Analysis of a Natural Immune Response against Tumor Antigens in a Melanoma Survivor: Lessons Applicable to Clinical Trial Evaluations

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Abstract

The long-term survival of some patients with metastatic melanoma may be attributable in part to cellular immune responses to melanoma antigens. However, little is known about the level of CTL reactivity in vivo that is required for immunological control of tumor progression. In the present report, T-cell responses were evaluated with lymphocytes obtained from tumor-involved nodes and peripheral blood of a long-term melanoma survivor. Using an ELISPOT assay, naturally occurring functional T cells, which recognize the peptide ALLAVGATK, plus two other HLA-A3 restricted peptides, were detected in a tumor-involved lymph node. The ALLAVGATK-reactive T cells were also evaluated by MHC-tetramers staining and were found to be CD8+ CD45RO+ L-selectin− CD11a+, suggesting that they are antigen experienced and have a memory phenotype. Unstimulated peripheral blood lymphocytes from the same patient demonstrated no detectable T-cell responses; however, a single stimulation with ALLAVGATK peptide in vitro resulted in a dramatic expansion of peptide-reactive CTLs. This patient, with evidence of tumor-reactive CTLs targeted to several tumor antigens in a tumor-involved lymph node and with evidence of a circulating memory T-cell response, has remained disease-free for 6 years, despite prior bulky nodal metastasis. In contrast, three HLA-A3+ patients with rapidly progressive metastatic melanoma had no detectable T-cell response in tumor-involved nodes or peripheral blood lymphocytes, even after peptide stimulation in vivo. The presented data are consistent with a systemic polyvalent immune response against tumor in this long-term survivor. These data provide an estimate of the level of CTL response that may be associated with protection from tumor recurrence.

Introduction

CTLs appear to be the key effector cells responsible for immune-mediated destruction of malignant tumors, both in animal models and in humans with cancer (1, 2). However, little is known about the frequency of CTLs (3) reactive to tumor-associated antigens in patients, nor is there a clear understanding of the level of CTL reactivity in vivo that is required for immunological control of tumor progression. In some studies, tumor antigen-reactive CTLs in peripheral blood were undetectable unless they were stimulated several times in vivo prior to measuring T-cell responses (3–6). In other studies, CTLs reactive to defined epitopes have been found at low frequencies of ~10^(-5) PBLs, and it has been the rare patient in whom higher CTL frequencies have been identified (7, 8). The biological significance of these low levels of CTL reactivity may be questioned, because PBLs of normal donors contain similar low levels of reactivity to the melanocytic tissue differentiation antigens tyrosinase and MART-1/Melan-A (3, 7, 9).

There is some evidence from human clinical trials that CTL reactivity against defined cancer-associated antigens in melanoma patients may mediate tumor destruction (4, 10–12). However, clinical responses have not always correlated with measures of immune response (4). Further progress in the development of immunotherapy for cancer will require that specific CTL responses to new treatments be characterized in a manner that correlates well with clinical outcome. Characterizing the naturally occurring immune response to tumor in long-term melanoma survivors may provide a guideline for the level of CTL response that is desired in patients undergoing immunotherapy. The aim of the present study is to characterize the naturally occurring CTL response against defined tumor antigens in a long-term melanoma survivor and several other HLA-A3+ patients and to assess whether that response correlated in any way with melanoma progression in the patients studied.

Materials and Methods

Patients and Clinical Histories. VMM18 is a Caucasian male diagnosed with melanoma of the back at age 44. Recurrent tumor detected in axillary lymph nodes 4.7 years later was...
treated by surgical resection. The patient had no additional surgery or other therapy and has remained clinically free of disease for >6 years since the diagnosis and treatment of metastatic disease. Lymphocytes were cryopreserved from the TINs and from peripheral blood. Clinical data for VMM18 and four other patients studied are presented in Table 1.

**LN and PBL Samples.** Patient LN and PBL samples were collected, and lymphocytes isolated from them were cryopreserved in 10% DMSO in liquid nitrogen as described previously (13). Lymph nodes available for evaluation included TINs and TFNs. The identification of a node as tumor free was based on standard histological evaluation on H&E-fixed sections, as performed by clinical pathologists at the University of Virginia. The clinical histories of patients and the types of nodes available are listed in Table 1.

Lymphocytes were subsequently thawed into complete RPMI 1640 with 10% heat-inactivated human AB serum, 2 mm l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Pen-Strep; Life Technologies, Inc., Grand Island, NY). The lymphocytes were washed and assayed either after overnight incubation in complete RPMI 1640 supplemented with IL-2 (20 units/ml) or 2–3 weeks after a single in vitro stimulation. *In vitro* stimulation with peptide was performed by incubating LN cells or PBLs at 2 × 10^6/ml in complete medium with the synthetic peptide ALLAVGATK (40 μg/ml) for 2 h at 37°C, 5% CO₂. The cells were then pelleted, resuspended in complete medium containing IL-2 (20 units/ml), and cultured for 14–21 days in 48-well plates (Costar, Cambridge, MA). Complete medium was replaced as needed, approximately twice per week. TIN samples were evaluated without any stimulation or after a short-term culture for 14–21 days of 2 × 10^6 LN cells, with the fresh autologous tumor cells contained in the TIN specimen, in complete medium containing IL-2 (20 units/ml) in 48-well plates.

**Cell Lines, HLA Typing, and Immunohistochemistry.**

The human melanoma cell lines VMM12 (HLA-A1, HLA-A3, HLA-B7, and HLA-B14), VMM15 (HLA-A1, HLA-A25, HLA-B8, HLA-B18), and VMM18 (HLA-A3, HLA-A31/33, HLA-B60, HLA-C3) were derived from TINs of patients at the University of Virginia (Charlottesville, VA). All three cell lines were gp100⁺, as determined by immunohistochemistry using the antibody HMB45 (BioGenex, San Ramon, CA). C1R-A3 (an HLA-A3 transfectant of the human lymphoblastoid cell line C1R) was kindly provided by P. Cresswell (Yale, New Haven, CT; Ref. 14). K562 is a natural killer cell target, and VMM68 EBV (HLA-A1101, HLA-A25, HLA-B35, HLA-B44, HLA-C4, HLA-C6) is an EBV-transformed lymphoblastoid line generated from a patient donor. HLA typing was performed by microcytotoxicity assay on autologous lymphocytes (One Lambda, Canoga Park, CA). Expression of HLA-A3 by tumor cells was confirmed by flow cytometry after staining with the monoclonal antibody HMB45 (BioGenex, San Ramon, CA). C1R-A3 (an HLA-A3 transfectant of the human lymphoblastoid cell line C1R) was kindly provided by P. Cresswell (Yale, New Haven, CT; Ref. 14). K562 is a natural killer cell target, and VMM68 EBV (HLA-A1101, HLA-A25, HLA-B35, HLA-B44, HLA-C4, HLA-C6) is an EBV-transformed lymphoblastoid line generated from a patient donor. HLA typing was performed by microcytotoxicity assay on autologous lymphocytes (One Lambda, Canoga Park, CA). Expression of HLA-A3 by tumor cells was confirmed by flow cytometry after staining with the monoclonal antibody GAP-A3 provided by P. Cresswell (Yale, New Haven, CT; Ref. 14).

**ELISPOT Assay.** Immunol 2 flat-bottomed plates (Dynatech, Chantilly, VA) were coated with anti-IFN-γ monoclonal antibodies (M-700A; Endogen, Woburn, MA). Lymphocyte samples from lymph node or peripheral blood (1.5 × 10^6

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**Table 1 Clinical histories and specimens evaluated**

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Time from diagnosis of melanoma to nodal metastases</th>
<th>Total no. of positive lymph nodes</th>
<th>% tumor cells GP100⁺</th>
<th>Current status of melanoma</th>
<th>Time from DX of LN metastasis to tumor progression or death</th>
<th>Source of LN cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>VMM18</td>
<td>4.7 yr</td>
<td>1⁰</td>
<td>90% (50, 10, 30)</td>
<td>No evidence of disease</td>
<td>No recurrence, alive and well at 6 yr</td>
<td>TIN</td>
</tr>
<tr>
<td>VMM39</td>
<td>0 yr</td>
<td>8</td>
<td>85% (60, 15, 10)</td>
<td>Died with melanoma in spleen, brain, LN</td>
<td>Progressed at 9 months, died at 11 months</td>
<td>TIN</td>
</tr>
<tr>
<td>VMM108</td>
<td>2 months</td>
<td>1</td>
<td>90% (30, 30, 30)</td>
<td>No evidence of disease</td>
<td>No recurrence, alive and well at 2 yr</td>
<td>TFN</td>
</tr>
<tr>
<td>VMM115</td>
<td>1.2 yr</td>
<td>16</td>
<td>100% (90, 5, 5)</td>
<td>Died with melanoma in periarteric nodes and visceral sites</td>
<td>Progressed at 3 months, died at 17 months</td>
<td>TIN and TFN</td>
</tr>
<tr>
<td>VMM116</td>
<td>0 yr</td>
<td>2</td>
<td>70% (50, 10, 10)</td>
<td>Progressive disease in lung</td>
<td>Progressed at 10 months, alive with disease at 2 yr</td>
<td>TFN</td>
</tr>
</tbody>
</table>

⁰ HMB45 staining (for gp100 expression) was performed by University of Virginia Department of Pathology as a clinical laboratory test on metastatic lymph node for VMM39, VMM108, VMM115, and VMM116. For VMM18, immunohistochemistry was performed on a cell block of the fresh cryopreserved tumor involved lymph node specimen. Also, for VMM115 tumor-involved lymph node cells, immunohistochemistry was repeated on a cell block of the same fresh cryopreserved tumor-involved lymph node specimen that was used for the immunological studies. Results were identical to those obtained with the original lymph node specimen. The percentage staining strongly positive (+++), moderately (++), and weakly (+) was estimated visually, and these percentages are noted in parentheses, respectively.

This patient had matted nodes in one nodal mass. Prior to these studies, all patients were treated with surgery only. No immunotherapy was performed prior to the collection of PBMCs or LNC.
cells/ml) were mixed with equal numbers of C1R-A3 cells alone or C1R-A3 cells pulsed with peptide (40 μg/ml) in the first row of the plate. Serial 1:3 dilutions of that mixture in the complete medium were made, such that cell numbers of stimulated PBLs ranged from 150,000 to 5,000 per well. Plates were then incubated at 37°C, 5% CO2 for 18 h. After extensive washing with washing buffer (0.025% Tween 20 in water), plates were incubated with a biotin-labeled secondary antibody to IFN-γ (M-701B; Endogen), then washed again, and incubated with avidin conjugated with alkaline phosphatase (13043E; PharMingen, San Diego, CA). After washing, plates were developed with the 5-bromo-4-chloro-3-indolyl phosphate substrate in 1% low melting agarose. The next morning, the number of blue spots corresponding to the number of cells secreting IFN-γ were counted in each well. Each sample was tested in triplicate at each of several dilutions of lymphocytes. The number of spots produced by lymphocytes incubated with C1R-A3 alone was compared with that produced by lymphocytes incubated with C1R-A3 loaded with peptide. The frequency of T cells reactive to peptide was calculated based on this difference. Statistical significance was tested by a standard t test.

Intracellular Staining for IFN-γ. Lymphocytes from peripheral blood or lymph nodes were incubated for 4 h in 96-well plates (Costar) at 0.25–0.5 × 10^6 cells/well in a volume of 300 μl of complete RPMI medium supplemented with the intracellular protein transport inhibitor monensin (2 μg/ml; GolgiStop; PharMingen, San Diego, CA). After washing, plates were developed with the 5-bromo-4-chloro-3-indolyl phosphate substrate in 1% low melting agarose. The next morning, the number of blue spots corresponding to the number of cells secreting IFN-γ were counted in each well. Each sample was tested in triplicate at each of several dilutions of lymphocytes. The number of spots produced by lymphocytes incubated with C1R-A3 alone was compared with that produced by lymphocytes incubated with C1R-A3 loaded with peptide. The frequency of T cells reactive to peptide was calculated based on this difference. Statistical significance was tested by a standard t test.

Generation of Melanoma-specific Cytotoxic T Cells. VMM18 CTLs were generated following the detailed protocol reported previously (15). Briefly, T-cell lines were established from the mixture of lymphocytes, and tumor was obtained from the fresh TIN, using a ratio of tumor cells:lymphocytes of 1:1. Cells were cultured in 24-well tissue culture plates (Linbro, Hamden, CT) in RPMI 1640 (Sigma) containing 10% FCS, Pen-Strept, and 20 units/ml recombinant IL-2 (Cetus, Emeryville, CA) and were maintained by repeated stimulation with irradiated (10 Gy) fresh cryopreserved autologous tumor cells or with the autologous tumor cell line at a tumor:lymphocyte ratio of 1:10 every 10–12 days. T-cell specificity for autologous melanoma was confirmed after 28 days of culture. Melanoma-reactive T lymphocyte lines were then expanded by a modification of E. Goulmy’s method as described (15).

Isolation of Naturally Processed HLA-A3-associated Peptides. HLA-A3-associated peptides were acid eluted from HLA-A3 molecules affinity-purified from melanoma cells, as described previously for HLA-A2-associated peptides (13, 16). Briefly, VMM18 melanoma cells cultured in cell factories (Nunc, Naperville, IL), were washed three times in cold PBS, pelleted, and then snap-frozen. Cell pellets were solubilized in detergent: 1% 3-[3-cholamidopropyl]dimethylammoniopropyl]l-propanesulfonate, 174 mg/ml phenylmethylsulfonyl fluoride, 5 mg/ml aprotinin, 10 mg/ml leupeptin, 16 mg/ml pepstatin A, 33 mg/ml iodoacetamide, 0.2% sodium azide, and 0.03 mg/ml EDTA for 1 h at 4°C. After centrifugation at 100,000 × g for 1 h at 4°C, the pellet of insoluble proteins was discarded, and the supernatant was filtered (0.2 μm) and then passed over a protein A-Sepharose column precoated with the monoclonal antibody GAP-A3. The HLA-A3 molecules and associated peptides, bound to GAP-A3, were then eluted from the column with 0.2 N acetic acid (pH 2.7). The eluate was brought to a final concentration of 10% acetic acid and boiled for 5 min. Finally, peptides were separated from the immunoglobulin, class I MHC molecules, and β2-microglobulin by centrifugation through Ultrafree-CL 5000-kDa filters (Millipore, Bedford, MA) at 2500 × g for 5 h. Filtrates containing purified peptides were concentrated using vacuum centrifugation and stored at −80°C.

HPLC Fractionation and Coelution of Naturally Processed and Synthetic Peptides. The extracted HLA-A3-associated peptides were fractionated by reversed-phase HPLC on a Brownlee narrowbore C-18 Aquapore column (2.1 mm × 3 cm, 3 μm) and eluted with a 40-min gradient of 0–60% (v/v) acetonitrile/0.085% trifluoroacetic acid in 0.1% trifluoroacetic acid. Fractions were collected at 1-min intervals. A synthetic peptide, ALLAVGATK, was eluted, in parallel experiments, under identical conditions to identify its elution point.

Cytotoxicity Assays. Cell-mediated lysis of target cells was determined using a standard 4-h 51Cr-release assay. Briefly, 51Cr-labeled target cells were plated at 1–2 × 10^3 cells/well in triplicate on 96-well V-bottomed plates (Costar) with the indicated ratio of effector cells in a final volume of 200 μl. Wells containing either culture medium or 1 N HCl in place of the effector cells served as background and total 51Cr-release controls, respectively. Percentage of specific 51Cr release was calculated as ([51Cr (experimental) − 51Cr (background)]/51Cr (total) − 51Cr (background)) × 100.

Reconstitution Assays with Synthetic or Naturally Processed Peptides. Isolated fractions of naturally processed peptides or aliquots of synthetic peptides were diluted in CTL assay medium (RPMI 1640, 10% FCS, and antibiotics) and preincubated for 2 h with 1–2 × 10^4 51Cr-labeled target cells in 100 μl/well in 96-well plates. Effector cells were added in 100 μl of assay medium for a final E:T ratio of 10:1–20:1, and the remainder of the 51Cr-release assay was performed as described above. Wells containing peptide and target cells but no CTLs were used as a control to rule out the possibility that the peptides themselves may be toxic.

ALLAVGATK-MHC Tetramers Staining. HLA-A*0301/ALLAVGATK (PE) tetramers were provided by National Institute of Allergy and Infectious Diseases MHC Tetramer Core Facility (Atlanta, GA). The specificity of tetramers was confirmed by titration on a VMM18 polyclonal CTL line. A PBL sample of normal donor was used as a negative control. Patient samples were evaluated after positive selection of CD8+ cells using Dynabeads (Dynal, Oslo, Norway). Cells were triple-stained with the tetramers (1:350 dilution), anti-CD8 antibody (MHCD 0804, Caltag), and monoclonal antibodies to each of the following cell surface antigens: CD45RO (31301A, 7 ram) and eluted with a 40-min gradient of 0-60% (v/v) acetonitrile/0.085% trifluoroacetic acid in 0.1% trifluoroacetic acid. Fractions were collected at 1-min intervals. A synthetic peptide, ALLAVGATK, was eluted, in parallel experiments, under identical conditions to identify its elution point.

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Pharmingen), L-selectin (MA-LECAM-1; Endogen), and CD11A (9320-01; Southern Biotechnology Associates). Goat F(ab′)2 antimouse IgGs labeled with TRI-COLOR (M35006; Caltag) were used as a secondary antibody to visualize CD45RO, L-selectin, and CD11A-positive cells.

**Results**

**HLA-A3-restricted VMM18 CTLs Recognize Multiple Tumor-associated Peptides.** VMM18 CTLs were generated by repeated stimulation of TIN lymphocytes with autologous tumor cells. Reactivity of the VMM18 CTLs against autologous VMM18 melanoma cells and HLA-A33 alloimmune melanoma tumor lines is demonstrated in Fig. 1A. They are nonreactive to an HLA-A3-negative melanoma VMM15, lymphoblastoid lines VMM68-EBV and C1R-A3, and the NK target K562. Because VMM18 and VMM12 only have the HLA-A3 class I MHC molecule in common, one or more shared antigens must be recognized by these CTLs in the context of HLA-A3.

HLA-A3 associated peptides were purified from VMM18 tumor cells by immunoaffinity chromatography. After HPLC fractionation, peptides from each fraction were added to C1R-A3 cells, and their ability to reconstitute an epitope for these CTLs was assessed by a chromium release assay. Prominent reactivity was obtained among the HPLC fractions 23–25, 36–37, and 47–49 of HLA-A3-associated peptides (Fig. 1B), indicating that at least three different naturally processed peptides were recognized. In addition, several more minor peaks were noted, which are of uncertain significance. We reported previously that the gp100 peptide ALLAVGATK is naturally processed and presented by VMM18 tumor cells (15). By tandem mass spectrometric evaluation, we have identified that peptide in fractions 36 to 37 represents the middle peak B in Fig. 1B. The other two peaks represent at least two different peptides (fractions 23–25, peak A; fractions 47–49, peak C), which are also recognized by these CTLs. The original sequences of these two peptides are submitted for publication in a separate paper.4 They are referred to as pA and pC, respectively.

**Lymphocytes from a VMM18 TIN Are Reactive to ALLAVGATK, pA, and pC Peptides.** TIN from long-term melanoma survivor VMM18 was evaluated directly for evidence of reactivity to the HLA-A3-restricted peptides ALLAVGATK (gp10017_25), pA, and pC. T cells reactive to these peptides were detected by ELISPOT assay in the cryopreserved TINs of patient VMM18 without prior ex vivo stimulation (Fig. 2). The number of peptide-reactive lymphocytes identified from LN cells incubated with C1R-A3/ALLAVGATK peptide was significantly greater than the number from LN cells incubated with C1R-A3 without peptide (P < 0.025). The number of spots attributable to specific reactivity against ALLAVGATK was 45 per 100,000 mononuclear cells (1/2222). Approximately 29% of the cells plated were CD8+ T cells (flow cytometry data not shown); thus, the frequency of ALLAVGATK-reactive CTLs among CD8+ cells was approximately 45 of 29,000 (1 of 644; 0.16%). Even higher frequencies were detected for pA- and pC-specific T cells, 88 and 146 per 105 cells, respectively. These correspond to approximately 0.3% and 0.5% of the CD8+ cells. Thus, almost 1% of the CD8+ cells in the TIN are reactive to these three HLA-A3-restricted peptides naturally presented by tumor. Using ALLAVGATK-A3 MHC class 1 tetramers, we found that 1.05 ± 0.2% of CD8+ T cells in the node are reactive to the ALLAVGATK peptide (Fig. 3). All of these tetramer+ cells express CD45RO, and at least 80% are negative for L-selectin (CD62L). The small percentage staining positive for L-selectin is not significantly above background and may overestimate the true percentage of L-selectin+ cells. Also, all of the tetramer+ cells expressed CD11a. These findings demonstrate that the tetramer+ cells are antigen experienced, consistent either with a memory or effector phenotype (18, 19). CD11a

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expression has been associated with the ability to travel through vascular endothelium (18). The level of CD11a expression was higher for the tetramer + cells than for the tetramer - cells (mean fluorescence intensity, 97.8 vs 65.9; data not shown), which is suggestive more of an effector phenotype than a memory phenotype (18).

Unstimulated lymphocytes from TFNs or TINs of four other patients were also evaluated by ELISPOT. This included two patients (VMM115 and VMM39) from whom TFNs were available, and two patients (VMM108 and VMM116) from whom TFNs were evaluated. None had detectable reactivity to the ALLAVGATK peptide only (data not shown) and 1.3% after testing with peptide only (data not shown) and 1.3% after testing with peptide only (data not shown). In all experiments, production of IFN-γ upon nonspecific activation with phorbol myristate acetate/ionomycin was detected in the positive control wells, corresponding to 4–9% of mononuclear cells plated.

**T Cells Recognizing ALLAVGATK Peptide Are Detected in Cultured LN Cells of Two Patients.** Subsequently, LN cells were incubated in medium containing IL-2 for 14 days, with the intent of expanding autologous tumor-reactive T cells without use of synthetic peptide. VMM18, VMM39, and two patients, VMM108 and VMM116, contained substantial numbers of viable tumor cells; therefore, these provided a natural source of tumor antigen. VMM108 and VMM116 LN cells were from a TIN of patient VMM18, with peptide only (data not shown) and 1.3% after testing with peptide only (data not shown). ALLAVGATK-specific CTLs using HLA-A3/ALLAVGATK tetramers (data not shown). All four tumors expressed gp100 antigen ALLAVGATK (data not shown). In all experiments, production of IFN-γ upon nonspecific activation with phorbol myristate acetate/ionomycin was detected in the positive control wells, corresponding to 4–9% of mononuclear cells plated.

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**No Reactivity to ALLAVGATK Was Detected in PBLS without Stimulation.** To compare the frequency of detection of tumor antigen in vivo, and occult tumor metastases in those nodes cannot be excluded. All four tumors expressed gp100 based on immunohistochemical evaluation of the TINs. An ELISPOT assay performed with these short-term cultures revealed CTL reactivity to the gp100 peptide ALLAVGATK in two patients, VMM18 and VMM108 (Fig. 4A). ALLAVGATK reactivity of VMM18 CTLs generated from short-term cultures of TINs was prominent at 1252 per 10^5 cells (Fig. 4A). On the other hand, the frequency of ALLAVGATK-reactive CTLs among cultured VMM108 LN cells was low but statistically significant (222 per 10^5, P = 0.02; Fig. 4A). No reactive CTLs were detectable from the cultured LN cells of patients VMM115, VMM116, and VMM39 (Fig. 4A and data not shown). We also evaluated short-term cultures of VMM18 lymphocytes by staining for intracellular IFN-γ production at 4 h after exposure to the ALLAVGATK peptide. By double staining with anti-CD8 and anti-IFN-γ antibodies, it was possible to enumerate specifically CD8 + CTLs producing IFN-γ in response to peptide antigen. This assay was performed by stimulating VMM18 lymphocytes with soluble peptide alone, without addition of antigen-presenting cells (3) other than the dendritic cells, macrophages, and B cells naturally present in the node. In the VMM18 TIN sample cultured for 2 weeks, 1.73% of the lymphocytes stimulated with ALLAVGATK peptide were CD8 + IFN-γ + (Fig. 5, C and D). There was a relatively high spontaneous release of IFN-γ by CD8 + cells without peptide, possibly because of the presence of tumor cells. The difference, however, represents 1 in 114 mononuclear cells (0.88%). This compares favorably to the ELISPOT assay on the same sample, where 0.64% peptide-specific cells were detected after testing with peptide only (data not shown) and 1.3% after testing with peptide pulsed on C1R-A3 (Fig. 4A).

**Fig. 2** LN cells from a TIN of VMM18 patient were tested by ELISPOT assay. HLA-A3-associated ALLAVGATK, pA, and pC peptides were presented on the lymphoid cells C1R-A3. Spots counted per 10^5 lymphoid cells plated represent the number of IFN-γ-secreting cells. Bars, 1 SD of the replicate values. The LN cells demonstrated detectable reactivity to ALLAVGATK, pA, and pC peptides, compared with their background response to C1R-A3 loaded with irrelevant peptide YLKXKIKNKL (17) from malaria CSP antigen (P = 0.029, 0.016, and 0.00007, respectively).

**Fig. 3** CD8 + lymphocytes isolated from a TIN of patient VMM18 by positive selection (purity, 95.5%) were tested for the presence of ALLAVGATK-specific CTLs using HLA-A3/ALLAVGATK tetramers. The cells were stained for T-cell receptor expression, CD8 expression, and for expression of L-selectin (CD62L), CD45RO, or CD11a and then evaluated by flow cytometry. The top bar represents the mean ± 1 SD for three determinations of the percentage of cells tested that are tetramer + and CD8 +. Each of the other bars represents a single determination. All values shown have been corrected for background staining of 0.34% with a tricolor-conjugated secondary antibody only. The numbers of tetramer + cells are more than 5 SDs above the background staining.
ALLAVGATK-specific CTLs Were Detected in VMM18 PBLs after One in Vitro Stimulation with Peptide. CTL responses in PBLs after one stimulation with peptide and in vitro culture for 2 weeks in the presence of IL-2 (20 units/ml), without additional antigen exposure during that interval, were also evaluated. This approach is effective at expanding antigen-reactive CTLs with minimal ex vivo manipulation. As detected by ELISPOT assay, VMM18 PBLs did show a dramatic expansion of antigen-specific cells in each of two separate assays, to 112 and 186 per 10^5 cells (0.11–0.19%). Results of one of those assays are shown in Fig. 4B. The blood for those assays was harvested at different time points, about 2 and 4 years after surgical removal of the TINs.

When these 14-day cultures of peptide-stimulated PBLs were evaluated by staining for intracellular IFN-γ production, a discrete population of CD8^+ IFN-γ^+ cells was identified as 0.31% of the counted cells. Subtracting the 0.04% spontaneous background leaves 0.27% CD8^+ IFN-γ^+ cells responding to the ALLAVGATK peptide (Fig. 5, A and B). This correlates well with 0.19% IFN-γ-secreting cells determined by ELISPOT with the same sample.

**Discussion**

In this report, we have identified naturally occurring immune responses to three melanoma-associated peptide antigens by direct evaluation of lymphocytes obtained from the TIN of a long-term melanoma survivor. Using the ELISPOT assay, we were able to enumerate those peptide-specific T cells directly, without ex vivo expansion (Fig. 2).

One of the recognized peptides represents a known HLA-A3-restricted CTL epitope of melanoma differentiation protein gp100 (ALLAVGATK, gp100_17-25; Ref. 15), and reactivity to this peptide was evaluated in more detail. CTLs reactive to that antigen were also detected in the peripheral blood of VMM18 patient after a single stimulation with the peptide in vitro, and this reactivity was evident up to 4 years after surgical resection of the metastatic tumor (Fig. 4B). The CTLs were undetectable in PBLs without stimulation, suggesting that their frequency in vivo is <1 per 10^5 circulating PBLs (Fig. 4B). Ex vivo culture of these PBLs for 2 weeks resulted in expansion to over 100 peptide-specific CTLs per 10^5 cells. Thus, these circulating
CTLs are capable of a marked expansion after a single in vitro stimulation with peptide, which is suggestive of a systemic memory T-cell response.

The number of antigen-specific T cells in the TIN is substantially higher than in the blood, with 0.16% of CD8+ cells secreting IFN-γ in response to ALLAVGATK (Fig. 2). As in the blood, these T cells also are capable of a strong proliferative response to tumor, as demonstrated by their expansion in short-term in vitro culture with IL-2 to 1–1.5% of the cultured T cells. Reactivity to each of two other recently identified HLA-A3-restricted peptides, pA and pC, was even greater than the response to the gp100 peptide. T cells recognizing each of three peptides were present simultaneously in the same TIN. The combined reactivity of ~1% of the CD8+ cells to HLA-A3-restricted antigens is evidence of a substantial polyvalent immune response. This has relevance to tumor vaccines, because there has been some concern that polyvalent responses to vaccination with multiple antigens at a single site may be hindered by immunodominance of individual epitopes. Although these data do not address potential competition of exogenously added peptides for MHC binding, the data do demonstrate that polyvalent responses to peptides presented by the same MHC molecule may occur within the same lymph node.

A major paradox of the results obtained with VMM18 is the finding of functional CTLs in the presence of growing tumor. Tumor-reactive CTLs analogous to those cultured from VMM18 have been generated from TINs of many patients (20); therefore, this paradox has general relevance. Possible mechanisms for tumor growth in the presence of tumor-reactive CTLs include tumor-mediated immune escape or dysfunction of the T cells. Mechanisms of immune escape include down-regulation of tumor antigen expression, MHC expression, or antigen processing. Tumors may also secrete immunosuppressive cytokines. We have not evaluated all of these issues in VMM18, but expression of HLA-A3 on the tumor cells has been confirmed, as has expression of gp100 (Table 1). Some CTLs recognizing melanoma antigens, detected by tetramers, have been found to be anergic (21). However, the peptide-specific HLA-A3-restricted CTLs in VMM18 TINs and PBLs were capable of responding rapidly to antigen, both by secretion of IFN-γ and by proliferation. In addition, the ALLAVGATK-reactive T cells identified by tetramers have a memory phenotype, and the expression of high levels of CD11a and low CD62L suggest that some may have an effector phenotype (Fig. 3). The number of ALLAVGATK-reactive cells identified by tetramers was ~6-fold higher than the number identified by ELISPOT. It is possible that the tetramer+ cells include a subset of functional CTLs and a subset of nonfunctional CTLs, but the relative homogeneity of the cells phenotypically argues against that idea and suggests instead that the differences simply reflect minor differences in assay sensitivity. Regardless, these data demonstrate the presence of naturally occurring CTLs in the TINs that are not anergized in vivo.

MART-1/Melan-A27–35-reactive CTLs in TINs or blood of unvaccinated patients with metastatic melanoma have been detected in several studies (21–24). However, neither the presence nor the level of CTL reactivity to that MART-1/Melan-A epitope correlated with the patients’ clinical disease course (22). CTLs reactive to the tyrosinase peptide YMDGTMSSQV have been identified in blood and metastatic nodes (21, 22, 25), and CTLs reactive to NY-ESO157–165 (SLLMWITQC) have been found in PBLs of patients with stage IV melanoma (26), but correlations with the clinical course were not described. CTL reactivity of unvaccinated patients to other melanoma peptides has not been studied extensively.

One patient vaccinated repeatedly with several melanoma peptides experienced clinical regression and was found to have cytotoxic reactivity against the MART-1/Melan-A peptides AAGIGILTV and EAAAGILTV (27). This reactivity was detected in PBLs prior to vaccination, was increased after vaccination, and was associated with vitiligo (27). This observation suggests that T-cell reactivity to melanoma antigens after vaccination may be associated with tumor regression. It has remained unclear, however, whether a naturally occurring T-cell response to defined melanoma antigens in unvaccinated patients has biological or clinical significance.

The present study suggests that a strong spontaneous polyvalent response to melanoma antigens including gp100 may be associated with improved prognosis. Further studies on spontaneous T-cell responses to additional tumor antigens will help to elucidate the relationship between these responses and outcome. It is likely that responses to single antigens are not adequate to confer protection against most tumors, but responses to multiple antigens potentially can synergize in providing some immune protection.

As clinical trials of melanoma vaccines are performed and evaluated, data are being generated on the number of reactive CTLs identified in PBLs after vaccination. There is, however, no consensus on the level of immune reactivity that may be biologically relevant for tumor control. We believe that T-cell responses in melanoma patients with long-term disease control can be useful indicators of the magnitude and character of immune responses that are biologically achievable and adequate for immunological control of tumor. In the long-term melanoma survivor studied in this report, the salient findings are: (a) a polyvalent immune response to melanoma antigens; (b) the presence of IFN-γ-producing CTLs in LNs, representing at least 1% of the CD8+ cells infiltrating tumor; and (c) a memory response to melanoma in the peripheral blood that was detectable after a single stimulation ex vivo. We can suggest that these characteristics are desirable and may serve as reasonable goals for future clinical trials of melanoma vaccines or other immune therapy.

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