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Survival and Tumor Localization of Adoptively Transferred Melan-A-Specific T Cells in Melanoma Patients

Norbert Meidenbauer,* Joerg Marienhagen,† Monika Laumer,* Sandra Vogl,* Jana Heymann,* Reinhard Andreesen,* and Andreas Mackensen²*

Adoptive T cell therapy has been successfully used for treatment of viral and malignant diseases. However, little is known about the fate and trafficking of transferred Ag-specific T cells. Using the tetramer (TM) technology which allows for detection and quantification of Ag-specific CTL, we assessed the frequency of circulating Melan-A-specific CTL in advanced melanoma patients during adoptive T cell therapy. Melan-A-specific CTL were generated from HLA-A2.1+ patients by in vitro stimulation of CD8+ T cells with dendritic cells pulsed with a mutated HLA-A2-binding Melan-A (ELAGIGILTV) peptide. Eight patients received three infusions of 0.25–11⁶ Melan-A-specific CTL i.v. at 2-wk intervals along with low-dose IL-2. The transferred T cell product contained a mean of 42.1% Melan-A-TM+ CTL. Before therapy, the frequencies of Melan-A-specific CTL in patients’ circulating CD8+ T cells ranged from 0.01 to 0.07%. Characterization of the TM frequencies before and at different time points after transfer revealed an increase of circulating Melan-A-specific CTL up to 2%, correlating well with the number of transferred CTL. An elevated frequency of TM+ T cells was demonstrated up to 14 days after transfer, suggesting long-term survival and/or proliferation of transferred CTL. Combining TM analysis with a flow cytometry-based cytokine secretion assay, unimpaired production of IFN-γ was demonstrated in vivo for at least 24 h after transfer. Indium-111 labeling of Melan-A-specific CTL demonstrated localization of transferred CTL to metastatic sites as early as 48 h after injection. Overall, the results suggest that in vitro-generated Melan-A-specific CTL survive intact in vivo for several weeks and localize preferentially to tumor. The Journal of Immunology, 2003, 170: 2161–2169.

Immunotherapy with autologous tumor-specific T cells for solid tumors is much more difficult since specific tumor-associated Ags (TAA)³ have only been defined in a minority of tumors. In malignant melanoma, several TAA have been recently identified and used as targets for immunotherapy (4). Melan-A, belonging to the group of melanocyte differentiation Ags, is an ideal target Ag, because of its high immunogenicity and preferential expression on melanoma cells (5). Regression of Melan-A-positive metastases or loss of Melan-A expression on tumor cells has been demonstrated in melanoma patients after vaccination with a Melan-A peptide or Melan-A peptide-pulsed dendritic cells (DC), showing the clinical potential of immunotherapies targeting this Ag (6).

A major drawback of this treatment modality was the fact that Ag-specific T cells had to be generated from tumor-infiltrating lymphocytes (TIL) and often only a relatively low number of cells could be produced. Recently, adoptive T cell therapy as a therapeutic option has regained interest by improved in vitro stimulation techniques, allowing the generation of high numbers of Ag-specific T cells (7, 8). However, the knowledge about the fate of Ag-specific T cells after transfer into patients with solid tumors is still very limited. Yee et al. (9) reported on T cell infiltration into both skin and tumor tissue after adoptive transfer of a Melan-A-specific CTL clone.

The tetramer (TM) technology allows for direct visualization of Ag-specific T cells by flow cytometry (10). The tetrameric complex is constructed of four synthetic and biotinylated peptide-loaded HLA molecules which are linked by a PE-labeled streptavidin molecule. In contrast to “indirect” assays such as limiting dilution analysis or ⁵¹Cr release assays requiring in vitro stimulation, this method allows direct monitoring of very low numbers of peptide-specific T cells without the need for in vitro sensitization (11).

The availability of large numbers of peptide-specific CTL provided us with the opportunity to study the in vivo localization of adoptively transferred T cells. In the current study, we investigated whether infused Melan-A-specific CTL labeled with indium-111 (¹¹¹In) oxine can traffic and localize at tumor sites. We and others have shown that ¹¹¹In can be used for short-term visualization and tracking of transferred cellular components (12–14). TM staining and ¹¹¹In labeling for Melan-A-specific CD8+ T cells were used in a clinical setting to monitor the function, survival, and tumor localization of ex vivo-generated adoptively transferred Ag-specific T cells in patients with advanced malignant melanoma.
Materials and Methods

Patients and treatment schedule

HLA-A2+ patients with advanced malignant melanoma, who were refractory to standard therapeutic regimens, were eligible for treatment. The trial was designed and conducted in accordance with the Declaration of Helsinki. The therapy protocol was approved by the Institutional Ethics Committee and registered with the regulatory state authority. All patients gave written informed consent before enrolling in the study. HLA-typing PBMCs were isolated from whole blood by gradient centrifugation. HLA-A2+ patients received at least three i.v. infusions of 0.25–11 × 10^9 Melan-A-specific T cells suspended in 100 ml of 5% human serum albumin. The adoptive transfer was repeated at 2-wk intervals. Immune monitoring was performed before T cell transfer and at least at 1, 3, 6, and 24 h after T cell infusion. In some patients, additional blood samples were obtained at various time points. PBMC were isolated by Ficoll density gradient centrifugation.

Peptides

The HLA-A2-binding peptide used in the clinical study was prepared under GMP conditions by Clinalfa (Laufenlingen, Switzerland); modified Melan-A26–35, A27L (ELAGIGILTV; designated as Melan-A26–35L). Wild-type (wt) Melan-A26–35 (EAAGIGILTV) and gp100260–288 (YLEGPGPTVA) HLA-A2-binding peptides, used for in vitro studies, were prepared by Bachem (Heidelberg, Germany). The identity of each peptide was confirmed by mass spectral analysis. The peptides were >98% pure as assessed by high-pressure liquid chromatography analysis. The endotoxin level was <0.1 endotoxin units/ml.

Media and reagents

Lymphocytes and monocytes were cultured in RPMI 1640 supplemented with 300 mg/L l-glutamine, 50 μM 2-ME, 1 mM sodium pyruvate, 40 μg/ml streptomycin, 40 μg/ml penicillin, 1% MEM vitamins, and 1× non-essential amino acids (=standard culture medium, M'). The following recombiant human cytokines and proteins were used at concentrations indicated in parentheses: IL-1β (10 ng/ml), IL-4 (1000 U/ml), TNF-α (10 ng/ml), TGF-β (5 ml; all from CelleGixen, Freiburg, Germany), GM-CSF (500 U/ml; Essex Pharma and Novartis Pharma, Basel, Switzerland), and PGE2 (1 μg/ml; Pharmacia and Upjohn, Erlangen, Germany).

Cells

Melanoma cells line and T2 cells were maintained in standard medium M' plus 10% FCS (Invitrogen, Karlsruhe, Germany). Expression of HLA-A2 and Melan-A was assessed by FACS analysis using an anti-HLA-A2-specific mAb (BD7.2; American Type Culture Collection, Manassas, VA), and by RT-PCR, respectively, as described previously (15). T2 cells were HLA-A*0201 human lymphoid cells that are defective in Ag processing, but effectively present exogenously supplied peptides (16).

mAbs and flow cytometry

Surface marker analysis of PBMC and DC was performed using a FACSCalibur (BD Biosciences, San Jose, CA) and the CellQuest software (BD Biosciences). The following mAbs were used: CD4-allophycocyanin, CD14-PerCP, CD11b-PE, CD19, CD20, and CD56+ cells with magnetic cell sorting using a mini-MACS device (Miltenyi Biotec, Bergisch Gladbach, Germany). The resulting population consisted of >80% CD8+ T cells and was used as responder cell population. DC were generated from monocytes cultured by countercurrent elutriation. Monocytes were then cultured with standard medium (M') plus 2% autologous serum supplemented with human GM-CSF, IL-4, and TGF-β. On day 6, fresh complete medium containing GM-CSF, IL-4, TNF-α, IL-6, IL-1β, and PGE2 was added to the culture (18). The culture was continued for an additional 48 h. The monocyte-derived DC were then harvested and pulsed for 2 h at 37°C with the appropriate peptide (30 μg/ml) and human β2-microglobulin (10 μg/ml) in serum-free M' medium. Briefly, 2–5 × 10^6 effector cells/ well and 5 × 10^3 peptide-pulsed autologous DC/well were cocultured in 96-well round-bottom plates in 225 μl of M' medium/well supplemented with 10% human AB serum and 1–2% T cell growth factor (19). Medium exchange was performed twice a week and effector cells were restimulated weekly with fresh peptide-pulsed DC. After four cycles of stimulation, phenotypic and functional analysis of T cells was performed.

Radiolabeling of Melan-A-specific CTL

For labeling, 1–4 × 10^7 Melan-A-specific CTL were washed twice in PBS and resuspended in 1 ml of 111In oxine (Amer sham, Braunschweig, Germany) with a total radioactivity of 2 μCi/10^6 T cells. After a 30-min incubation at room temperature while gently rocking, the radiolabeled cells were washed twice in PBS and resuspended in 50 ml of 5% human serum albumin. Whole body as well as static images were obtained by a double-headed whole body scanner (Siemens, Erlangen, Germany).

Chromium release assay

The cytotoxic activity of T cell lines was measured by a conventional 4-h 51Cr release assay using triplicate cultures in V-bottom plates. Target cells analyzed included HLA-A2+, Melan-A+, or HLA-A2-Melan-A+ melanoma cell lines, and peptide-pulsed T2 cells. E:T ratios were 25:1, 5:1, and 1:1 on 2000 target cells/well. The percentage of specific cytotoxicity was calculated conventionally.

TM staining

PE-labeled HLA-A*0201 TM that had been folded around ELAGIGILTV (Melan-A26–35L) or SLNYTVALT (HIV gag) were synthesized by Beckman Coulter (Fullerton, CA). To minimize nonspecific staining, each TM was titrated and used at the lowest concentration that showed a clearly distinguishable positive population in Melan-A-specific CTL generated as described above and in PBL of a HIV-infected individual, respectively. PBMC or in vitro-generated T cells were incubated for 30 min at 37°C on a cell shaker with the indicated TM, then mAbs for surface staining were added. Cells were incubated in the dark for 30 min at 4°C, washed twice, and then fixed with 0.5% paraformaldehyde (PFA; Merck Eurolab, Darmstadt, Germany).

For combination of TM and annexin V staining, cells were washed once with Flow buffer and once with annexin V buffer (BD Pharmingen) after incubation with surface mAbs. Cells were resuspended in 100 μl of annexin buffer and 1 μl of annexin V was added. After incubation of 20 min at room temperature in the dark, the cells were fixed with 0.5% PFA.

The following Ab panels were analyzed on PBMC at the indicated time points before and after transfer: 1) CD3-FITC, CD4-PerCP, CD8-allophycocyanin; 2) CD3-FITC, Melan-A-TM-PE, CD4-PerCP, CD8-allophycocyanin; 3) CD3-FITC, PBS, CD4-PerCP, CD8-allophycocyanin; 4) Annexin VFITC, Melan-A-TM-PE, CD4-PerCP, CD8-allophycocyanin; 5) CD3-FITC, HIV gag-TM-PE, CD4-PerCP, CD8-allophycocyanin; and 6) isotype controls.

Compensation was checked before each acquisition. Events (1 × 10^8) were routinely collected using a FACSCalibur set on a maximal flow rate of 1000 events/s. For analysis, TM-positive events were evaluated within a Boolean gate including cells within an extended lymphoid light scatter gate and a CD3+ gate, excluding CD14+ and annexin V+ events (20).

The frequency of circulating Melan-A-specific CTL is presented as a percentage of Melan-A+ T cells within total CD8+ TBL. The percent TM+ CD8+ T cells present in the circulation at 1 h after transfer, and the total number of circulating CD8+ T cells in a blood volume of 5000 ml.

Generation of Melan-A-specific CTL lines from PBL

PBMC were obtained from donors and patients by leukapheresis. Melan-A-specific CTL lines were generated as described previously (7, 17). Briefly, CD8+ T lymphocytes were enriched from PBMC by depletion of CD14+ monocytes, CD19, CD20, and CD56+ cells with magnetic cell sorting using a mini-MACS device (Miltenyi Biotec, Bergisch Gladbach, Germany). The resulting population consisted of >80% CD8+ T cells and was used as responder cell population. DC were generated from monocytes enriched by countercurrent elutriation. Monocytes were then cultured with standard medium (M') plus 2% autologous serum supplemented with human GM-CSF, IL-4, and TGF-β. On day 6, fresh complete medium containing GM-CSF, IL-4, TNF-α, IL-6, IL-1β, and PGE2 was added to the culture. The culture was continued for an additional 48 h. The monocyte-derived DC were then harvested and pulsed for 2 h at 37°C with the appropriate peptide (30 μg/ml) and human β2-microglobulin (10 μg/ml) in serum-free M' medium. Briefly, 2–5 × 10^6 effector cells/well and 5 × 10^3 peptide-pulsed autologous DC/well were cocultured in 96-well round-bottom plates in 225 μl of M' medium/well supplemented with 10% human AB serum and 1–2% T cell growth factor. Medium exchange was performed twice a week and effector cells were restimulated weekly with fresh peptide-pulsed DC. After four cycles of stimulation, phenotypic and functional analysis of T cells was performed.

Competition between TM and anti-CD3 Ab binding

To determine the specificity of the TM staining, we quantified the extent to which TM binding competed with CD3 binding on CD8+ cells using the formula described previously (20): percent competition = (CD3MrelCD3+CD8+TM−) − (CD3M+CD3+CD8+TM−)/CD3M+CD3+CD8+TM− × 100. CD3 M rel represents the log mean fluorescence intensity of CD3 staining of the population designated by the subscript.

IFN-γ secretion by Melan-A-specific T cells

T cell function was analyzed combining MHC-TM staining with the IFN-γ secretion assay (Miltenyi Biotec). Before activation, T cells were stained...
with PE-labeled Melan-A TM as described above. To induce IFN-γ secretion in Melan-A-specific CTL, responder T cells were stimulated in vitro for 2 h with autologous DC pulsed with the Melan-A peptide or gp100 peptide as control (30 μg/ml; responder/stimulator ratio = 3:1). Cells were then labeled for 5 min at 4°C with an IFN-γ-specific high-affinity capture matrix (Miltenyi Biotec), i.e., a bispecific Ab-Ab conjugate directed against CD45 and IFN-γ. After 45 min. of incubation at 37°C, the secreted cytokine bound to the affinity matrix was stained with an allophycocyanin-labeled anti-IFN-γ-specific mAb (Miltenyi Biotec), anti-CD8-PerCP mAb, and anti-CD3-FTTC mAb (BD Biosciences). As positive control cells were stimulated with staphylococcal enterotoxin B (SEB, Sigma-Aldrich, Taufkirchen, Germany). Cells were then washed and fixed with 1% PFA before flow cytometry analysis.

**IFN-γ ELISPOT assay**

The IFN-γ ELISPOT assay has been described previously (21). Melan-A-specific CTL were incubated either with peptide-loaded T cells or allo- geneic melanoma cells. Unstimulated T cells or T cells stimulated with 0.5 ng/ml PMA and 0.5 μM ionomycin (both from Sigma-Aldrich) served as negative and positive controls, respectively.

**Statistical analysis**

Regression analysis was performed using SigmaPlot software (SPSS, Chicago, IL). Competition between anti-CD3 binding and TM binding was compared between HIVgag-TM and Melan-A-TM using the two-tailed Student’s t test (SigmaPlot). The values of p < 0.05 were considered to be statistically significant.

**Results**

**Generation, phenotypic, and functional characterization of Melan-A-specific CTL**

We have previously demonstrated that in vitro stimulation of CD8⁺-purified lymphocytes with wt Melan-A26-35-pulsed DC (7) leads to generation of significant numbers of Melan-A-specific T cells. In this study, we wished to test whether the use of the peptide analog Melan-A26-35L vs the wt Melan-A26-35 peptide would improve the yield and the specificity of T cells suitable for clinical application, as suggested by Valmori et al. (22). Using our previously described in vitro sensitization protocol (7), we first performed a pilot study with cells of healthy donors. CD8⁺-purified T cells from five healthy donors were stimulated with Melan-A26-35L-pulsed autologous DC once a week for 4 wk. We were able to generate up to 55 × 10⁶ CD8⁺ T cells from 1 × 10⁶ cells, corresponding to a mean expansion of 159.7-fold (range, 32–353; Table I). The generated T cells were largely CD3⁺ CD8⁺ cells (89.7 ± 7.3%, mean ± SD) with >93% of the cells expressing the memory cell marker CD45RO and only 22.3 ± 9.3% expressing CD62 ligand. Additional analysis of CD27 and CCR7 in three donors showed that 27% ± 19% expressed CD27 and 28% ± 19% expressed CCR7. This staining pattern with a majority of cells negative for CD27 and CCR7 is characteristic for central memory or effector type cells, respectively. The CTL lines were highly activated as indicated by the expression of CD95 in 94.5 ± 4.5% and CD69 in 36.3 ± 10% of CD3⁺ CD8⁺ cells. Cells expressing CD4 or NK cell markers on the cell surface were nearly absent (<5% and <1%, respectively). The frequency of Melan-A-specific T cells as determined by Melan-A-TM staining was 42.6 ± 27.7% within total CD8⁺ lymphocytes. Experiments comparing stimulation of T cells with wt Melan-A26-35 and Melan-A26-35L peptide-pulsed DC revealed a higher proliferation rate as well as an increased frequency of Melan-A-specific T cells after stimulation with Melan-A26-35L (data not shown). Functional analysis of the T cells revealed a strong cytotoxic activity not only against T2 cells pulsed with the wt Melan-A26-35 peptide (Table I), but also against allogeneic HLA-A2⁺ melanoma cell lines expressing the Melan-A Ag (data not shown).

**Characteristics of Melan-A-specific CTL adoptively transferred to patients**

Eight patients with refractory malignant melanoma, included in a Phase I clinical protocol designed to evaluate the feasibility and toxicity of adoptively transferred Melan-A-specific CTL, received at least three i.v. infusions of in vitro-generated Melan-A-specific T cells at 2-wk intervals. All patients had extensive metastatic disease with lung and/or liver (patients 1–4 and 8), bone (patients 2 and 3), lymph node (patients 1, 4–6, and 8), and skin metastases (patients 1, 2, and 7); the majority had undergone multiple previous therapies including surgery, chemotherapy, chemomunotherapy, or immunotherapy. Immunohistochemical analysis of Melan-A expression in tumor biopsies obtained before adoptive transfer demonstrated abundant expression of Melan-A in all tumors (data not shown). A total of 24 T cell infusions were administered, averaging 3.31 × 10⁸ Melan-A-specific T cells per infusion, with a range from 1.1 to 22.7 × 10⁸ T cells/infusion. Each T cell infusion was accompanied by a 6-day course of s.c. IL-2 (3 × 10⁶ IU daily), The T cell transfer was well tolerated with the exception of chills and low-grade fever up to 38.5°C in five of eight patients that typically occurred within 6 to 8 h after infusion. Hematological effects, observed after T cell transfer, consisted of an increase in eosinophils up to 30% in five of eight patients, peaking 24 h after transfer (data not shown). Eosinophilia is a common side effect after IL-2 application and is linked primarily to production of IL-5. However, sufficient amounts of IL-5 could be also detected in the supernatant of the transferred T cell product (data not shown). No changes in total lymphocyte counts could be observed.

**Table 1. Phenotypic and functional characteristics of in vitro-generated Melan-A-specific CTL lines from healthy donors and melanoma patients**

<table>
<thead>
<tr>
<th>Donor/Patient</th>
<th>No. of T Cell Transfers</th>
<th>x-Fold Expansion of CD8⁺ Cells (day 28)</th>
<th>CD3⁺ CD8⁺ (mean %)</th>
<th>CD3⁺ CD8⁺ (mean %)</th>
<th>Melan-A-TM CD8⁺ (mean %)</th>
<th>Specific Lysis (mean %)</th>
<th>Total no. of Transferred T Cells (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donors 1–5⁴</td>
<td>3</td>
<td>159.7 (93)</td>
<td>89.7 (7.3)</td>
<td>4.0 (1.2)</td>
<td>42.6 (27.7)</td>
<td>45.8 (5.8)</td>
<td>0.25 (0.09)</td>
</tr>
<tr>
<td>Patient 1 ⁵</td>
<td>3</td>
<td>23.2 (9.8)</td>
<td>93.0 (7.0)</td>
<td>7.9 (5.6)</td>
<td>20.2 (10.7)</td>
<td>34.6 (6.8)</td>
<td>10.96 (2.6)</td>
</tr>
<tr>
<td>Patient 2 ⁵</td>
<td>3</td>
<td>215.6 (88)</td>
<td>95.3 (3.7)</td>
<td>2.6 (1.8)</td>
<td>68.1 (14.9)</td>
<td>47.3 (17)</td>
<td>0.85 (0.93)</td>
</tr>
<tr>
<td>Patient 3 ⁵</td>
<td>3</td>
<td>59.8 (30)</td>
<td>86.6 (8.1)</td>
<td>16.6 (6.4)</td>
<td>32.5 (25.6)</td>
<td>34.3 (15)</td>
<td>4.71 (3.59)</td>
</tr>
<tr>
<td>Patient 4 ⁵</td>
<td>3</td>
<td>88.6 (45)</td>
<td>90.3 (5.5)</td>
<td>3.5 (2.4)</td>
<td>40.0 (25.4)</td>
<td>34.3 (11)</td>
<td>0.39 (0.56)</td>
</tr>
<tr>
<td>Patient 5 ⁵</td>
<td>3</td>
<td>118.3 (71)</td>
<td>88.6 (13)</td>
<td>14.0 (21)</td>
<td>14.8 (5.6)</td>
<td>47.0 (4.3)</td>
<td>0.83 (0.23)</td>
</tr>
<tr>
<td>Patient 6 ⁵</td>
<td>3</td>
<td>60.8 (18)</td>
<td>96.6 (1.6)</td>
<td>1.5 (1.4)</td>
<td>51.7 (11.8)</td>
<td>49.0 (7.2)</td>
<td>2.93 (1.79)</td>
</tr>
<tr>
<td>Patient 7 ⁵</td>
<td>3</td>
<td>145.1 (52)</td>
<td>96.9 (1.7)</td>
<td>2.0 (2.4)</td>
<td>45.9 (22.1)</td>
<td>48.3 (3.3)</td>
<td>5.52 (2.10)</td>
</tr>
<tr>
<td>Patient 8 ⁵</td>
<td>3</td>
<td>303 (53)</td>
<td>92.1 (0.1)</td>
<td>0.5 (0.3)</td>
<td>63.3 (8.4)</td>
<td>53.8 (3.8)</td>
<td>3.31 (3.80)</td>
</tr>
<tr>
<td>Patients 1–8 ⁵</td>
<td>3</td>
<td>126.8 (96)</td>
<td>92.5 (3.6)</td>
<td>6.0 (6.3)</td>
<td>42.1 (20.2)</td>
<td>43.6 (8.1)</td>
<td>3.31 (3.80)</td>
</tr>
</tbody>
</table>

Data are expressed as the mean percentage (± SD) of positive lymphocytes at day 28 of in vitro culture.

Numbers express the mean number (± SD) of total CD8⁺ Melan-A-TM⁺ T cells infused.

Data indicate the mean results (± SD) for donors 1–5 and patients 1–8.
after T cell transfer and IL-2 injection. Clinical responses consisted of one partial response, one mixed response with shrinkage of one metastatic deposit, and one no change (12 mo) of eight patients.

In the patients, the frequency of CD3<sup>+</sup>CD8<sup>+</sup> cells in the T cell product was comparable to the frequencies observed in normal donors, with slightly higher percentages of CD3<sup>+</sup>CD8<sup>+</sup> lymphocytes (Table I). To our surprise, we did not observe any clinically significant decrease in the ability of the T cells to proliferate as it would be expected in patients with advanced disease that had received different chemotherapeutic regimens compared with healthy individuals. The median expansion rate of all 24 T cell cultures in the patients’ group was 126.8 (range, 23.2–303), compared with 159.7 (range, 32–533) in healthy adults (Table I). Nevertheless, large interindividual differences in the ability of CD8<sup>+</sup> T cells to proliferate could be observed in patients and controls (Table I).

The frequency of Melan-A-specific T cells in the patients’ T cell cultures, as determined by Melan-A-TM analysis, was also comparable to healthy individuals (Table I). Even though there were differences in some patients from transfer to transfer in the expansion rate and the frequency of Melan-A-TM T cells in the culture, the number of transfused Melan-A-specific T cells was reasonably stable in the individual patient.

Since the TM assay measures only the specificity of the TCR, but does not allow any conclusion about the function of the T cells, we performed different assays measuring T cell function, such as the 51Cr release assay, IFN-γ ELISPOT assay, or cytokine secretion assay in response to specific targets. In all T cell products, transferred to the patient, a significant specific activity against T2 cells pulsed with the wt Melan-A peptide was measured at E:T ratios between 1:1 and 25:1, showing the high cytotoxic potential of T cells generated ex vivo (see Table I for a ratio of 25:1). There was no difference between the cytotoxic activity of T cells generated from patients compared with T cell lines generated from healthy individuals (Table I).

IFN-γ ELISPOT measured specific secretion of the T<sub>1</sub>-cytokine IFN-γ after resensitization with Melan-A-pulsed stimulator cells (data not shown). Another method to test for cytokine secretion of Ag-specific cells is the combination of TM analysis with an IFN-γ secretion assay as recently described by Pittet et al. (23). As shown in Fig. 1, 56.3% of Melan-A-TM<sup>+</sup> T cells secreted IFN-γ upon stimulation with Melan-A-pulsed autologous DC. Upon stimulation with the control peptide, gp100, there was no increase in the IFN-γ secretion compared with the unstimulated T cells. Of note is a down-regulation of Melan-A-TM staining with specific stimulation, showing a reduction in the frequency of Melan-A-TM<sup>+</sup> T cells in the stimulated samples compared with unstimulated controls. This phenomenon has already been described and provides indirect evidence for the specificity of the generated T cells (24, 25).

**In vivo tracking of Melan-A-specific CTL after adoptive transfer to patients**

Only limited data are available in the literature on the fate of adoptively transferred Ag-specific T cells in the peripheral blood after transfer. Using the TM technology, which is able to monitor frequencies of specific T cells of <0.1%, we followed the fate of Melan-A-specific CTL after therapy.

To ensure specific staining, it was necessary to exclude apoptotic cells and monocytes, since both populations bind TM in an unspecific manner (20). Gating based on annexin V for labeling of apoptotic cells resulted in nearly identical frequencies of TM<sup>+</sup>CD8<sup>+</sup> T cells ($r^2 = 0.95$). Another proof for specific staining revealed a reduction of CD3 expression in Melan-A-TM<sup>+</sup> cells compared with T cells nonspecifically stained with HIVgag-TM (7.3 ± 2.8% vs 1.4 ± 2.3%, $p < 0.001$), allowing us to neglect the spurious HIVgag-TM<sup>+</sup> cells for further analysis.

PBMC obtained immediately before and 1 h, 3 h, 6 h, 24 h, and 14 days after transfer (i.e., immediately before the subsequent transfer) were stained with Melan-A-TM (Figs. 2 and 3). In some patients, additional samples were obtained between 48 h and 5 days after transfer.

The preinfusion frequency of Melan-A-TM<sup>+</sup> cells within CD8<sup>+</sup> PBL of all patients was <0.1%. One hour after infusion, a marked increase in the frequency of Melan-A-TM<sup>+</sup> CD8<sup>+</sup> T cells could be observed (Figs. 2 and 3). Assessment of the fraction of transfused Melan-A-specific CTL that appear in the circulation at 1 h after T cell transfer revealed a mean percentage of 5.78% (range, 1.0–18.4%) of the transferred CTL that can be detected in the circulation.

At 3 h and in most cases also at 6 h after T cell transfusion, the frequency of circulating Melan-A-TM<sup>+</sup> cells decreased in the majority of the tested patients (Figs. 2 and 3). Between 6 and 24 h after infusion, the frequency of Melan-A-TM<sup>+</sup> CD8<sup>+</sup> T cells rose again to a level in the range of the 1 h value. Of note, we observed an additional increase in the frequency of circulating Melan-A-TM<sup>+</sup> cells in the blood samples obtained later than 24 h after transfer. (Figs. 2, A and B, and 3A). Additional blood samples obtained between 48 and 144 h after transfer (patients 1–3) revealed the highest frequency of Melan-A-TM<sup>+</sup> cells in these patients at these time points (Fig. 2, A and B). Since the frequency of Melan-A-TM<sup>+</sup> T cells had decreased again at 14 days after transfer, these findings suggest the disappearance of the transfused T cells from the peripheral blood mainly in the second week after infusion.

Concerning the kinetics of the frequency of circulating Melan-A-specific T cells after transfer, we found a nearly identical pattern in the individual patient after each transfer with marked interindividual differences (Fig. 3A).

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**FIGURE 1.** IFN-γ secretion-based detection of Melan-A-specific CD8<sup>+</sup> T cells after in vitro stimulation with Melan-A<sub>26–35L</sub> peptide-pulsed DC. CD8-purified T cells from a melanoma patient were stimulated once a week with Melan-A<sub>26–35L</sub> peptide-pulsed DC. Seven days after the fourth stimulation (day 28), T cells were incubated with autologous DC pulsed with the specific peptide Melan A<sub>26–35L</sub> or irrelevant gp100 peptide. Additional controls included unstimulated T cells (negative control) or T cells unspecifically stimulated with SEB (positive control). After 4 h, cells were labeled as described in Materials and Methods and then directly analyzed by flow cytometry. For analysis of IFN-γ secretion, cells were gated for CD3<sup>+</sup>Melan-A-TM<sup>+</sup> cells (left dot blot). The right four dot blots show staining with allopurinyl conjugated anti-IFN-γ mAb and PerCP-conjugated anti-CD8 mAb. Numbers in the upper right quadrants represent percentages of IFN-γ<sup>+</sup>CD8<sup>+</sup> cells within CD3<sup>+</sup>Melan-A-TM<sup>+</sup> T cells. A representative of two independent experiments is shown.
A

pre transfer  1h post  3h post  6h post

24h post  48h post  14 days post

CD8-APC

B

pre transfer  1h post  3h post  6h post

24h post  72h post  14 days post

CD8-APC

C

pre transfer  1h post  3h post  6h post

6h post  24h post  14 days post

CD8-APC

On day 14 after transfer (before subsequent transfer), PBMC were obtained to monitor the survival of transferred Melan-A-specific T cells. Since the frequency was generally very low, we regarded all values more than the double baseline value as evidence for elevated levels of Melan-A-specific T cells due to therapy. In patients 1, 2, 5, and 8, an increased frequency of Melan-A-specific T cells could be detected for >14 days, at least after one T cell transfer (Fig. 3B). In patient 8, the frequency of Melan-A-TM$^+$ cells at 28 days after the first transfer (before the third retransfer) was ~15.8 times higher than the preinfusion value, suggesting long-term survival and/or proliferation of adoptively transferred CTL (Fig. 3B).

Functional characterization of transferred Ag-specific T cells

There is a considerable amount of data on immunosuppression in cancer patients caused by interaction of tumor cells with immune cells, leading to dysfunctional or dying lymphocytes (26). Lee et al. (27) showed complete anergy of tumor-specific T cells in a melanoma patient, while the function of CMV-specific T cells was not altered. Since the TM technology alone does not provide any data on the function of the TM$^+$ cells, we used IFN-$\gamma$ secretion upon peptide-specific stimulation as a surrogate marker for function of the transferred T cells. As already demonstrated in Fig. 1, ~60% of the in vitro-stimulated Melan-A-TM$^+$ T cells responded to specific restimulation by secretion of IFN-$\gamma$. Ex vivo analysis of Melan-A-specific T cells obtained from patient PBMC at 24 h after transfer revealed specific IFN-$\gamma$ secretion in 41.7% of the Melan-A-TM$^+$ cells after specific in vitro restimulation with Melan-A-pulsed DC (Fig. 4). Control samples obtained from unstimulated PBMC or PBMC stimulated with an irrelevant peptide (gp100) showed a frequency of 6% IFN-$\gamma$-secreting cells (Fig. 4). Similar results were obtained at 1 h after transfer (data not shown), indicating that Melan-A-specific CTL maintained functional activity in vivo.

Characterization of radiolabeled Melan-A-specific CTL

Pilot studies had been performed to determine the influence of incubation time and of increasing doses of $^{111}$In on the labeling efficiency of Melan-A-specific CTL. Labeling doses of 20 $\mu$Ci/10$^7$ T cells was optimal when incubated for 30 min (data not shown). Functional analysis ($^{51}$Cr release assay) comparing the cytotoxic activity of labeled and unlabeled Melan-A-specific CTL showed no significant decline in the ability of the labeled CTL to lyse Melan-A-expressing target cells when labeling was performed with 20 $\mu$Ci/10$^7$ T cells (data not shown). Cell death attributed to radiolabeling was assessed by trypan blue dye exclusion and was found to be negligible after $^{111}$In labeling. The viability of radiolabeled CTL during 8 days in culture was >90%.

Three of the eight patients with metastatic melanoma treated in the Phase I clinical trial with Melan-A-specific CTL received one infusion of 100–400 $\times$ 10$^6$ $^{111}$In-labeled T cells along with low-dose IL-2 (3 $\times$ 10$^6$ IU s.c. per day for 6 days). Two of the three patients received unlabeled CTL 1 h before receiving radiolabeled CTL. The frequency of radiolabeled Melan-A-specific T cells as determined by Melan-A-TM staining was between 24.7 and 54.6% within the total CD8$^+$ lymphocytes. The mean concentration of radioactivity infused was 15.6 $\mu$Ci/10$^7$ cells. The distribution and localization of the CTL were evaluated using serial whole body and static gamma camera imaging.

In vivo distribution and tumor localization of adoptively transferred $^{111}$In-labeled Melan-A-specific T cells

Representative whole body gamma camera images from patients 7 and 8 following adoptive transfer of $^{111}$In-labeled Melan-A-specific T cells are presented in Fig. 5. At 10 min and 1 h after injection, gamma camera images showed a predominant accumulation of radioactivity in lung and, to a lesser extent, in spleen and liver. Lung uptake cleared substantially during the first 24 h after infusion. The activity in the liver and spleen revealed a marked...
increase in the first 24 h. Retention of the tracer in the liver and spleen was observed for as long as 72 h.

All three patients who underwent gamma camera imaging had evidence of uptake of $^{111}$In-labeled Melan-A-specific CTL in sites of metastases including tumors in lymph nodes and s.c. tissues. Uptake of $^{111}$In-labeled CTL was observed as early as 48 h after receiving radiolabeled T cells. One representative set of scans obtained from a melanoma patient (patient 7) is shown in Fig. 6. An abnormal focus of uptake correlated with known areas of metastasis as imaged on positron emission tomography scan (Fig. 6A). Patient 7 showed uptake of radiolabeled CTL in an involved s.c. metastasis at the left popliteal fossa (Fig. 6B) at 48 h. Of note, a tumor biopsy obtained from patient 7 two months before T cell transfer from the metastatic lesion, where $^{111}$In-labeled CTL have accumulated, demonstrated abundant expression of Melan-A (data not shown). Computerized gamma camera imaging of patients 6 and 8 revealed an increased uptake in a retroperitoneal and para-aortal lymph node mass as early as 48 h after infusion of $^{111}$In-labeled CTL, respectively (data not shown).

Overall, this study provides evidence for the first time that radiolabeled Melan-A-specific CTL generated from PBL can localize to tumor.

Discussion

The adoptive transfer of in vitro-induced and -expanded tumor-specific CTL provides a promising approach to the immunotherapy of cancer. Antitumoral effects of adoptively transferred CTL were previously demonstrated in a variety of animal models (28, 29) and also in humans (30). However, these reports did not lead to a broad application of this treatment modality. One major obstacle to a widespread application of adoptive T cell therapy was a requirement for large numbers of CTL thought to be needed to achieve and maintain antitumor effects. Another obstacle is the difficult, time-consuming, and costly process of cloning and expanding specific T cells of TIL. In recent years, the characterization of TAA and the use of DC as APC for specific in vitro stimulation has helped to overcome these difficulties. We and others showed previously that the coculture of autologous lymphocytes and Ag-pulsed DC led to T cell numbers sufficient for adoptive immunotherapy (7, 8).

For this study, a Melan-A-derived, HLA-A2-binding peptide, belonging to the family of melanocyte differentiation Ags, was selected as target Ag. The use of this peptide has several advantages: 1) its overexpression on the majority of melanoma allows treatment of nearly all HLA-A2$^+$ patients eligible for the clinical
FIGURE 4. IFN-γ secretion-based detection of circulating Melan-A-specific CD8⁺ T cells 24 h after adoptive transfer of Melan-A-specific CTL. PBMC obtained from a melanoma patient 24 h after infusion of Melan-A-specific T cells were incubated with autologous DC pulsed with Melan \( A_{26-35} \) peptide or irrelevant gp100 peptide. Additional controls included unstimulated PBMC (negative control) or PBMC unspecifically stimulated with SEB (positive control). After 4 h, cells were labeled as described in Materials and Methods and then directly analyzed by flow cytometry. For analysis of IFN-γ secretion, cells were gated for CD3⁺ Melan-A-TM⁺ cells (left dot blot). The right four dot blots show staining with allophycocyanin-conjugated anti-IFN-γ mAb and PerCP-conjugated anti-CD8 mAb. Numbers in the upper right quadrants represent percentages of IFN-γ⁺CD8⁺ cells within CD3⁺ Melan-A-TM⁺ PBL at 24 h after T cell transfer. A representative of two independent experiments is shown.

The fate of the transferred T cells in the patient is a crucial question for the success of adoptive therapy. Functionally active transferred T cells have to survive long enough in the circulation to be able to migrate into the tumor and eradicate the malignant cells. By frequent analyses of Melan-A-specific T cells during the first 72 h after transfer, we obtained a clear kinetics pattern in the frequency of circulating Melan-A-TM⁺ cells in the treated patients.

Immediately (1 h) after transfer the frequency of Melan-A-TM⁺ cells increased dramatically, followed by a decline at 3–6 h after infusion. At 24–72 h after transfer, a second increase of Melan-A-reactive T cells could be observed in the majority of the patients. The decline and subsequent rebound of transferred CTL in the peripheral blood most probably reflect entrapment of T cells in the lung, migration to spleen and liver, and recirculation of the transferred T cells, as indicated by our radioimaging studies. Another explanation for this phenomenon would be proliferation of the transferred cells, as suggested by Heslop et al. (35). This question cannot be definitely answered in our study, since there is no possibility to differentiate between resting and proliferating specific T cells based on TM staining.

However, our data clearly demonstrate survival of the transferred T cells for several weeks (Fig. 3B). Results from other clinical trials applying adoptive T cell therapy are quite inconsistent, ranging from not detectable CTL immediately after transfer (36) to...
survival of CTL for several months (35, 37). The different results might be attributed to differences between autologous and allogeneic T cell therapy. After allogeneic bone marrow transplant, donor lymphocytes are able to survive and proliferate because of a constant immunostimulatory milieu in the host. The use of different assays for detection of transferred T cells may be another reason for inconsistent results. Limiting dilution assay, which has an ~10-fold lower sensitivity than TM analysis (38), failed to detect adoptively transferred tyrosinase-specific CTL after transfer (36), whereas gene-transduced EBV-specific T cells could be tracked down by highly sensitive semiquantitative PCR for several months (35).

Another reason for impaired survival of specific T cells may be the lack of help by CD4+ T cells or inappropriate concentrations of cytokines such as IL-2. Helper function provided by CD4+ T cells was shown to be required for persistence of adoptively transferred CMV-specific T cells (1, 39), whereas application of IL-2 seemed to prolong survival of tumor-specific T cells after adoptive transfer (9). In line with these data, we observed a decrease of circulating Melan-A-specific T cells at the second week after transfer, correlating with termination of low-dose IL-2 injection.

Senescence of long-term expanded cells might be a specific problem for adoptive T cell therapy, since the polyclonal expansion of adult T cells in vitro is limited to 30–40 cell divisions due to loss of telomeres. This might explain the short survival of transferred T cell clones (40) compared with T cell lines used in our study.

In terms of therapeutic efficacy, a high frequency of circulating tumor-specific T cells is only a first step for successful adoptive therapy. TM analysis does not address the function of the transferred T cells, making it theoretically possible to detect high frequencies of therapeutically ineffective Ag-specific T cells. The phenomenon of paradoxical coexistence between tumor and tumor-specific T cells was already addressed in several studies (24, 41). One explanation for the divergence of TM staining and clinical response might be the enhanced affinity of the TM complex to the TCR which might allow binding on a nonphysiological low level (42). This theory is supported by the fact that TM binding gives ~4-fold higher frequencies than other assays like the ELISPOT assay which relies on the functional status of the T cells (38). Another explanation is provided by Lee et al. (27) who have demonstrated selective anergy of tyrosinase-specific T cells in a patient with malignant melanoma. Therefore, we assessed the function of the transferred Melan-A-specific T cells by using specific IFN-γ secretion as surrogate marker for T cell activity. To be able to monitor the functional state of the transferred T cells at low frequencies, we combined the TM analysis with the IFN-γ secretion assay as previously described (23). As shown in Fig. 4, we could demonstrate that a significant proportion of the transferred T cells was able to secrete IFN-γ for at least 24 h after infusion, suggesting unimpaired T cell function for this period of time. We have demonstrated, that the IFN-γ-negative fraction of Melan-A-specific CTL may consist of IL-4-secreting Tc2 cells (17). Enriched IFN-γ+ Tc1 and IL-4+ Tc2 cells are both functionally active, exhibit comparable cytotoxic activity, and can be stained with Melan-A TM.

To characterize traffic and tumor localization of transferred Melan-A-specific CTL, we applied the 111In-labeling method of others who successfully labeled and trafficked TIL (13, 43). The half-life of 65 h is long enough for in vivo distribution, but short enough to minimize radiation exposure to the patient. The distribution of 111In-labeled Melan-A-specific CTL was similar to the distribution of PBL which first appeared in the lung and after 24 h accumulated in liver and spleen (44). This is supported by several reports including a recent study with adoptively transferred tyrosinase-specific CTL (36, 40). The most important difference, of course, was the localization of Melan-A-specific CTL to sites of tumor as early as 48 h after transfer. Previous studies have demonstrated that TIL, expanded in vitro with IL-2, preferentially localize to tumor sites (13, 43). However, TIL consists of a special population of T cells that have already entered the tumor, which may result in sensitization to TAA and subsequent remigration after in vitro expansion. Specific traffic of PBL-derived tumor-reactive CTL to tumor sites, as demonstrated in our study, may be based on in vitro priming of T cells with tumor-associated peptides such as Melan-A. Previous studies of lymphocyte traffic in animal models have demonstrated that T cell localization to tumor is mediated by Ag-specific interactions (45, 46).

In summary, we demonstrated that Melan-A-specific CTL generated by in vitro stimulation with peptide-pulsed DC survive physiologically intact in vivo for several weeks, which is longer than in comparable trials (40). The combination of TM analysis with the cytokine secretion assay demonstrated unimpaired functional activity of the transferred T cells in the peripheral blood for at least 24 h after infusion. Furthermore, our study provides evidence for the first time that radiolabeled Melan-A-specific CTL generated from PBL can localize preferentially to tumor. Strategies to provide help to transferred CD8+ T cells, such as concurrent adoptive transfer of tumor-specific CD4+ Th cells, which may improve CTL survival and the therapeutic potential, are in progress.

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References


homologous properties than initial frequencies are crucial. J. Immunol. 163: 5535.


