Detection and Long-Term In Vivo Monitoring of Individual Tumor-Specific T Cell Clones in Patients with Metastatic Melanoma

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We investigated the presence of individual melanoma-specific T cell clones in patients with metastatic melanoma. Ten patients were examined for the presence of melanoma-reactive T cells using dendritic cells loaded with autologous tumor cells. Their specificity was tested using nonradioactive cytotoxicity test. Individual immunodominant T cell clones were identified by the clonotypic assay that combines in vitro cell culture, immunomagnetic sorting of activated IFN-γ+ T cells, TCRβ locus-anchored RT-PCR, and clonotypic quantitative PCR. All patients had detectable melanoma-reactive T cells in vitro. Expanded melanoma-reactive T cells demonstrated specific cytotoxic effect against autologous tumor cells in vitro. Three patients experienced objective responses, and their clinical responses were closely associated with the in vivo expansion and long-term persistence of individual CD8+ T cell clones with frequencies of 10^-6 to 10^-3 of all circulating CD8+ T cells. Five patients with progressive disease experienced no or temporary presence of circulating melanoma-reactive T cell clones. Thus, circulating immunodominant CD8+ T cell clones closely correlate with clinical outcome in patients with metastatic melanoma. The Journal of Immunology, 2007, 178: 6789–6795.

Adoptive immunotherapy using tumor-infiltrating lymphocytes, tumor-specific T cells, or tumor-specific vaccination strategies using dendritic cells (DC) loaded with tumor Ags demonstrated objective partial response in 30–50% of patients with metastatic melanoma (MM) and other solid tumors in the absence of serious vaccine toxicity (1–8). Such tumor regression largely exceeds the rate of spontaneous regression of melanoma metastases and it is very likely due to the effect of adoptive immunotherapy. Because most of the patients demonstrate only a short-term response in various adoptive immunotherapy trials, the optimal number of tumor-reactive T cells that are believed to be central for the anticancer effect in vivo has not been well defined yet. This may be caused in part by our limited methodological approaches to monitor Ag-specific T cells in vivo and in part by changing the repertoire of tumor-specific T cell clones that occur in vivo at various stages of cancer treatment. Relatively limited information exists about the frequency of naive T cell precursors that are able to recognize a given Ag in association with MHC class I or MHC class II. In humans, for example, the relative frequency of CTL precursors that recognized a MAGE-3 peptide presented by HLA-A1 was estimated to be 3 × 10^-7 of the blood CD8+ T cells (9). Assuming a mean CTL precursor frequency of ~10^-6 of tumor Ag-specific CD8+ T cells, a significant increase of this frequency, for example a 40-fold increase, translates into 0.00004 of the CD8+ T cells following vaccination. This result is beyond the limit of detection of all immunological methods, including tetramer, unless the frequency of Ag-specific cells is increased through an in vitro restimulation assay (10). Direct methods can only detect substantially larger T cell populations with frequencies of >0.001 of Ag-specific T cells. Such responses are observed against viral peptides and quite frequently exceed even 0.01 of Ag-specific T cells in case of CMV or HIV peptides (11, 12). In cancer immunotherapy, there is no proof that a cancer vaccine has to stimulate a large number of T cells to initiate tumor rejection. Thus, we should take into account that T cell responses to tumor Ags may be low level and that negative results obtained with most ex vivo assays may not exclude the beneficial effect of tumor-specific T cells in vivo.

Previously, we were able to identify and quantify in vivo individual alloreactive and leukemia-reactive donor T cell clones in patients undergoing allogeneic HLA-matched hematopoietic stem cell transplantation who developed acute graft-vs-host disease (GVHD) and demonstrated their GVHD- and graft-vs-leukemia (GVL) specificity without prior knowledge of GVHD- and GVL-specific Ags (13–15). Using the clonotypic assay based on selection of Ag-reactive T cells and further molecular analysis of their TCRβ repertoire (14, 15), we were able to identify the most immunodominant Ag-specific T cells and detect them by clone-specific primers and probes quantitatively in vivo with a threshold frequency of 0.00001 T cells. Such a novel and sensitive approach to the detection of Ag-specific T cells does not require the prior knowledge of the particular Ag and can be used for long-term monitoring of individual tumor-specific T cells in cancer patients. In this study, we used the clonotypic assay in patients treated for MM and correlated their clinical outcome with individual tumor-specific T cell clone monitoring.
Materials and Methods
Patient characteristics and cell collection

Ten consecutive patients with MM (American Joint Committee on Cancer stage IV) were enrolled in the study and treated according to the Czech Oncology Society guidelines with dacarbazine or temozolomide, fotemustin alone or fotemustin alone. M, Male; F, female; sd, stable disease; PR, partial response; PD, progressive disease; MR, mixed response.

Table I. Characteristics of patients with metastatic melanoma

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age at Diagnosis (years)</th>
<th>No. of Metastatic Sites</th>
<th>No. of Therapeutic Regimens</th>
<th>Response/Survival (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>46</td>
<td>1</td>
<td>1 (A)</td>
<td>sd/60</td>
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<tr>
<td>2</td>
<td>F</td>
<td>67</td>
<td>2</td>
<td>2 (D;A)</td>
<td>PR/22</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>58</td>
<td>3</td>
<td>1 (A)</td>
<td>PR/27</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>76</td>
<td>2</td>
<td>1 (A)</td>
<td>PD/12-died</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>64</td>
<td>5</td>
<td>2 (D;A)</td>
<td>PD/4-died</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>26</td>
<td>4</td>
<td>3 (D;AI;T)</td>
<td>PD/16-died</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>62</td>
<td>4</td>
<td>2 (TFCV;A)</td>
<td>PD/7-died</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>64</td>
<td>3</td>
<td>2 (A,F)</td>
<td>PD/8-died</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>54</td>
<td>1</td>
<td>2 (AF)</td>
<td>MR/14</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>70</td>
<td>3</td>
<td>3 (D;ALT)</td>
<td>PD/7</td>
</tr>
</tbody>
</table>

*Therapeutic regimens: dacarbazine (D); IFN-α2a (A) and/or IL-2 (I); temozolomide (T), fotemustin (F), cisplatin (C), vinblastine (V) in combination or temozolomide alone or fotemustin alone. M, Male; F, female; sd, stable disease; PR, partial response; PD, progressive disease; MR, mixed response.

Flow cytometric analysis

Cells were washed with PBS and incubated with FITC-conjugated, PE-conjugated, or allophycocyanin-conjugated Abs for 15 min on ice. Cell surface phenotype was analyzed by flow cytometry using anti-CD4-FITC, anti-CD8-FITC, anti-CD3-APC (Immunotech), and anti-IFN-γ-PE mAbs (Miltenyi Biotec). After washing with cold PBS, cells were fixed with 1% formaldehyde for 15 min.

FIGURE 1. Sorting melanoma-reactive IFN-γ+ T cells. Melanoma-reactive IFN-γ+ T cells were sorted immunomagnetically. Representative data from patient no. 3 are shown. A. The presorted cell population (gated on CD3+ cells) with the presence of both CD3+CD4+ and CD3+CD8+ T cells. B. Enrichment of CD3+IFN-γ+ T cells.
parafomaldehyde (Sigma-Aldrich) and analyzed using a Cytomics FC 500 flow cytometer (Beckman Coulter).

**Tumor-specific reactivity of CTLs in vitro**

Harvested IFN-γ-positive CTLs were cultured in complete medium containing X-VIVO 15 with 50 mg/l gentamicin, 2 mM L-glutamine, 25 mg/ml HEPES (BioWhittaker), 10% heat-inactivated human AB-serum (Sigma-Aldrich), and 500 IU/ml IL-2 (Prolenkin; Chiron) for 3–4 wk. PHA (5 μg/ml) (Sigma-Aldrich) was added on day 1. Cultures were usually started with 10^5 melanoma-reactive IFN-γ-positive T cells, and when possible the single-cell culture was initiated in parallel. Complete culture medium was replaced twice weekly, and feeder cells (irradiated PBMC from healthy volunteers) were added once weekly starting at the second week of expansion. CTL assay with Calcein-AM (Molecular Probes) was performed as standard Cr51 release assay but is nonradioactive and requires fewer cells. A modified version of the SMART method (SMART Race cDNA Amplification Kit; BD Clontech) as described previously (15). TCRβ primer (5’-GCTTCTGATGGCTCAAACACAGCGACCTC-3’) was used to obtain TCRβ PCR products from the 5’ end to the start of the TCRβ region. The PCR product was ligated into the pGent-Easy vector (Promega) and used for Escherichia coli transformation. Clones were defined by the presence of at least two identical DNA sequences (clonotypes) of the TCRβ CDR3. For each PCR product, at least 50 colonies were selected, amplified by PCR with vector-specific primers, and used for direct sequencing to obtain TCRβ CDR3 sequences corresponding to sorted, activated melanoma-reactive T cells.

**In vivo quantitative monitoring of individual immunodominant T cell clones**

The most immunodominant T cell clones were selected based on the frequency of their unique DNA sequence of the TCRβ CDR3 region (>10% of the same clonotype among all bacterial colonies sequenced). Clone-specific primers and probes were designed for the TCRβ CDR3 with Primer Express version 1.5. Clonotypic quantitative PCR (qPCR) was performed using an ABI PRISM 7700 on PBMC lysates or biopsy DNA as described previously (15). qPCR was a 50-cycle 2-step reaction using Platinum Taq (Invitrogen Life Technologies), with the annealing/extension phase at 65°C. A standard curve was plotted and template copies were calculated. Plasmid template for the qPCR standards was the same as that used in the sequencing of the CDR3. Each primer/probe set was tested against the target plasmids for the individual’s other primer/probe sets, and redesigned if necessary to ensure no cross-reactivity. Each set was also tested with PBMC derived from five different healthy donors, and we found no amplification with any of the 12 primer/probe sets. The high specificity and sensitivity (1 in 100,000 cells) of clonotypic qPCR has been shown previously (17, 18). Samples were analyzed in triplicate.
Correlations were performed by Spearman’s rank test, the Wilcoxon matched pairs test, and the Mann-Whitney U test using Prism 3.0 software.

**Results**

**Patients with MM have circulating melanoma-reactive CD4+ and CD8+ T cells**

DC loaded with irradiated autologous tumor cells were used for activation of T lymphocytes in 10 patients with MM. Patient characteristics are shown in Table I. After two rounds of stimulation, low frequencies of IFN-γ-producing CD4+ (0.9–3.2%, median 1.3%) and CD8+ (0.6–2.4%, median 1.0%) T cells were detected (Fig. 1A). Melanoma-reactive IFN-γ-producing T cells were immunomagnetically separated. The percentage of activated CD3+ IFN-γ+ T cells before magnetic separation was 1.8–4.4% (median 2.4%) and after the procedure reached 59.7–88.4% (median 76.2%) of all CD3+ T cells (Fig. 1B). Sorted CD3+ IFN-γ+ T cells (0.52–2.39 × 10⁶ cells) were further expanded for 3–4 wk until at least 10⁸ viable T cells were reached. After the expansion, 96.7–99.5% (median 99.0%) of cells were CD3+ T cells with a prevalence of 72.8–86.4% (median 81.1%) CD3+ CD8+ cells, remaining T cells were CD4+.

**Expanded melanoma-reactive CTLs demonstrate specific cytotoxic potential**

A specificity of expanded melanoma-reactive CTLs was tested against the original autologous melanoma cells in five patients (nos. 1, 3, 4, 7, and 8) with sufficient numbers of remaining tumor cells. Unselected T cells showed specific cytotoxicity to autologous tumor cells in all five individuals, reaching 21–39% (median 33%) at 20:1 E:T ratio and 14–25% (median 21%) at 1:1 E:T ratio. Representative data from two independent experiments (patient nos. 1 and 3) are shown at Fig. 2A. In patient no. 3, we were also able to grow and expand individual T cell clones in 15 of 96 wells that were started as a single-cell suspension, and in 6 of 15 expanded clones we were able to confirm the presence of the same immunodominant clone (described in the next paragraph) as in the cell culture initiated with 10⁵ sorted cells. This particular clone demonstrated the same cytotoxic potential to autologous melanoma cells as the melanoma-reactive IFN-γ+ CD3+ CD8+ T cells expanded from 10⁵ cells in a single well. This particular clone from patient no. 3 recognized specifically autologous tumor cells but not the allogeneic tumor cells from patient no. 1 (Fig. 2A).

Cytotoxicity was blocked by an anti-HLA class I Ab, demonstrating the dominant role of CD8+ CTLs (Fig. 2B). Negligible reactivity against autologous DC and both negative controls (myeloma cell line ARH77 and allogeneic PBMC) was noticed (Fig. 2).

**Identification of immunodominant T cell clones in vitro**

We next analyzed the clonal composition of the sorted IFN-γ+ melanoma-reactive T cells and defined them by the unique sequences of their TCRβ CDR3 region in patient nos. 1–8. TCRβ loci of the sorted cells were amplified by template switch-anchored RT-PCR, as described in Materials and Methods, to ensure that all melanoma-reactive TCRβ sequences were amplified without bias to particular TCRβ V families. Thus, the melanoma-reactive clonotypes were represented in the anchored RT-PCR product with a relative frequency reflecting that found in the original sorted cell population. Sequencing of 50 bacterial colonies containing TCRB CDR3 from each sort was performed to identify the number and frequency of individual T cell clones in each sorted population. We have previously demonstrated the specificity and sensitivity of this approach to analyze the clonality of HIV-, GHVD-, and GVL-specific T cells (15, 17). We have also demonstrated that only highly immunodominant T cell clones (forming at least 10% of all in vitro-identified clonotypes) are also detectable in vivo (15, 17).

Table II shows the number of successfully sequenced bacterial colonies (in vitro-identified clonotypes) are also detectable in vivo (15, 17). We have also demonstrated that only highly immunodominant T cell clones (forming at least 10% of all in vitro-identified clonotypes) are also detectable in vivo (15, 17). Table II shows the number of successfully sequenced bacterial colonies and the number of different melanoma-reactive T cell clones identified for each patient. The frequency and amino acid sequences of CDR3 regions that occurred in >10% of bacterial colonies in vitro are also shown. These are likely to represent the most “dominant” T cell clones and their presence in peripheral blood of melanoma patients were further studied in vivo. We observed considerable heterogeneity in the clonality of melanoma-reactive T cells among the patients. Notably, melanoma-reactive CD8+ T cells from patients nos. 4–8 who died due to rapid cancer progression were highly polyclonal, comprising between 28 and 35 (median 31) individual clonotypes, whereas melanoma-reactive CD8+ T cells from patients with at least some measurable clinical response were far more oligoclonal, comprising between 5 and 22 (median 9) individual clonotypes. Indeed, within these oligoclonal repertoires, only one or two CD8+ clones stood out as being clearly dominant in frequency for each patient with at least some treatment response. In one case (patient no. 3), we were able to...
identify a single immunodominant CD8⁺ T cell clone (Clone 3A, Table II) that represented 6 of 15 in vitro-expanded clones initiated from a single-cell culture. This particular clone was the same as the most immunodominant CD8⁺ T cell clone identified from the culture expanded in vitro from 10⁵ melanoma-reactive IFN-γ⁺ T cells in a single well, and we were able to monitor this clone in vivo for 24 mo as described in the next section.

Melanoma-reactive CD4⁺ T cells were rather polyclonal in all patients regardless of their clinical response, comprising between 15 and 32 individual clonotypes with no clearly dominant clones (Table II, top).

Long-term persistence of immunodominant CD8⁺ T cell clones correlates with better clinical outcome

Having identified individual immunodominant CD8⁺ T cell clones in vitro, we next quantified the dominant clones at multiple time points after the patient was diagnosed with MM in peripheral blood. We have also examined the presence of these clones in tumor biopsies performed at the time of diagnosis and, in some cases, at the time of further metastatic spread of melanoma. We designed clone-specific PCR primers and probes specific for the TCRβ CDR3 region of each dominant melanoma-reactive clone, the sequence of which had been detected in at least 10% of bacterial colonies from the anchored RT-PCR product (Table II, bottom)—a total of 18 individual clones. We performed clonotypic quantitative real-time PCR on each sample from each recipient. Because a single T cell contains one productively rearranged TCRβ locus of a particular sequence, the copy number detected in each PCR was directly equivalent to the absolute number of melanoma-reactive clone cells present in the sample. Fig. 3 shows the levels of each immunodominant clone in the peripheral blood of seven patients characterized and numbered as shown in Table I. Notably, subjects 1–3 who are long-term survivors of MM have demonstrated a long-term persistence of the most dominant CD8⁺ T cell clones in vivo in their peripheral blood. In contrast, subjects 4–7 had only temporary or no (subject 8) detectable immunodominant CD8⁺ T cell clones in their peripheral blood. Quantification of absolute numbers of melanoma-reactive CD8⁺ T cell clones showed in general some variability reaching from the threshold levels of qPCR 0.000001–0.001 of CD8⁺ T cells in peripheral blood. It revealed a long-term persistence with minimal or mild expansion in long-term survivors but disappeared shortly after diagnosis of MM in nonsurvivors.

Because we did not see any dominant (comprising >10% of identical TCRβ CDR3 in DNA sequenced bacterial colonies in vitro) CD4⁺ T cell clones, we tested the presence of the two most frequent melanoma-reactive CD4⁺ T cell clones in their peripheral blood. Quantification of absolute numbers of melanoma-reactive CD8⁺ T cell clones showed in general some variability reaching from the threshold levels of qPCR 0.000001–0.001 of CD8⁺ T cells in peripheral blood. It revealed a long-term persistence with minimal or mild expansion in long-term survivors but disappeared shortly after diagnosis of MM in nonsurvivors.
Finally, we examined the presence of immunodominant CD8\(^+\) clones in original tumor-infiltrated lymph node biopsies by the clone-specific PCR and demonstrated the presence of these clones in all survivors (subjects 1–3) but only in three of six nonsurvivors studied (in subjects 4, 6, and 7). Subjects 6 and 8 were rebiopsied for a new metastatic involvement (12 and 6 mo, respectively, after their initial lymph node biopsy), but none of their immunodominant CD8\(^+\) T cell clones were detected. Subjects 9 and 10 were not studied by the clone-specific qPCR.

**Discussion**

By using DCs loaded with irradiated autologous tumor cells, we were able to identify circulating melanoma-reactive T cells in patients with MM. After stimulation, Ag-reactive T cells activate and produce IFN-γ. Both populations of CD4\(^+\) and CD8\(^+\) melanoma-reactive T cells were present. Such cells can be enriched immunomagnetically and further expanded in vitro (16, 19). To generate melanoma-specific CTLs for further study, we expand IFN-γ\(^+\) melanoma-reactive T cells that contain most of the immunodominant T cell clones. Despite the advanced stage of tumor spread, it was possible to expand functional melanoma-reactive T cells. For the most part, CD8\(^+\) CTLs were expanded and demonstrated a tumor-specific cytotoxic effect. Cytotoxicity against autologous melanoma cells was blocked by an anti-HLA class I Ab, indicating that the effect was most likely mediated through CD8\(^+\) HLA class I interaction. This finding confirms an important role for CD8\(^+\) CTLs in specific cytotoxicity that is in agreement with studies using a defined melanoma-specific peptide HLA class I-restricted Ag (9, 10).

In this study we identified in vitro, and quantified in vivo, individual immunodominant melanoma-reactive CD8\(^+\) T cell clones by developing an approach that combines in vitro cell culture, immunomagnetic sorting of activated IFN-γ\(^+\) T cells, TRβ\(^+\) locus-anchored RT-PCR, and clonotypic qPCR. In published studies, the identification of clonal T cell responses has been accomplished by flow cytometry using mAbs directed against TRβ\(^+\) V regions, by CDR3 spectratyping, or by microplate hybridization assay. These studies were able to demonstrate that the TRβ repertoire is highly variable; for example, monoclonal, oligoclonal, or polyclonal CTL responses can be induced depending on the tumor Ag used (20–22). Interestingly, either monoclonal or polyclonal responses can be elicited by the same peptide derived from a MAGE-3.A1 tumor-specific Ag in different individuals (21, 22). Yet, using these techniques does not enable detailed analysis of individual T cell clones because, in the case of flow cytometry, the series of Abs are not complete enough to analyze all TRβ\(^+\) V region families; in the case of spectratyping and microplate hybridization assay, TRβ\(^+\) V region families, but not individual T cell clones within these families, can be identified (23–25). Our approach obviated skewing of the clonotype population by prolonged stimulation and propagation. Furthermore, the anchored RT-PCR step used to amplify TRβ\(^+\) CDR3 regions of all T cell clones present in the sorted population avoided the incomplete coverage of, and bias to, particular TRβ\(^+\) V regions conferred by using TRβ\(^+\) V-specific PCR primers. This approach required no prior knowledge of either the specific target Ag or the MHC-restricting element, and when combined with qPCR, it allowed for the sensitive and specific quantification of melanoma-reactive T cell clones in the patient at any point and in any blood or tissue sample. In agreement with spectratyping and microplate hybridization studies (23–25), we were able to detect oligoclonal or monoclonal expansions of melanoma-reactive CD8\(^+\) T cell clones. The sensitivity of clone-specific qPCR reached the threshold of 1 × 10\(^{-9}\), thus allowing us to monitor low-frequency clones. It has been shown that the frequency of naive T cells recognizing melanoma-specific peptide Ags such as MAGE-A3\(_{68–176}\), gp100, NA17, LAGE-1, or MAGE-A10 exists in vivo with the frequencies of 1 × 10\(^{-7}\) to 1 × 10\(^{-8}\) (10). The Melan-A/MART-1\(_{28–36}\) is a remarkable exception with a very high naive T cell frequency of ~5 × 10\(^{-4}\) of CD8\(^+\) T cells (26). Recently, Speiser et al. (27) demonstrated that human CD8\(^+\) T cell clones specific to the Melan-A/MART-1 persisted for >1 year in a patient with melanoma, reaching up to 2.5% of the circulating CD8\(^+\) T cells. At the time of the melanoma-specific T cell clonal expansion, the disease stabilized but subsequently progressed with loss of Melan-A-specific T cell clones (27). In agreement with that study, we were able to demonstrate that the immunodominant CD8\(^+\) T cell clones in five patients with progressive disease were only temporarily present (or were undetectable in one case) in the circulation, and all of these patients subsequently died of their disease. Melanoma-reactive clones were also undetectable in tumor biopsies taken at the time of tumor progression shortly before death. In contrast, all three patients with objective responses had melanoma-reactive CD8\(^+\) T cells present in the circulation for 18–60 mo with frequencies of 10\(^{-6}\) to 10\(^{-3}\). These low-frequency CD8\(^+\) T cell clones can probably be expanded as a response to tumor growth or as a response to melanoma treatment. Such observation further confirms the importance of individual tumor-reactive CD8\(^+\) T cell clones in cancer survival. Thus, prolonged persistence of melanoma-reactive CD8\(^+\) T cell clones is associated with better survival.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


