, despite the association of these T-cell clones with specific clinical responses, the functional specificity of these T cells was not established. Further studies were therefore undertaken to quantify the expansion of individual T-cell clones in vivo, isolate and expand these clones in vitro, determine their origin from either recipient or donor, and test the ability of these cells to specifically recognize patient myeloma cells.

To detect individual T-cell clones in patient samples, we designed clonotypic PCR primers and probes based on the CDR3 sequence of five T-cell clones associated with clinical GVM or GVHD. By PCR analysis, GVM-associated clones were detected in patient peripheral blood before DLI. Because these T-cell clones were not detected in blood samples by inspection of CDR3 patterns, this confirmed the increased sensitivity of this method and also suggested that analysis of CDR3 patterns was not likely to identify all clones that were expanding in response to either GVM or GVHD. Using clone-specific primers and probes, we also developed a semiquantitative method to examine the kinetics of expansion of these GVM clones after DLI. These experiments showed that the GVM clones began to expand 1–3 months after DLI, and this coincided with the ability to detect these clones as monoclonal CDR3 peaks in TCR repertoire patterns. These GVM clones reached peak levels in peripheral blood at 5–6 months post-DLI and subsequently regressed. Nevertheless, these clones remained detectable at levels that exceeded pre-DLI levels for at least 18–21 months after DLI. The initial expansion of these clones 1–3 months post-DLI is consistent with the initiation of myeloma response at the same time after DLI. The persistence of these clones at relatively high levels for prolonged periods is also consistent with the long time intervals (6–12 months) required for patients to achieve maximal response after DLI. Thus, measurements of the relative expansion of these putative GVM clones confirmed the temporal association of these clones with the GVM response in vivo.

In contrast, GVHD-associated clones were not detectable before DLI and first appeared 5–6 months after DLI. These clones expanded rapidly, reaching relatively high levels 6 months post-DLI. One of these clones (p1–15) persisted at very high levels for 21 months post-DLI. The other GVHD-associated clone (p1–20) also remained detectable for 21 months, but at relatively low levels. Because these clones were not detectable before DLI, it is possible that they were...
when normal numbers of CD3+ cells have recovered after BMT, these cells are predominantly CD8+, and the number of circulating CD4+ T cells often remains low for >1 year after transplant (38, 42). It is possible that the addition of CD4+ T cells with DLI facilitated the expansion and functional activation of circulating CD8+ T-cell clones resulting in tumor regression. A critical role for CD4+ T cells in maintaining cytotoxic CD8+ T-cell viability has been reported in different models of chronic viral infections and antitumor immunity (43–45). Mattloubian et al. (43) demonstrated that CD4+ T cells play a critical role in chronic infections, which require long-term maintenance of CD8+ CTL to resolve the infection. The role of CD4+ T-cells was less critical in acute infections, which did not require sustained activity of CD8+ CTL for more than 2 weeks (43). Under conditions in which CD4+ T-cell help is lacking, CD8+ T cells specific for viral antigens can persist in blood without being able to provide antiviral effector function (46). Similarly, adoptive transfer of activated Th1-type CD4+ T cells in tumor-bearing mice resulted in de novo generation of endogenous CD8+ T cells with antitumor specificity and concomitant tumor destruction (45). In this model system, effective antitumor immunity required the expression of both MHC class I and class II molecules on host cells, suggesting that CD4+ T cells might enhance the ability of host antigen presenting cells to activate endogenous CD8+ T cells.

Clinical responses after DLI are often associated with the development of GVHD. This observation suggests that the antigenic targets of the DLI and GVHD responses are similar. In particular, these targets would likely be minor histocompatibility antigens that are expressed on normal, as well as malignant recipient cells (47). Alternatively, some patients have had documented responses after DLI without clinical evidence of GVHD. This observation suggests that the GVM response might be directed against tumor-associated antigens in at least some individuals. Both of the patients we studied developed GVHD, as well as tumor responses, but the onset of tumor response and GVHD occurred at different times post DLI. Because tumor responses occurred before GVHD, we postulated that GVM-associated clones would be specific for antigens selectively expressed by recipient myeloma cells. This was confirmed by the functional studies of clone p2–13.1, which showed that these cells were specifically reactive with recipient CD138+ myeloma cells and were not reactive with CD138 negative hematopoietic cells from the same marrow sample. Clone p2–13.1 cells were also not reactive with PBMC or EBV-transformed B cells derived from either recipient or donor (data not shown). This pattern of reactivity essentially excludes the possibility that this clone is reactive with a recipient minor histocompatibility antigen. More importantly, this pattern of reactivity suggests that this clone is reactive with a tumor-associated antigen.

Although the peptide specificity of these T-cell clones has not been determined, our functional studies provide direct evidence that myeloma-specific T-cell clones can at least contribute to antitumor responses after DLI. Several lines of evidence have previously supported the presence of specific antitumor immune responses in patients with myeloma. T-cell clonal expansions have been described by several groups in myeloma patients in different stages of disease, and their immunoregulatory role has been suggested (48–50). Other investigators have also demonstrated the presence of idiotype-specific T cells in the peripheral blood of myeloma patients (51, 52), as well as the ability of the M protein idiotype to induce an autologous CTL response (53, 54). In addition to tumor idiotype as a potential target antigen, it is also possible that other tumor-associated antigens might be recognized after DLI. In patients with relapsed CML who have responded to DLI, previous studies from our laboratory have demonstrated the presence of high titer antibody responses to a variety of novel tumor-associated antigens. Recent studies from our laboratory...
suggest that antibody responses to tumor-associated antigens also develop in patients with myeloma who respond to DLI. Further studies to identify these novel myeloma-associated antigens are currently underway.

In summary, the present studies demonstrate that donor-derived CD8+ T-cell clones with antitumor specificity can be identified in the peripheral blood of patients with relapsed myeloma after allogeneic BMT. The infusion of additional donor CD4+ T cells appears to induce the expansion of these T-cell populations, thus mediating control of disease and clinical remission. These observations suggest an alternative mechanism of action for infusion of donor lymphocytes and provide the basis for further studies investigating the complexity of donor-host interactions after allogeneic BMT.

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Expansion of Tumor-specific CD8+ T Cell Clones in Patients with Relapsed Myeloma after Donor Lymphocyte Infusion

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