Intrathecal Cytotoxic T-Cell Immunotherapy for Metastatic Leptomeningeal Melanoma

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Abstract
A 49-year-old patient with primary, recurrent melanoma on the lower extremity developed metastatic leptomeningeal melanoma that did not respond to treatment with radiation therapy or intrathecal interleukin 2 (IL-2). Disease was characterized by neurological symptoms, including loss of hearing, loss of short-term memory, and gait disturbance. CD8+ CTLs were generated in vitro using autologous dendritic cells pulsed with peptides from the melanoma-associated antigens tyrosinase (145–156), Melan-A/MART-1 (26–35), and gpl00/Pmel17 with defined HLA-restricted peptides from the gpl00, Melan-A/MART-1, tyrosinase melanoma antigens to stimulate CD8+ CTLs yielding the greatest lytic activity. CD8+ CTLs possessed a type 1 cytokine profile, expressing tumor necrosis factor α and IFN-γ but not IL-4. Infusions of CTLs were supported with systemic low-dose IL-2 administration. 111In labeling and computerized gamma imaging were used to monitor the distribution of CTLs up to 48 h after infusion. Intra-arterial delivery via the right carotid artery was followed by redistribution of the CTLs to the lungs, liver, and spleen within 16 h. In contrast, delivery via an indwelling Ommaya reservoir resulted in prolonged retention of CTLs within the brain for at least 48 h after infusion. Marked but transient elevations in tumor necrosis factor α, IFN-γ, and IL-6 in the cerebrospinal fluid were observed within 4 h of CTL infusion. There was no evidence of tumor progression throughout the treatment period, and clinically the patient showed some resolution of neurological symptoms.

Introduction
Although the immunogenic potential of melanoma has been recognized for some time, clinical trials involving the use

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3The abbreviations used are: DC, dendritic cell; PBMC, peripheral blood mononuclear cell; CSF, cerebrospinal fluid; MRI, magnetic resonance imaging; GM-CSF, granulocyte/macrophage-colony stimulating factor; TNF, tumor necrosis factor; IL, interleukin; NK, natural killer; PMA, phorbol myristate acetate; CNS, central nervous system.
3.3 mm) was removed in November 1995. Additional local recurrences, diagnosed as melanoma, were treated by wide local excision, followed by high-dose IFN therapy. IFN treatment was curtailed because of excessive neurological side effects. Further local recurrences were excised in April and August 1997, followed by local radiation therapy. In April 1997, the patient was enrolled on a vaccine trial with an anti-idiotype antibody (TriGem), targeting disialoganglioside G_{2}^{o}. After an initial weekly induction phase, the patient remained on s.c. monthly vaccine therapy until March 1999, receiving a total of 2.7 mg (27 doses) of TriGem (TriGem Pharmaceuticals, Inc., South San Francisco, CA) admixed with QS-21 adjuvant (Biopharmaceuticals, Inc., Worcester, MA).

The patient remained disease free and asymptomatic until December 1998, when she presented with symptoms of dizziness and difficulties with balance and hearing. A MRI scan of the brain showed nodular enhancement along the corpus callosum in the lateral ventricles, two enhancing left parietal lesions, and involvement of both internal auditory canals. CSF cytology was consistent with melanoma. Whole-body scanning did not reveal disease at any other site, and serum chemistries were normal. In January 1999, she was treated with daily intrathecal IL-2 via an Ommaya reservoir receiving a total of 10 doses (1 \times 10^{6} \text{units/m}^{2}/\text{dose}). She failed to respond and suffered acute toxic side effects. In February 1999, she underwent radiation therapy, receiving a total of 3750 cGy in 15 fractions to the whole brain and a total of 1250 cGy in 5 fractions to the ventricles. A repeat MRI in April 1999 indicated disease progression and a new lesion in her left parietal region (Fig. 1). The parietal lesions were treated with gamma knife stereotactic radiosurgery in April 1999, followed by intrathecal GM-CSF in May 1999. Five doses of intrathecal GM-CSF were administered at 200 \mu g/m^{2}/\text{dose/day}. The patient continued to have persistent neurological symptoms, and hence from June to November 1999, she was treated under a Food and Drug Administration-approved, melanoma antigen-specific CTL immunotherapy protocol (IND #8422), as described in this report.

**Media and Reagents.** DC and CTL culture medium was AIM-V (Life Technologies, Inc., Grand Island, NY). Recombinant human GM-CSF (Leukine; 3.6 \times 10^{6} \text{units/mg}) was from Immunex Corp. (Seattle, WA), and recombinant human IL-4 (2.9 \times 10^{6} \text{units/\mu g}) and TNF-\alpha (1.1 \times 10^{5} \text{units/\mu g}) were from R&D Systems (Minneapolis, MN). Recombinant human IL-2 for in vitro T-cell culture was provided by the Biological Response Modifiers Program, National Cancer Institute, whereas the IL-2 for patient treatment was from Chiron (Emeryville, CA). Prostaglandin E_{2} was from Sigma Chemical Co. (St. Louis, MO). The HLA-A1-restricted tyrosinase peptide 145–156, and the HLA-A2-restricted Melan-A/MART-1 peptide 26–35 and gp100 peptide 209–217 (24–27) were synthesized by Genemed Synthesis, Inc. (South San Francisco, CA). PCR primers were synthesized by Life Technologies, Inc.

**Reverse Transcription-PCR Analysis.** RNA from melanoma cells isolated from the patient’s CSF, and from the MEL-24 cell line (American Type Culture Collection, Rockville, MD) was extracted with TRizol (Life Technologies, Inc.), according to the manufacturer’s instructions. RNA was reverse-transcribed into cDNA using the SuperScript One-Step RT-PCR System (Life Technologies, Inc.). The Melan-A/MART-1 external primer sequences were 5'-ATG CCA AGA GAA GAT GCT TAC-3' and 5'-AGC ATG TCT CAG GTG TCT GC-3', yielding a 384-bp product (28), and the internal primer sequences were 5'-CAC GGC CAC TCT TAC ACC AC-3' and 5'-GGA CCA TTG GGA ACC ACA GG-3', yielding a nested product of 252 bp (29). The tyrosinase external primer sequences were 5'-TTG GCA GAT TGT CTG TAG CC-3' and 5'-AGG CAT TGT GCA TGC TGC TT-3', yielding a 284-bp product (30, 31), and the internal primer sequences were 5'-GTC TTT ATG CAA TGG AAC GC-3' and 5'-GCT ATC CCA GTA AGT GGA GCA TTG GGA ACC ACA GG-3', yielding a nested product of 252 bp (29).

**DCs.** DCs were generated as described previously (32), with minor modifications. Briefly, PBMCs were allowed to adhere to six-well culture dishes (5 \times 10^{6} \text{PBMCs/well}) at 37°C in AIM-V medium. After 2 h, nonadherent cells were removed, and the adherent cells were cultured in AIM-V (3 ml/well) plus...
1000 units/ml GM-CSF and 500 units/ml IL-4. An additional 1000 units/ml GM-CSF and 500 units/ml IL-4 were added to the cells every 3 days, and 1000 units/ml TNF-α and 1 μM prostaglandin E2 were added on day 6 (33). Mature DCs were collected on day 8 and pulsed with 50 μg/ml peptide for 1.5 h at 37°C, after which the DCs were washed once with AIM-V medium prior to stimulation of T cells.

Generation of Peptide-specific CD8⁺ CTLs. Peptide-pulsed DCs were cultured with autologous PBMCs at ratios of 20–30:1 PBMCs:DCs, at a final concentration of 10⁶ PBMCs/ml in AIM-V medium supplemented with 10 units/ml IL-2. Cultures were fed every 3 days by half changes of AIM-V medium supplemented with 10 units/ml IL-2. Responder T cells were restimulated every 7–14 days with peptide-pulsed DCs in AIM-V medium plus 100 units/ml IL-2. After two rounds of stimulation in vitro, CD8⁺ T cells were positively selected with anti-CD8 Dynal beads (Dynal, Inc., Lake Success, NY), according to the manufacturer’s instructions. Resulting populations were >95% CD8⁺, as determined by flow cytometric analysis. To achieve the cell numbers needed for immunotherapy, CD8⁺ CTL cultures were expanded by stimulation with 0.5 μg/ml anti-CD3 monoclonal antibody (OKT3) and irradiated (2500 cGy) autologous PBMCs in AIM-V medium plus 100 units/ml IL-2.

Cytotoxicity Assays. Standard ⁵¹Cr-release assays were performed essentially as described (34). Briefly, autologous EBV-transformed lymphoblastoid cells (LCL) were pulsed with peptide (50 μg/ml, 1 h at 37°C), subsequently labeled for 1 h with 50 μCi Na₂⁵¹CrO₄ (New England Nuclear, Boston, MA), and washed three times before use. NK-sensitive K562 target cells were similarly ⁵¹Cr-labeled. Target cells were plated at 1 × 10⁵/well in 96-well-round-bottomed plates with CD8⁺ effector T cells at the ratios indicated. ⁵¹Cr released into the supernatant after target cell lysis was measured on a Cobra Auto Gamma counter (Packard, Meriden, CT). Assays were performed in triplicate wells, and the percentage of target cell lysis was calculated as described (34).

Intracellular Cytokine Assays. Flow cytometric analysis of intracellular cytokine expression by CD8⁺ T cells was performed as described (34). CD8⁺ T cells were treated with or without 50 ng/ml PMA (Sigma) and 500 ng/ml ionomycin (Sigma) in 24-well plates at 37°C. After 3 h of incubation, 10 μg/ml Brefeldin A (Sigma) were added to each well. After 6 h of incubation, the cells were collected, washed, and fixed with 2% paraformaldehyde in PBS for 10 min, washed once more with PBS, and permeabilized with 0.5% saponin (Sigma) and 1% BSA (Sigma) in PBS. Nonactivated and activated CD8⁺ T cells were then stained with FITC-anti-IFN-γ and phycoerythrin-anti-IL-4 (Becton Dickinson, San Jose, CA) for 30 min at room temperature, washed twice with 0.5% saponin and 1% BSA in PBS, and once with 0.5% BSA in PBS, and fixed a second time with 2% paraformaldehyde in PBS. Fluorescence was recorded with a FACScan (Becton Dickinson), and data were analyzed with WinMDI software (kindly provided by Joe Trotter, Scripps Research Institute, La Jolla, CA).

Indium Labeling. CD8⁺ T cells were labeled with ¹¹¹In oxine (Amersham Life Science, Arlington Heights, IL) as described (20). Briefly, 10⁶ T cells were spun down and resuspended directly in 1 ml of ¹¹¹In oxine (approximate volume, 1 ml) and incubated at room temperature for 20 min. Labeling was terminated by the addition of 2 ml of autologous serum, followed by one wash with excess PBS. T cells were then resuspended in 5–10 ml, injection-grade saline plus 2% autologous serum. Incorporated radiolabel was measured with a nuclear medicine dose calibrator prior to infusion. Incorporated activity averaged 750 μCi. Digital gamma camera images after T-cell transfer to the patient were recorded with a Siemens Diacam (Siemens, Chicago, IL) at 1, 16, 24, and 48 h after infusion.

Cytokine ELISA. CSF samples collected via the Ommaya reservoir were centrifuged to remove cellular components and then frozen at 20°C. ELISAs for TNF-α, IFN-γ, IL-6, and IL-10 were conducted with ELISA kits (CalTAG, Burlingame, CA), according to the manufacturer’s instructions.

Safety Tests. In accordance with Food and Drug Administration requirements, lymphocyte cultures were tested for bacterial contamination by Gram’s stain and microbiological culture. Mycoplasma tests were performed by PCR assay (Stratagene, La Jolla, CA), and the presence of endotoxins was assessed by the Limulus amebocyte lysate test by an independent laboratory (ViroMed Biosafety, Camden, NJ). All cultures tested negative for bacterial and Mycoplasma contamination, and the levels of endotoxin were less than 0.03125 EU/ml.

Results

Antigen Expression by Melanoma Tumor Cells. Numerous studies have shown that the melanoma differentiation antigens tyrosinase, gp100, and Melan-A/MART-1 are frequently expressed in metastatic lesions (8–10). Accordingly, we tested for expression of these antigens in tumor cells recovered from the patient’s CSF. Melanoma cells were found in very low numbers (<100/ml) in CSF drawn via the Ommaya reservoir, and thus the cellular fractions from several CSF samples were pooled, and the RNA was extracted and subject to nested set PCR. The outer primers gave PCR products for gp100, tyrosinase, and Melan-A from RNA extracted from the MEL-24 melanoma cell line (Fig. 2A, Lanes 1, 5, and 9). However, an initial round of PCR using the outer primers failed to detect tyrosinase or gp100 expression and revealed only faint expression of Melan-A (Fig. 2A, Lanes 2, 6, and 10) in the patient-derived RNA. In contrast, a second round of PCR using nested primers resulted in amplification of products for all three antigens from the patient-derived RNA sample (Fig. 2B, Lanes 2, 5, and 8).

Generation of Melanoma Antigen-specific CD8⁺ CTLs. The patient’s class I HLA type (A1, A2, B7, B62) included both the A1 and A2 alleles, which afforded us a wide choice of potential T-cell epitope targets within the gp100, tyrosinase, and Melan-A/MART-1 antigens found to be expressed in tumor cells from the CSF. The HLA A1-binding tyrosinase 145–156 peptide and the HLA A2-binding gp100 200–217 and Melan-A/MART-1 26–35 peptides were selected for DC-based stimulation of specific CD8⁺ CTLs. Cytotoxicity was assessed after two rounds of in vitro stimulation, followed by purification of CD8⁺ T cells. The inaccessibility of autologous tumor cells from this patient did not permit direct assessment of potential antitumor T-cell cytotoxicity. For this reason, we tested CD8⁺ CTL function against autologous LCLs pulsed with peptides. Melan-A/MART-1 peptide-specific CTLs showed the highest level of lysis (up to 75% at an E/T ratio of 20:1) against peptide-pulsed autologous LCLs (Fig. 3A). CTLs
CD8+ CTLs used for the initial intra-arterial transfer (1 x 10^8 T cells). In view of the documented importance of type 1 cytokines in T-cell-mediated tumor regression in vivo (35, 36), we evaluated peptide-specific CD8+ T cells for cytokine expression. Flow cytometric analysis of intracellular IFN-γ and IL-4 expression by peptide-specific CD8+ T cells revealed a strong bias in favor of an IFN-γ+/IL-4- type 1 cytokine profile in all cases (Fig. 4). Intermediate IFN-γ+/IL-4+ CD8+ T cells constituted a small minority (typically <5%), and type 2 IFN-γ-/IL-4+ CD8+ T cells were also infrequent, although almost 10% of the gp100 peptide-specific CD8+ T cells exhibited this phenotype (Fig. 4D).

Adoptive Transfer of Melanoma Peptide-specific CD8+ CTLs. A summary of the treatment regimen for T-cell immunotherapy, including the dates of T-cell transfer, the route of delivery, and the number of T cells transferred, is provided in Table 1. For the first treatment, T cells were given systemically, through catheterization of the right carotid artery for suborbital delivery. The CD8+ CTLs used for the initial intra-arterial transfer (1 x 10^8 T cells) were produced by stimulation with DCs loaded with a mixture of the three melanoma antigen peptide epitopes described above, and thus it is not possible to ascertain the numbers of T cells specific for any given peptide. For subsequent intrathecal treatments via the Ommaya reservoir, peptide-specific CD8+ CTLs were prepared in separate cultures stimulated with DCs pulsed with the respective peptides, and the T cells were pooled at the time of delivery. For the first intrathecal treatment, 8.33 x 10^7 Melan-A-specific T cells and 2.22 x 10^7 tyrosinase-specific T cells were delivered, giving a total of 1.055 x 10^8 CTLs. On the second intrathecal treatment, the dose was increased to 2.3 x 10^8, of which 8.5 x 10^7 T cells were specific for gp100, 1.1 x 10^7 were Melan-A specific, and 3.5 x 10^7 were tyrosinase specific. For the final intrathecal treatment, the dose was further escalated to 1.8 x 10^9 T cells, of which 1.05 x 10^8 were gp100 specific, 1.47 x 10^8 were Melan-A specific, and 1.5 x 10^7 were tyrosinase specific. In this last case, a large proportion (>90%) of the tyrosinase peptide-stimulated T cells were CD4+, which we elected to transfer, rather than purify the relatively small number of CD8+ T cells in the culture. In all other cases, purified CD8+ T cells were used for immunotherapy.

Concurrent with the adoptive T-cell therapy, systemic IL-2 was administered in de-escalating doses for in vivo T-cell support (Table 1). The decrescendo IL-2 regimen (30 x 10^6 units to 44 x 10^6 units) was administered by continuous i.v. infusion over 30-96 h. The 96-h schedule was associated with hypotension and hypoxemia. In contrast, the 30-h schedule was well tolerated and was adopted for the last three rounds of intrathecal T-cell immunotherapy.

In Vivo Localization of Transferred T Cells. For the first transfer via the right carotid artery, 1 x 10^8 T cells were injected.
extent in the liver and spleen. By 16 h after infusion, nearly all detectable radiolabel was resident in the liver and spleen. In contrast, intrathecal infusion of \(^{111}\)In-labeled T cells via an indwelling Ommaya reservoir resulted in prolonged retention of labeled cells within the cranium, with a low level of migration along the spinal column (Fig. 5B). The smaller spot above the main area of cellular accumulation most probably represents signal from the Ommaya reservoir, which was located on the right side of the cranium. There was minimal systemic recirculation, with very few cells localized in the lungs, liver, or spleen at any time point tested. In the light of these results, all subsequent treatments were by the intrathecal route.

**In Vivo Cytokine Expression.** To assess activity of transferred T cells, the patient’s CSF was tapped via the Ommaya reservoir at the time of treatment and at 4, 24, 48, and 114 h after treatment. ELISA quantification revealed a marked elevation in IFN-γ and TNF-α levels at 4 h after transfer, followed by a rapid return to baseline levels within 24 h (Fig. 6, A and C). IL-6 was also elevated in the CSF at 4 h after transfer and was sustained for at least 24 h (Fig. 6B). Surprisingly, very high levels of IL-10 (>2000 pg/ml) were detected in the CSF before adoptive transfer of T cells, and IL-10 levels remained unchanged throughout the monitoring period (Fig. 6D). Similarly high levels of IL-10 were also detected in the patient’s serum (data not shown). No other cytokines were detected in the serum.

**Discussion**

Immunotherapeutic treatment of malignant disease involving the brain presents a unique set of challenges. Of the approaches described, the majority have involved administration of intrathecal IL-2, either alone or in combination with lymphokine-activated killer cells (37–41). Response to treatment of multidrug-resistant intracranial metastatic melanoma with a combination of systemic IL-2, IFN-α, and 5-fluorouracil has also been documented (42). Of particular interest in the context of the present study, intrathecal injection of autologous CTLs via an Ommaya reservoir has been shown to induce regression

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**Table 1** Treatment regimen for infusion of autologous peptide-specific CTLs

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<th>Date of in vitro T-cell stimulation</th>
<th>Date of T-cell infusion</th>
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<th>No. of cells infused</th>
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<td>9/2/99</td>
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<td>1.8 × 10⁹</td>
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*The IL-2 was initially administered as 22 × 10⁶ units over the initial 24 h, 11 × 10⁶ units over the subsequent 24 h, and followed by 11 × 10⁶ units over the final 48 h for a total of 44 × 10⁶ units of IL-2. Because of the associated hypotension and hypoxemia, the subsequent dosages were altered such that 12 × 10⁶ units of IL-2 was given over the first 6 h, followed by 12 × 10⁶ units over the next 12 h, followed by 6 × 10⁶ units over the final 12 h, for a total of 30 × 10⁶ units IL-2.*

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*Fig. 4 Two-color flow cytometric analysis of intracellular IFN-γ and IL-4 expression by CD8⁺ T cells specific for gp100 peptide 209–217 (A and D), Melan-A/MART-1 peptide 26–35 (B and E), and tyrosinase peptide 145–156 (C and F). T cells were unstimulated (A–C) or activated with PMA and ionomycin (D–F).*
CTL Immunotherapy for Leptomeningeal Melanoma

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Fig. 5 Computerized gamma imaging of $^{111}$In-labeled melanoma antigen-specific CD8$^+$ T cells adoptively transferred by intra-arterial infusion via the right carotid artery (A) or intrathecal infusion via an indwelling Ommaya reservoir (B). Images were obtained at 1, 16, 24, and 48 h after infusion.

of end-stage malignant glioma with minimal treatment-associated toxicity (43), thus illustrating the safety and potential efficacy of intrathecal CD8$^+$ T-cell immunotherapy for the treatment of malignant disease with CNS involvement.

In this report, we describe the application of melanoma antigen-targeted CD8$^+$ CTLs for intrathecal immunotherapy of advanced metastatic leptomeningeal melanoma. This approach was adopted because computerized gamma imaging of $^{111}$In-labeled CTLs showed that systemic administration of T cells, even when locally delivered via catheterization of the carotid artery, failed to result in accumulation of T cells at the site of disease. T cells rapidly washed out of the cranium and were almost totally resident in the liver and spleen within 24 h of transfer. From these observations, we conclude that the blood-brain barrier represents an insurmountable obstacle to cellular immunotherapy of metastatic disease within the brain. To circumvent this problem, we took advantage of an indwelling Ommaya reservoir that had been placed for prior intrathecal IL-2 therapy. Gamma imaging showed that intrathecal transfer of CD8$^+$ CTLs resulted in extended cranial localization, at least through the 48-h monitoring period, with minimal wash-out to systemic sinks for lymphocyte accumulation. However, the extent to which the T cells localized to tumor cannot accurately be determined from our imaging studies. Similarly, because control studies could not be done, it is unclear whether antigen nonspecific T cells would have shown equally good retention within the CNS. Intrathecal transfer of CD8$^+$ T cells was well tolerated by the patient, even at the highest dose administered (>10$^9$ T cells).

Evidence for in vivo T-cell activation was provided by detection of a rapid onset peak in cytokine levels, in particular IFN-$\gamma$, in the patient's CSF within 4 h of intrathecal CD8$^+$ T-cell transfer. TNF-$\alpha$ production in the CSF may also be a direct result of CD8$^+$ T-cell activation in vivo, although it should be noted that TNF-$\alpha$ can be secreted by many other cell types, particularly resident monocytes and macrophages. More prolonged detection of IL-6 in the CSF up to 48 h after treatment may also be indicative of downstream inflammatory processes. The rapid decline in IFN-$\gamma$ and TNF-$\alpha$, which are not detectable later than 24 h after transfer, is unlikely to be a reflection of loss

Fig. 6 Evaluation of CSF cytokine levels after intrathecal C-8$^+$ T cell infusion. ELISAs for TNF-$\alpha$ (A), IL-6 (B), IFN-$\gamma$ (C), and IL-10 (D) were performed on CSF samples drawn via the Ommaya reservoir immediately preceding treatment (0 h) and at 4, 24, 48, and 114 h after intrathecal administration of autologous melanoma antigen peptide-specific CD8$^+$ T cells. Assays were performed in duplicate, and the results are expressed as the mean cytokine titer (pg/ml). Results are representative of at least two individual experiments.

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of T-cell viability, because the indium scans clearly indicate prolonged residence of viable cells.

One of the more intriguing observations was the detection of very high levels of IL-10, both in the CSF and in the patient's serum, both prior to T-cell immunotherapy and throughout the posttreatment monitoring period (up to 114 h). IL-10 is widely regarded as an immunosuppressive cytokine (44) and can also down-regulate the transporter associated antigen processing (45) and inhibit HLA class I and class II expression (46, 47). IL-10 may thus play a key role in tumor evasion of T-cell immunosurveillance and may also represent a barrier to T-cell immunotherapy. In contrast, however, IL-10 has also been reported to increase cytotoxic potential in cervical tumor-specific CD8+ CTLs (48) and may thus enhance cytotoxic effector function in vivo. In this case, the impact of IL-10 on intrathecal CD8+ T cell immunotherapy is open to conjecture.

For almost 2 years (April 1997 to March 1999), the patient was also treated with the TriGem vaccine, an anti-idiotype antibody, targeting the disialoganglioside GD2. Preliminary results from the TriGem vaccine trial (n = 47) in advanced melanoma patients show promise for clinical rejection. Proc. Natl. Acad. Sci. USA, 91: 3515-3519, 1994.


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