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Ex Vivo Characterization of Multiepitopic Tumor-Specific CD8 T Cells in Patients with Chronic Myeloid Leukemia: Implications for Vaccine Development and Adoptive Cellular Immunotherapy

Monique Nannagé,* Michal Abel,2* Anne-Sophie Michallet,2† Stéphanie Delluc,† Marion Lambert,* Stéphane Giraudier,‡ Roland Kratzer,§† Gabrielle Niedermann,§ Loredana Saveanu,† François Guilhot,‖ Luc Camoin,# Bruno Varet,** Agnès Buzyn,†‡ and Sophie Caillat-Zucman3*§

Identification of tumor-associated Ags is a prerequisite for vaccine-based and adoptive immune therapies. Some tumor-associated Ags elicit specific CD8 T cells in patients with chronic myeloid leukemia (CML). Here, we characterized ex vivo responses of CD8 T cells from CML patients to extrajunction bcr-abl peptides and telomerase 540–548 hTert, PR1, and WT1 peptides. CML-specific CD8 T cells were present in most treated patients and were usually multiepitopic: WT1, hTert, PR1, and WT1 tetramer+ cells were detected in 85, 82, 67, and 61% of patients, respectively. The breadth and magnitude of these responses did not differ significantly according to treatment or disease status. CML-specific tetramer+ CD8 T cells had a predominantly memory phenotype, an intermediate perforin content, and low intracellular IFN-γ accumulation in the presence of the relevant peptide. However, in short-term culture with HLA-matched leukemia cells, the patients’ memory T cells were specifically reactivated to become IFN-γ-producing effector cells, suggesting that CD8 T cell precursors with lytic potential are present in vivo and can be activated by appropriate stimulation. In conclusion, this study shows that multiepitopic tumor-specific CD8 T cell responses occur naturally in most CML patients, opening the way to new strategies for enhancing anti-CML immunity, in particular in patients with minimal residual disease. The Journal of Immunology, 2005, 174: 8210–8218.

I dentification of tumor-associated Ags has paved the way for new immunotherapies of human cancers. However, the number of tumor-associated Ags is still somewhat limited, and studies of their ability to elicit functional antitumor immune responses in patients have been rather disappointing. Chronic myeloid leukemia (CML) is an attractive candidate disease for immunotherapy, because it is a slow growing malignancy that may allow time to generate an immune response against malignant cells. The observation that infusions of donor lymphocytes into patients who have relapsed after haematopoietic stem cell transplantation (HCT) can re-induce complete molecular remission (1) strengthens the idea that CML cells are sensitive to tumor-specific T cell effectors. Furthermore, treatment with imatinib, which leads to prolonged minimal residual disease, allows vaccination strategies to be designed for patients with a very low tumor burden.

Recent studies have identified a variety of Ags that elicit specific CD8 T cell responses against leukemia cells. In CML, the Philadelphia (Ph) chromosome 9:22 translocation leads to the production of a chimeric bcr-abl tyrosine kinase (2, 3). Peptides derived from the bcr-abl junction region can act as neoantigens and, therefore, as targets of specific immune responses. An HLA-A3-associated tumor-specific breakpoint peptide has been eluted from primary CML cells, and specific CTLs have been detected in HLA-A3 CML patients (4). HLA-A2-restricted junction peptides can elicit CTL responses in vitro, but whether or not they are naturally processed is controversial (5–8). Of note, peptides from outside the junction region also have the potential to elicit specific in vitro responses by CTL from healthy donors and CML patients (9).

Other studies have focused on Ags derived from normal tissue proteins that can behave as tumor Ags in CML. Proteinase 3, a differentiation Ag associated with granule formation, is aberrantly expressed in tumor cells. PR1, an HLA-A2-restricted peptide derived from proteinase 3, elicits CTLs that kill myeloid leukemia cells but not normal marrow cells (10–12) and are present at significant frequencies in CML patients in remission (7, 13–15). The product of the Wilms’ tumor gene WT1, a transcription factor expressed at low levels by immature CD34+ progenitor cells, is...
also overexpressed in the leukemia cells of almost all CML patients. The HLA-A2-restricted WT1–126 peptide elicits CTLs that specifically lyse HLA-A2 leukemia CD34+ cells (12, 16–18). In addition, the ribonucleoprotein telomerase (hTert), expressed by >85% of human cancers, can elicit CTLs that lyse tumors of various histologic types (19, 20) but not normal cell types in which telomerase activity is detected, such as CD34+ peripheral blood lymphocytes. There is currently no information on the role of hTERT in CML.

Here, we report a detailed study of multiepitopic CD8 T cell responses to bcr-abl, hTert, PR1, and WT1 epitopes in CML patients. Although these specific CD8 T cells are usually unable to function ex vivo as fully differentiated effectors without additional stimulation, such CML-associated epitopes may serve as candidates for vaccine strategies, or as tools to selectively stimulate tumor Ag-specific T cells in vitro to produce IFN-γ and to acquire antitumor cytotoxicity; this approach would be particularly useful for treating patients with minimal residual disease.

Materials and Methods

Patients

The study was approved by the Hôpital Necker Ethics Committee. PBMC were obtained from 33 HLA-A*0201 CML patients, with their informed consent, then separated by Ficoll-Hypaque centrifugation (Amersham Biosciences), and cryopreserved in liquid nitrogen until use. Four patients had received IFN-α2b therapy, 17 patients were receiving imatinib, and 12 patients had received myeloablative HLA-identical HCT from a sibling donor, five HLA-A*0201-negative CML patients, with their informed consent, then separated by Ficoll-Hypaque centrifugation (Amersham Biosciences), and cryopreserved in liquid nitrogen until use. Four patients had received IFN-α2b therapy, 17 patients were receiving imatinib, and 12 patients had received myeloablative HLA-identical HCT from a sibling donor, five HLA-A*0201-negative CML patients.

Peptides and peptide binding assay

Peptides were synthesized by NeoSystems. TAP-deficient T2 cells (American Type Culture Collection) were grown for 18 h at 37°C in serum-free medium, then 106 T2 cells were pulsed with 10 nM peptide in the presence of 100 ng/ml β2-microglobulin (Sigma-Aldrich) for 18 h at 37°C. After washing, cells were stained with the HLA-A2-specific Ab BB7.2 (American Type Culture Collection) and with FITC-conjugated goat anti-mouse Ig. HLA-A2 expression was measured with a FACSCalibur flow cytometer and the mean fluorescence intensity (MFI) was recorded. For each peptide, a specific fluorescence index (FI) was calculated as FI = (MFI with peptide − MFI without peptide)/MFI without peptide.

Generation of peptide-specific CD8 T cells

PBMC from HLA-A*0201+ healthy donors and CML patients were enriched in CD8+ populations by magnetic bead selection with the MACS system (Miltenyi Biotec). CD8 T cells were cultured in 24-well plates (5 × 105 CD8 T cells/well) in complete medium supplemented with 10% heat-inactivated human AB serum. Autologous PBMC were irradiated at 33 Gy, pulsed with 20 mM peptide for 3 h, washed, and added to CD8 T cells at a ratio of 3:1 in the presence of 10 ng/ml IL-7 and 100 pg/ml IL-12. Cells were further stimulated on days 7, 14, and 21 in the presence of 10 ng/ml IL-7 (Sigma-Aldrich). Human rIL-2 (20 IU/ml) was added on days 3, 10, and 17.

MHC class I tetramers, Abs, and flow cytometry

HLA-A2 tetramers were produced as described (21). Each tetramer was titered individually by staining the relevant peptide-specific CD8 T cell line and was used at the optimum concentration (5–10 μg/ml). For staining, 106 PBMC were incubated at 37°C for 30 min in the dark with PE-labeled tetramer, then with combinations of the following Abs: CD8-allophycocyanin, CD3-FITC, CD45RA-PerCP, and CCR7- or CD27-FITC (all from BD Pharmingen) for 15 min at 4°C. Small lymphocytes were gated according to forward/side scatter profiles, then CD3+CD8αβ cells were selected, and staining with 7AAD (BD Pharmingen) was used to exclude dead cells (gating strategy is shown in Figure 1). Data were collected on a FACSCalibur flow cytometer within 1 h after staining and analyzed using Cell Quest software (BD Biosciences).

Intracellular cytokine assay

The frequency of Ag-specific T cells was determined by intracellular IFN-γ staining. PBMC (2 × 106) were cultured in the presence or absence of peptide, then pulsed with 20 mM peptide for 4 h, washed, and stained with PE-conjugated anti-IFN-γ (BD Pharmingen) and with PE- and FITC-conjugated Abs for CD3 and CD8, respectively. After washing, cells were stained with 7AAD (BD Pharmingen) and analyzed using FACSCalibur flow cytometer.
of 10 μg/ml peptide. Two hours after Ag stimulation, 5 μg/ml brefeldin A (Sigma-Aldrich) was added to block cytokine secretion. Following overnight culture, cells were harvested, washed, permeabilized, and stained with allophycocyanin-labeled anti-CD8 and FITC-labeled anti-human IFN-γ mAbs (BD Pharmingen). A response was considered positive if the percentage of CD8 T cells producing IFN-γ in the presence of the cognate peptide was at least twice that observed in the absence of peptide (with a minimum of 0.05% peptide-specific IFN-γ-producing CD8 T cells).

**IFN-γ ELISPOT assay**

The number of IFN-γ-producing T lymphocytes was determined as described by Scheibenbogen et al. (22). Briefly, peptide-specific CD8 T cell lines were cultured in triplicate in 96-well ELISPOT plates (Millipore) at 5 x 10⁶ cells/well with HLA-A²− or A²+ heterologous leukemia cells for 20 h at 37°C with 5% CO2. Pokeweed mitogen was used as a positive control. Spots were counted by an automated ELISPOT reader (Zeiss). A response was considered positive when spot numbers in triplicate assays in the presence of HLA-A²+ leukemia cells significantly exceeded the cutoff value, corresponding to the number of nonspecific spots in the presence of HLA-A²− leukemia cells (cutoff = mean ± 3 SD).

**Elution, purification, and identification of peptides from leukemia cells**

Peptides were isolated from leukemia cells, loaded onto C18 SepPak columns (Waters) and eluted with 60% acetonitrile in water as described by Ostankovitch et al. (23). Peptides were then fractionated by reversed phase HPLC (PerkinElmer/Cetus). Elution with increasing concentrations of acetonitrile (0% for 5 min, 0–10% for 5 min, 10–20% for 50 min, 20–60% for 30 min, and 60–100% for 5 min) was monitored by measuring UV absorption at 214 nm. Individual fractions containing peaks corresponding to the specific retention time of the eight synthetic peptides were collected, lyophilized, and analyzed by mass spectrometry (MS). Mass analysis was performed in reflector mode with a Voyager DE-Pro MALDI-TOF MS (Perseptive Biosystems). Monoisotopic masses above the background noise were calculated after external calibration with a mixture of five peptides.

**Statistical analysis**

The nonparametric Kruskal-Wallis test was used to identify significant differences in the mean number of tetramer-positive T cells among the three patient groups. The Mann-Whitney U test was calculated to identify significant differences in the perfirn content of tetramer+ cells between CML patients and healthy controls. Statistical significance was assumed when p (two-tailed) was <0.05.

**Results**

**Peptide binding assay and generation of HLA-A2 tetramers**

The peptides used here were selected on the basis of previous reports as being able to bind HLA-A2 and elicit specific CTL responses in vitro or in vivo (Table I). The strength of interaction between the peptides and HLA-A2 molecules was checked in a conventional binding assay with T2 cells. PR1, WT1, hTert, and two extrajunction peptides (bcr74 and bcr79) bound efficiently to T2 cells, with an index of fluorescence intensity of at least 1, compared with an index of 4.9 for the reference high-binder peptide CMV pp65. Two other extrajunction peptides (bcr73 and bcr77) were intermediate binders. The b3a2 and b2a2 junction peptides had very low binding indices. HLA-A2 tetramers were obtained for all peptides, except for the two junction peptides that gave rise to unstable soluble A2/peptide monomers, as shown by the gel filtration profile (not shown). There was a good correlation between the capacity of a given peptide to bind HLA-A2 molecules on T2 cells, its ability to generate large amounts of stable tetramers, and the peptide binding score determined by a HLA class I ligand prediction computer program (http://syfpeithi.bmi-heidelberg.com). The specificity of each tetramer was determined by positive staining of a relevant peptide-specific CD8 T cell line and negative staining of an irrelevant CD8 T cell line.

**Frequencies of tetramer-positive CD8 T cells in healthy individuals and patients**

A series of 10 HLA-A2-negative individuals were analyzed for each tetramer, allowing us to define a detection limit for positive staining in A²+ individuals (cutoff = mean frequency of tetramer+ cells in A2-negative subjects ± 3 SD; Table II), as described by Pittet et al. (24). Because all peptides selected for this study were derived from nonmutated self proteins, we first analyzed whether specific T cell precursors were present in a series of 10 HLA-A²+ healthy individuals. Using the chosen cutoffs, no PR1 tetramer+ lymphocytes were detected in these controls, as previously reported (13). However, in a few cases, we found low frequencies of CD8 T cells specific for WT1 (n = 1), hTert (n = 2), bcr74 (n = 2), and bcr77 (n = 2) (0.06–0.27% of CD8+ T cells). We also analyzed five Ph1-negative HLA-A²+ AML patients and found an average of 0.044% bcr-abl tetramer+ CD8 T cells (range 0.02–0.07%). Using the pp65 CMV tetramer as a positive control, we found specific CD8 T cells in all HLA-A²+ CMV-seropositive healthy subjects (mean 1.40%, range 0.05–6.3%).

Higher frequencies of CML-specific T cells were found in the patients (Table II). Positive responses to at least one CML-associated Ag peptide were detected in 31 of 33 patients. Twenty-one patients (64%) had responses to at least four different epitopes.

**Table I. Characteristics of synthetic peptides and results of peptide binding assays**

<table>
<thead>
<tr>
<th>Peptide (location)</th>
<th>Amino Acid Sequence</th>
<th>Score (SYFPEITHI)a</th>
<th>MFI Indexb</th>
<th>Tetramer</th>
<th>Referencec</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR1 (169–177)</td>
<td>VLQELNVTU</td>
<td>28</td>
<td>0.97</td>
<td>+</td>
<td>11</td>
</tr>
<tr>
<td>WT1 (126–134)</td>
<td>RPMFPFAVPY</td>
<td>30</td>
<td>1.2</td>
<td>+</td>
<td>18</td>
</tr>
<tr>
<td>hTert 540 (540–548)</td>
<td>GLKFINIWHN</td>
<td>30</td>
<td>3.3</td>
<td>+</td>
<td>19</td>
</tr>
<tr>
<td>b3a2 junction (926–934)</td>
<td>SSKALRQPV</td>
<td>12</td>
<td>0.24</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>b2a2 junction (897–905)</td>
<td>LTINKEEAL</td>
<td>20</td>
<td>-0.10</td>
<td>–</td>
<td>6</td>
</tr>
<tr>
<td>bcr73 (684–692)</td>
<td>FLSSINEEI</td>
<td>21</td>
<td>0.6</td>
<td>+</td>
<td>9</td>
</tr>
<tr>
<td>bcr74 (714–722)</td>
<td>FMVELVEGA</td>
<td>21</td>
<td>1.3</td>
<td>+</td>
<td>9</td>
</tr>
<tr>
<td>bcr77 (881–889)</td>
<td>MLTSNCVXL</td>
<td>23</td>
<td>0.5</td>
<td>+</td>
<td>9</td>
</tr>
<tr>
<td>bcr79 (912–920)</td>
<td>FLVIIVHIAGA</td>
<td>23</td>
<td>1.2</td>
<td>+</td>
<td>9</td>
</tr>
<tr>
<td>pp65 CMV</td>
<td>NLVPWVAVTV</td>
<td>30</td>
<td>4.9</td>
<td>+</td>
<td>9</td>
</tr>
<tr>
<td>tyr (368–376)</td>
<td>YMDGTMNGVQ</td>
<td>22</td>
<td>ND</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

*a The PR1, WT1, and hTert peptides and the b3a2 and b2a2 junction peptides were selected on the basis of previous reports. Four extrajunction bcr-abl peptides were chosen from the results of peptide binding/stabilization assays and generation of peptide-specific T cell lines in healthy donors (9) or CML patients. The HLA-A2-restricted CMV peptide pp65 was used as a positive control in CMV-positive individuals. The melanoma-associated HLA-A2-restricted tyrosinase peptide was used as a negative control in 10 CML patients.

b Peptide binding scores determined with the MHC class I ligand prediction computer-based program SYFPEITHI (http://syfpeithi.bmi-heidelberg.com).

MFI index = (MFI with peptide – MFI without peptide)/MFI without peptide.

c Reference to the original paper in which the epitope was first described.
CMV tetramer<sup>+</sup> T cells were detected in 24 of 27 CMV-seropositive patients (mean 1.21%, range 0.03–6.29% of CD8<sup>+</sup> T cells). Responses to the melanoma-associated HLA-A2-restricted tyrosinase peptide, used as negative control, were always below the cutoff (0.001–0.02% of CD8<sup>+</sup> T cells), indicating that increases in CML-specific tetramer<sup>+</sup> cells did not reflect global CD8<sup>+</sup> T cell dysfunction in the CML patients. Examples of tetramer staining are shown in Fig. 1. To summarize, responses to the WT1 and hTert peptides were the most frequent (respectively, 28 of 33 patients, range of tetramer<sup>+</sup> CD8<sup>+</sup> T cells 0.08–9.3%); and 27 of 33 patients, range 0.11–13.2%) (Table II). PR1-specific responses were detected in 22/33 patients (range 0.09–2.43%), a frequency compatible with that previously described (7, 14, 15). Responses to bcr-abl were also very frequent, as cells from 82% of patients recognized at least one of the four extrajunction peptides. The most immunogenic of these peptides was bcr74 (24 of 33 patients, range 0.12–14.9% of CD8<sup>+</sup> T cells) and bcr77 (18 of 31, range 0.17–4.27%).

Only two patients (unique patient number (UPN)5 and UPN27) had no CML-specific CD8<sup>+</sup> T cells, and it is noteworthy that neither had responded to their treatment at the time of this study: patient UPN5 did not enter CCR with imatinib, whereas patient UPN27 relapsed early after HCT and died.

HLA tetramers have been proposed to distinguish CD8<sup>+</sup> T cells with high and low TCR affinity, based on fluorescence intensity (25, 26). As already described, bright pp65 tetramer<sup>+</sup> cells were present in the majority of CMV immune individuals (mean MFI = 300, range 50–1269). By comparison, staining with tumor-specific tetramers was significantly weaker (mean MFI = 107, range 20–388, p = 0.02 by two-sided Mann-Whitney U test), as is often the case with tumor-associated Ags (24, 27–29), but the responses were clearly heterogeneous (see examples in Fig. 1).

**Breadth and magnitude of T cell responses according to CML treatment**

We then examined whether the nature of CD8<sup>+</sup> T responses depended on treatment status (Table II, Fig. 2). Seventeen patients received imatinib as first-line therapy (group 1). Of these, 12 were in CCR at the time of the study and all 12 had CD8<sup>+</sup> T cells directed...
to at least one tumor Ag. Two patients were in hematologic relapse: UPN3 (accelerated phase) had responses to hTert, WT1, and bcr77, whereas UPN4 (blast crisis) responded to all seven epitopes studied. Two patients with cytogenetic or molecular relapse (UPN16 and UPN17) had significant frequencies of PR1, WT1, and hTERT tetramer<sup>+</sup> cells and variable responses to bcr-abl peptides. No response was found in UPN5, a patient with a partial cytogenetic response (35% Ph<sup>+</sup> cells). There was no correlation between the breadth or magnitude of CML-specific CD8 T cell responses and the clinical response to imatinib. PR1-specific responses were relatively frequent (12 of 17 patients) but usually weak (mean 0.14% of PR1 tetramer<sup>+</sup> cells, range 0.09–0.21%). Responses to hTert and WT1 were frequent (n = 14 of 17 patients each) and of medium magnitude (mean 0.30% and 0.26% of tetramer<sup>+</sup> cells, respectively). The bcr77 peptide was the most immunogenic bcr-abl peptide (n = 9), followed by bcr74 (n = 8); bcr79 and bcr73 were rarely recognized.

Four patients received IFN-α as first-line treatment (group 2). Patient UPN21 had been in hematologic remission without treatment for 3 years, although he had 50% of Ph<sup>+</sup> cells. The other three patients were in CCR and had been free of IFN-α treatment for at least 3 years at the time of the study (range 3–8 years). All four patients had responses to hTert, WT1, and at least one bcr-abl peptide. Patient UPN18, who was in complete molecular remission (confirmed by repeated RT-PCR analysis) and had been treatment-free for 5 years had strikingly high frequencies of tetramer<sup>+</sup> cells: 45% of his circulating CD8 T cells were dedicated to a CML epitope.

Twelve patients received myeloablative allogeneic HCT during the chronic phase of the disease, usually following failure of IFN-α treatment (group 3). Patient UPN27 relapsed rapidly after grafting (at the time of the study) and died 3 mo later. He had no detectable CML-specific responses. The remaining 11 patients were in complete (n = 10) or partial (UPN33) cytogenetic or molecular remission at the time of this study (mean 44.7 mo after HCT), and all exhibited CD8 T cell responses to at least one CML peptide.

Overall, the magnitude and specificity of responses did not differ significantly among the three patient groups, except that the magnitude of PR1-specific responses was significantly smaller in imatinib-treated patients (mean 0.15%, range 0.11–0.21%) than in patients treated with IFN-α (0.80%, range 0.18–1.7%, p = 0.022 by two-sided Mann-Whitney U test) or HCT (mean 0.66%, range 0.21–2.43%, p = 0.0003).

**Phenotypic analysis of tetramer<sup>+</sup> cells**

We then analyzed tetramer<sup>+</sup> cells for their expression of CD45RA, CD27, and CCR7, characterizing the naive, memory, and effector phenotypes (30, 31), in patients from whom enough material was available (6 patients from group 1, 3 from group 2, and 10 from group 3). As expected, CMV tetramer<sup>+</sup> T cells were predominantly Ag-experienced effector cells (CD45RA<sup>−</sup>CCR7<sup>−</sup> (CD45RA<sup>−</sup>CD27<sup>−</sup>) or effector memory cells (CD45RA<sup>−</sup>CD27<sup>+</sup>) (data not shown). By contrast, all the maturation stages were represented among CML-specific cells, as shown in Fig. 3a. Among CML-specific tetramer<sup>+</sup> CD8 T cells, 51% stained positively for CD45RA (range 10–79%), 75% for CD27 (range 32–93%), and 43% for CCR7 (range 6–86%), in keeping with an early memory phenotype rather than a differentiated effector phenotype. This phenotypic profile was similar to that of the corresponding entire CD8 T cell population. No significant difference in the expression of CD45RA, CD27, or CCR7 on tetramer<sup>+</sup> cells was found between 13 patients with complete cytogenetic responses and 6 patients with molecular/cytogenetic relapses.

The potential of CML-specific CD8 T cells to produce perforin was analyzed in 13 patients from whom enough material was available. For each patient, we studied intracellular perforin accumulation according to the CML-associated peptide(s) that gave the most significant results by tetramer staining. Most patients had relatively low percentages of perforin-containing tetramer<sup>+</sup> cells (median 42%, range 5–76%). As expected, perforin was mainly expressed by the subset of tetramer<sup>+</sup> cells with a CD45RA<sup>−</sup>CCR7<sup>−</sup> or CD45RA<sup>−</sup>CD27<sup>−</sup> effector phenotype, and...
FIGURE 3. Phenotypic and functional characterization of leukemia-specific tetramer$^+$ CD8 T cells in CML patients. a, The CD45RA, CCR7, and CD27 phenotype of CD8$^+$ tetramer$^+$ cells was analyzed in 19 patients subgrouped according to their clinical status. For each marker, we studied 6 patients with molecular/cytogenetic relapse (left column) and 13 patients in complete cytogenetic remission (right column). Phenotypic analyses across all assessable tetramer responses are shown. Each circle represents an individual experiment with a given tetramer. Horizontal bars represent the median value for tetramer$^+$ cells that stained positively for each phenotypic marker. The two patient groups were compared with the two-sided Mann-Whitney nonparametric test. b, Percentage of perforin-containing lymphocytes in 5 patients with cytogenetic/molecular relapse (left part) and 8 patients in complete cytogenetic remission (right part). Values are expressed as a fraction of tetramer$^+$ cells (open circles) or total CD8 T cells (black diamonds). The cutoff for perforin staining was established on the total CD3$^+$CD8$^{b^+}$ lymphocyte population. c, Intracellular perforin staining in hTert-specific (left panel), and CMV-specific (middle panel) tetramer$^+$ CD8 T cells in a representative patient. Lymphocytes were gated on CD3$^+$CD8$^{b^+}$ cells and are shown as tetramer vs perforin dot plots. The numbers indicate the percentage of perforin-containing tetramer$^+$ cells. Perforin staining of the total CD8 T cells is also shown (right panel). d, Tumor-specific T cell responses detected by IFN-$\gamma$ ELISPOT assay. Top panel, CD8 T cells from a hTert-specific CD8 T cell line derived from patient UPN14 were cultured with medium alone (negative control), leukemia cells from one HLA-A2$^+$ and one HLA-A2$^-$ CML patient, or pokeweed mitogen (PMA, positive control). The effector-to-target cell ratio was 1:1. Bottom panel, CD8 T cells from a bcr79-specific T cell line derived from patient UPN29 were cultured with medium alone, autologous leukemia cells, or leukemia cells from three HLA-A2$^+$ CML patients (CML-1, CML-2, CML-3), or from two HLA-A2$^-$ CML patients (CML-4, CML-5). The effector-to-target cell ratio was 20:1. Results are the mean number of specific spot-forming cells (SFC) per triplicate well. A response was considered positive if the number of spots in the presence of HLA-A2$^+$ leukemia cells was above the cutoff value, based on the number of nonspecific spots in the presence of HLA-A2-negative leukemia cells (cutoff = mean $\pm$ 3 SD).

Detection of tumor-specific T cell responses by IFN-$\gamma$ production

To gain further insight into the effector properties of tumor-specific CD8 T cells, we analyzed their capability to produce IFN-$\gamma$. First, we tested whether CD8 T cells accumulated intracellular IFN-$\gamma$ in response to a few hours of contact with CML peptides in 9 patients selected for their elevated frequencies of tetramer$^+$ CD8 T cells. In unstimulated circulating cells, a mean of 0.04% of the total CD8 T cell population contained intracellular IFN-$\gamma$. Only one patient showed significant IFN-$\gamma$ production in response to hTert peptide (23% of hTert tetramer$^+$ cells stained positively for IFN-$\gamma$, compared with 45% of CMV tetramer$^+$ cells) (data not shown).

We then analyzed hTert- and bcr79-specific T cell lines derived from patients UPN14 and UPN29, respectively, for their capacity to produce IFN-$\gamma$ in short-term culture with autologous or heterologous HLA-A2$^+$ leukemia cells. The IFN-$\gamma$ ELISPOT assay served as a read-out system to quantify responses at the cellular level. The mean number of spots was low in unstimulated cells (background, range 0–10 spots). In both lines, memory T cells were specifically reactivated to IFN-$\gamma$-producing effector cells in the presence of A2$^+$ leukemia cells, with frequencies of 30 to 240 IFN-$\gamma$-specific spots/10$^4$ CD8 T cells (Fig. 3d). This suggests that quiescent leukemia-specific CD8 T cells can be amplified in vitro to produce IFN-$\gamma$ following short-term specific stimulation.

The frequency and phenotype of CML-specific tetramer$^+$ cells can fluctuate with the clinical course

Serial analyses were performed in two patients who underwent HCT (UPN22 and UPN23, Fig. 4). Both patients entered complete cytogenetic remission but developed moderate then extensive chronic graft-vs-host disease (GVHD); the GVHD finally resolved, concomitantly with complete molecular remission. Interestingly, clinical GVHD coincided with a rise in the number of CML-specific CD8 T cells. In patient UPN22, hTert-specific CD8 T cells were detected first in samples drawn 12 and 18 mo after HCT (0.44 and 0.19%, respectively). These hTert tetramer$^+$ cells expressed a central memory phenotype. At this time the patient had moderate
GVHD. Six months later, he entered molecular remission but developed extensive GVHD. Surprisingly, frequencies of CD8 T cells directed against 6 of the 7 tumor epitopes studied here were high, representing a total of 6.6% CML-specific tetramer + cells (i.e., 1 in 15 CD8 T cells). The hTert response increased 9-fold (3.9% of tetramer + cells), and these hTERT tetramer + cells shifted toward a more highly differentiated effector memory phenotype. Patient UPN23 was first analyzed 29 mo after grafting, when he was in cytogenetic remission and had moderate GVHD. No CML-specific CD8 T cells were detected, contrasting with a very strong CMV-specific response (5.3% of tetramer + cells). He entered complete molecular remission 10 mo later but developed extensive GVHD; significant tumor-specific CD8 T cell responses were found at this time (0.13% WT1 tetramer + cells, 0.53% hTert, 0.25% bcr74). In addition, hTert-specific cells showed unusually high perforin content (80% perforin-containing hTert tetramer + cells, compared with 97% for CMV tetramer + cells and 87% for the total CD8 population, Fig. 4). Thus it appears that marked and reversible shifts in the proportion of memory-type circulating CML-specific CD8 T cells can be associated with particular clinical events such as GVHD.

Identification of CML-associated peptides on leukemia cells

To determine whether the peptides studied here were actually expressed on leukemia cells, eluates of circulating leukemia cells obtained from two patients at diagnosis were analyzed by MS. MS product ion spectra characteristic of three peptides corresponding to hTert (mass detected 1140.71 Da), PR1 (1014.59 Da), and bcr73 (1051.50 Da) were found in patient UPN15, whereas four peptides corresponding to PR1 (1014.59 Da), bcr79 (999.58 Da), bcr73 (1051.55 Da), and bcr74 (994.50 Da) were found in patient UPN29 (Fig. 5). No evidence of peptides WT1 or bcr77 was found. These data show that the leukemia cells of these patients presented at least 6 of the 8 studied CML peptides at their surface and suggest that the proteolytic machinery in CML cells properly generates these peptides. Prediction of proteasome-mediated digestion of polypeptides encompassing the candidate epitopes, in particular determination of their correct COOH-terminal cleavage, was therefore incorporated in our analysis. A proteasome cleavage prediction algorithm ([http://flieder.biol.biologie.uni-tuebingen.de/paproc2/paproc2a.html]) suggested that PR1, hTert, bcr73, bcr74, and bcr79 could be properly cleaved by the proteasome, arguing for their presence on tumor cells containing a proteasome. Interestingly, WT1 and bcr77, which were never detected on leukemia cells by MS, were not predicted to be cleaved by the proteasome.

Discussion

This study shows the existence of naturally occurring CD8 T cells directed against various CML-associated epitopes expressed by leukemia cells, as well as their memory phenotype and lack of functional capacities ex vivo without additional stimulation. These results have important implications for the design of novel peptide-based vaccine strategies and for in vitro expansion of Ag-specific T cells for adoptive immunotherapy.

Circulating T cells directed against at least seven different tumor-associated self peptides were frequently detected in CML patients, accounting for up to 1 in 15 peripheral CD8 T cells (45% in one patient with prolonged remission after IFN-α treatment). In addition to the previously described WT1- and PR1-specific responses in treated CML patients (7, 26), we identified responses to two further Ags. First, we found that hTert, previously implicated in immune responses to various cancers, also induced specific CD8
T cell response in almost 80% of CML patients, potentially making it a near-universal tumor-associated Ag. Second, we showed that >80% of HLA-A2 patients had CD8 T cell responses to peptides derived from the extrajunction region of the bcr-abl chimeric protein, i.e., from the normal sequence of the ubiquitous bcr protein. In a vaccine trial based on various bcr-abl-derived fusion peptides given to patients with chronic-phase CML, CD8 responses specific to the HLA-A2-restricted junction peptide b3a2 were never detected in HLA-A2 patients, whereas all A3 and A11 patients had positive responses to the cognate junction peptides (32). Thus, at least in HLA-A2 individuals, it appears that the immunogenicity of the bcr-abl fusion protein is not related to the breakpoint region itself, as frequently suggested, but rather to extrajunction self peptides. Such self peptides may behave as cryptic epitopes during CML, either because of their overexpression by leukemia cells, or because of delocalization of bcr protein to the cytoplasm, modifying its processing. As almost half of the Caucasian population is HLA-A2, these data should be taken into account in the design of peptide vaccine trials with bcr-abl as candidate Ag. Of great interest in this context are the recent results from Bocchia et al. (33) showing that a vaccine consisting of b3a2 breakpoint peptides restricted by the HLA-A3, A11, B8, or class II HLA molecules (but no HLA-A2-restricted peptide) could reduce persistent disease in 16 patients on conventional treatment for CML. Most patients showed consistent reduction of their residual disease after vaccination, with half of them reaching complete remission. This early promise of efficacy emphasizes the need for further investigation on vaccine development against CML-specific Ags.

We successfully eluted hTert, PR1, and extrajunction bcr73, bcr74, and bcr79 peptides from HLA-A2 CML cells, identifying them by MS, suggesting that these peptides can be generated by the proteasome and bind efficiently HLA-A2 molecules. To our knowledge, this is the first evidence that hTert and extrajunction bcr-abl peptides are expressed on CML cells and can therefore potentially be recognized by specific CD8 T cells. It is unclear why we failed to detect WT1 and bcr77 peptides in eluates of CML leukemia cells, whereas the corresponding tetramer+ cells were present in the patients. It is possible that tetramer+ cells are specific for peptides that undergo posttranscriptional modifications in leukemia cells and are therefore not detectable at their surface. Alternatively, the peptides may be present on leukemia cells but at levels below the detection limit of mass spectrometry. Finally, WT1 and bcr77 peptides, although not generated by the proteasome in leukemia cells, might be digested by the immunoproteasome in dendritic cells. We are currently testing these hypotheses.

It is not known whether CML-specific CD8 T cells are capable of being activated in vivo to kill residual autologous leukemia cells, and further longitudinal studies will be necessary to determine whether the existence and nature of CML-specific T cell responses have any impact on disease progression. However, these mechanisms clearly do not operate optimally in vivo, because the disease would never occur if they did. In CML patients, the coexistence of leukemia cells and tumor-specific circulating CD8 T cells could be due to gradual escape of tumor cells from T cell recognition as a result of immune editing (34), or exposure of CD8 T cells to immune suppressor molecules secreted within the tumor microenvironment. We show here that the primary reason for the lack of tumor immune responsiveness is inefficient T cell function. Indeed, the majority of tumor-specific CD8 T cells detected in CML patients were unable to function as fully differentiated effectors ex vivo. Although perforin accumulation alone does not comprehensively portray cytotoxic T cell functions, we found that low levels of perforin were constitutively expressed by CML-specific T cells compared with CMV-specific T cells. Moreover, most CML-specific tetramer+ cells had phenotypic characteristics of memory T cells and were not able to secrete IFN-γ ex vivo. Such functional unresponsiveness of tumor Ag-specific CD8 T cells has already been shown for metastatic melanoma and ascribed to a selective anergy in vivo (35). However, we show that some of CML-reactive memory cells could be reactivated in vitro to produce IFN-γ in the presence of HLA-matched leukemia cells. These data suggest that it might be possible to improve the functional capacity of leukemia-specific cells for therapeutic purposes. Furthermore, we found that CML-specific CD8 T cell responses were frequently multiepitopic, a feature that may compensate for the low frequency and amplitude of individual responses. Such polyclonal responses would be valuable for maintaining a sufficient pool of T cells specific for tumor Ags, reducing the risk of immune escape variants, and establishing long-lasting antitumor immunity after active vaccination (36). Indeed, the prolonged molecular remission observed many years after IFN-α treatment in patient UPN18 suggests that if sufficient numbers of low-avidity CTLs directed against different tumor Ags could be elicited, minimal residual disease might be effectively controlled. Future trials of candidate vaccines or adoptive cellular immunotherapy should thus be optimized not only for the Ag type and dose, but also for the number of target epitopes.

We found no difference in the nature and efficiency of CML-specific CTL responses according to treatment status, apart from the already described weak PR1 responses in imatinib-treated patients (15). This tends to endorse the different peptides as candidate Ags for immunotherapy, whatever the first line of therapy. Likewise, we found no relation between clinical status and the frequency or phenotype of CML-specific T cell responses. However, intriguingly episodes of severe GVHD coincided with a striking expansion of tumor-specific CD8 T cell responses in two patients who were studied longitudinally after grafting. It is tempting to speculate that this rise in CML-specific tetramer+ cells resulted from in vivo activation of these cells during GVHD-associated biological events, although we cannot formally demonstrate it. The cytokine storm associated with GVHD is known to induce expression of cell surface markers on effector and regulatory T cells (37) and might enhance the expansion of pre-existing Ag-specific T cells and modify their phenotype. In the absence of appropriate stimulation and/or addition of growth factors, T cells might retain a quiescent phenotype that cannot be activated by a relatively indolent tumor microenvironment (38). Although these two patients had durable molecular remissions of CML, the correlation between CML-specific CD8 T cell expansion and a graft-vs-leukemia effect will remain speculative pending longitudinal analysis of a sufficient number of patients.

In conclusion, we show that multiepitopic tumor-specific CD8 T cell responses occur naturally in treated patients with CML and offer new insights into the specificity and functionality of such responses. Although we obtained no direct evidence that the tetramer+ CD8 T cells identified in these CML patients can exert lytic functions in vivo, their ability to proliferate and to produce IFN-γ in vitro suggests that appropriate stimulation and/or addition of growth factors may overcome this ineffectiveness in vivo, notably in patients with minimal residual disease.

Disclosures
The authors have no financial conflict of interest.

References


