

## EVALUATION OF PEPTIDE VACCINE IMMUNOGENICITY IN DRAINING LYMPH NODES AND PERIPHERAL BLOOD OF MELANOMA PATIENTS

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**Many peptide epitopes for cytotoxic T lymphocytes (CTLs) have been identified from melanocytic differentiation proteins. Vaccine trials with these peptides have been limited mostly to those associated with HLA-A2, and immune responses have been detected inconsistently. Cases of clinical regression have been observed after peptide vaccination in some trials, but melanoma regressions have not correlated well with T-cell responses measured in peripheral blood lymphocytes (PBLs). We vaccinated stage IV melanoma patients with a mixture of gp100 and tyrosinase peptides restricted by HLA-A1 (DAEKSDICTDEY), HLA-A2 (YLEPGPVTA and YMDGTSQV) and HLA-A3 (ALLAVGATK) in an emulsion with GM-CSF and Montanide ISA-51 adjuvant. CTL responses were assessed in PBLs and in a lymph node draining a vaccine site (sentinel immunized node, SIN). We found CTL responses to vaccinating peptides in the SIN in 5/5 patients (100%). Equivalent assays detected peptide-reactive CTLs in PBLs of 2 of these 5 patients (40%). CTLs expanded from the SIN lysed melanoma cells naturally expressing tyrosinase or gp100. We demonstrated immunogenicity for peptides restricted by HLA-A1 and -A3 and for 1 HLA-A2 restricted peptide, YMDGTSQV. Immune monitoring of clinical trials by evaluation of PBLs alone may underestimate immunogenicity; evaluation of SIN provides a new and sensitive approach for defining responses to tumor vaccines and correlating these responses with clinical outcomes. This combination of an immunogenic vaccine strategy with a sensitive analysis of CTL responses demonstrates the potential for inducing and detecting anti-tumor immune responses in the majority of melanoma patients.**

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**Key words:** immunotherapy; melanoma; tumor vaccine; cytotoxic T lymphocyte; cytotoxic T-lymphocyte epitope

Dozens of peptide epitopes for cytotoxic T lymphocytes (CTLs) have been identified from human melanoma. A current challenge is to determine the immunogenicity of these peptides *in vivo*. Several HLA-A2–restricted peptides from melanocytic differentiation proteins (MDPs) have been evaluated in vaccine trials, but little evaluation has been done on the immunogenicity of MDP-derived peptides restricted by other common MHC molecules. We have identified peptide epitopes from the MDPs tyrosinase and gp100, restricted by the common class I MHC molecules HLA-A1 and HLA-A3. These are the HLA-A1–restricted peptide tyrosinase<sub>240–251S</sub>, with a substitution of S for C at residue 244 (DAEKSDICTDEY), and the HLA-A3–restricted gp100<sub>17–25</sub> peptide (ALLAVGATK).<sup>1,2</sup> In the present work, we describe the clinical evaluation of these peptides, in addition to new approaches for determining the immunogenicity of 2 HLA-A2–restricted peptides from tyrosinase and gp100.<sup>3,4</sup>

In peptide-based vaccine trials, immune responses have been detected inconsistently.<sup>5–8</sup> This may be due to poor vaccine immunogenicity or to inadequate immune-response monitoring. Immunogenicity depends in part on presentation by effective antigen-presenting cells. Intradermal administration of GM-CSF induces maturation of epidermal Langerhans cells (LCs), followed by their migration to draining lymph nodes.<sup>9,10</sup> In a murine model, HIV peptides administered in an emulsion with Montanide ISA-51

adjuvant were most immunogenic when GM-CSF was included in the emulsion.<sup>11</sup> Thus, an attractive approach to vaccination is to administer melanoma peptides in an emulsion with GM-CSF-in-adjuvant with the intent of presenting the peptides on LCs that are maturing *in situ*.

Occasional marked clinical regressions of melanoma have been observed after peptide vaccination,<sup>8,12–14</sup> but tumor regressions have not correlated well with T-cell responses measured in peripheral blood lymphocytes (PBLs).<sup>8,12,14</sup> This has led to a paradoxical perception that the clinical response to a vaccine may be unrelated to the immune response to that vaccine. We hypothesize, instead, that discordance between clinical and immunological responses may reflect inadequate immune monitoring. Detection of immune responses in PBLs is complicated by patterns of T-cell trafficking and by dilutional factors. Responses in lymph nodes may better reflect immunogenicity at the site of vaccination. New technology permits identification and harvest of a lymph node draining a skin site using lymphoscintigraphy with Tc<sup>99m</sup> sulfur colloid.<sup>15</sup> This procedure is used for mapping sentinel nodes draining sites of primary cutaneous melanomas,<sup>16</sup> for the purpose of detecting metastatic disease. Here, we report a novel application of this technology for identification of a node draining a cutaneous vaccination site.

A possible concern with vaccines using MDP-derived peptides is that pre-existing tolerance to differentiation proteins may cause deletion of T cells with high affinity for the MDP peptides. Thus, responding T cells may have such low affinity that they recognize only target cells pulsed with high concentrations of peptide and not tumor cells that express the relevant epitopes at lower copy numbers. When evaluating T-cell response to peptide vaccines, it is important to confirm that responding CTLs lyse human melanoma cells.

In the current report, we present data responding to 3 hypotheses: (i) the 4 HLA-A1, -A2 and -A3 restricted peptides described above are immunogenic in humans when administered in an emulsion with GM-CSF; (ii) immune responses identified in PBLs underestimate vaccine immunogenicity compared with evaluation

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of a sentinel immunized node (SIN); and (iii) CTLs responding to MDP peptides are not limited to the low-affinity type but have adequate affinity to lyse melanoma cells.

## MATERIAL AND METHODS

### Patients

Patients with stage IV melanoma with evaluable unresected metastases who were serologically typed as HLA-A1<sup>+</sup>, -A2<sup>+</sup> or -A3<sup>+</sup> and whose melanoma cells expressed the gp100 and/or tyrosinase MDPs by immunohistochemistry were studied, with informed consent and institutional review board and Food and Drug Administration (FDA) approval (under IND 7593). Patients ranged in age from 40 to 58 years. Primary tumor sites were skin of the back (2 patients), right calf (1 patient) and mucous membrane sites (2 patients, vulvar and anorectal). Sites of metastatic tumor included viscera (lung and/or liver) in 3 patients and were limited to skin and lymph nodes in 2 patients. Immunohistochemistry prior to vaccination demonstrated that metastatic tumor expressed tyrosinase in all 5 patients, expressed gp100 strongly in 3 patients (VMM115, VMM119 and VMM150) and expressed gp100 weakly in 1 patient (VMM193). HLA-A region alleles for each patient were VMM115 (A1, A3), VMM119 (A2, A68), VMM150 (A1, A68), VMM193 (A2) and VMM204 (A2, A24).

### Immunization protocol

Patients received a vaccine comprising 4 melanoma peptides [100 µg each of the HLA-A1-restricted peptide tyrosinase<sub>240-251S</sub> (DAEKSDICTDEY), the HLA-A2-restricted peptides tyrosinase<sub>368-376D</sub> (YMDGTMSQV) and gp100<sub>280-288</sub> (YLEPGPVTA) and the HLA-A3-restricted peptide gp100<sub>17-25</sub> (ALLAVGATK) and 190 µg of the HLA-DR-restricted tetanus helper peptide AQYIKANSKFIGITEL. This peptide represents peptide p2 of tetanus toxoid (residues 830-844) plus an amino-terminal alanine residue to prevent formation of pyroglutamate from the N-terminal glutamine residue. Tetanus peptide p2 is a promiscuous binder to HLA-DR molecules.<sup>17</sup> Vaccines were administered with 225 µg GM-CSF-in-Montanide ISA-51 adjuvant. GM-CSF was kindly provided by Schering-Plough (Kenilworth, NJ). Montanide ISA-51 was purchased from Seppic (Fairfield, NJ). Each patient was immunized at days 0, 7, 14, 28, 35 and 42 (weeks 0, 1, 2, 4, 5 and 6), for a total of 6 immunizations. The first 3 vaccinations were divided between 2 injection sites (primary and replicate), and the last 3 vaccinations were delivered to the primary injection site only. At each injection site, half was administered s.c. and half i.d. Patients also received out-patient IL-2 (Chiron, Emeryville, CA) daily for 6 weeks at a dose of  $3.0 \times 10^6$  IU/m<sup>2</sup> s.c. on days 7-49.<sup>18,19</sup>

### SIN resection

To provide a replicate immunization site at which a node draining that site can be tested, patients were vaccinated at 2 sites for the first 3 injections. The primary vaccination site was in an arm, and each vaccine in that arm was administered to the same skin location. The replicate immunization site was on a thigh in 4 patients and on an arm in 1 patient who had previously had bilateral inguinal node dissections. The lymph node draining these replicate immunization sites (SIN) was localized by lymphatic mapping with Tc<sup>99m</sup> sulfur colloid at approximately 1 week after the third immunization. Selective biopsy of the SIN was performed (by CLS) under local anesthesia with the intra-operative aid of a sterile hand-held gamma probe (Care Wise, Morgan Hill, CA). Patients consented to this procedure as part of the initial informed consent process for the trial and again by a separate operative consent immediately prior to the procedure. No patients refused SIN biopsy. The incision was routinely 1 inch long, and the node was removed in the out-patient clinic. There were no infections or complications at these surgical sites. Once removed, the SIN was dissociated mechanically into a single-cell suspension and cryopreserved in human AB serum and DMSO. Tissues were processed by the Tissue Procurement Facility at the University of Virginia (Charlottesville, VA).

### Testing for immunological responses

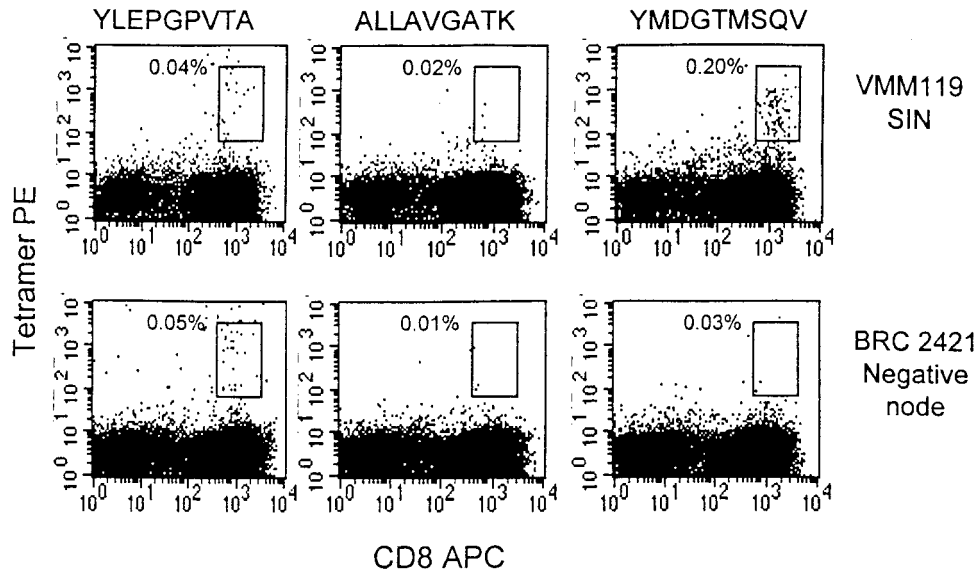
**Cell lines.** C1RA1 and C1RA3 are human EBV-transformed B-cell (EBV-B) lines that lack expression of class I MHC molecules, except that they have been transfected with the genes for human HLA-A1 and HLA-A3, respectively. These cell lines were kindly provided by Dr. P. Cresswell. T2 (ATCC, Manassas, VA) is a mutant human T/B cell hybrid that lacks the transporter associated with antigen processing (TAP) but expresses HLA-A2. K562 is a natural killer cell target. VMM5 (HLA-A2, -A11), VMM14 (HLA-A1, -A25, -B8, -B48), VMM15 (HLA-A1, -A25, -B8, -B18), VMM18 (HLA-A3, -A31/33, -B60, -C3) and VMM115 (HLA-A1, -A3) are melanoma cell lines established at the University of Virginia from tumor-involved nodes of melanoma patients. All express gp100 and tyrosinase, as shown by immunohistochemistry. DM331 and DM6 are melanoma cell lines established at Duke University and kindly provided by Drs. T. Darrow and H. Seigler. DM6 (HLA-A2) expresses gp100 and tyrosinase, and DM331 (HLA-A1, -A2) does not express either gp100 or tyrosinase. SkMel24 (HLA-A1, -A2; ATCC) is an amelanotic melanoma that is gp100-negative and tyrosinase-negative.<sup>20</sup> HLA typing was performed by microcytotoxicity assay on autologous lymphocytes (One Lambda, Canoga Park, CA).

**Peptides.** Class I MHC-associated peptides included HLA-A1-associated DAEKSDICTDEY (tyrosinase<sub>240-251S</sub>), HLA-A\*0201-associated YMDGTMSQV (tyrosinase<sub>368-376D</sub>), YLEPGPVTA (gp100<sub>280-288</sub>), KTWGQYWQV (gp100<sub>154-162</sub>), AAGIGILTV (MART-1/MelanA<sub>27-35</sub>), YLKKIKNSL (malaria CSP<sub>334-342</sub>)<sup>21</sup> and HLA-A3-associated ALLAVGATK (gp100<sub>17-25</sub>). Peptides were synthesized and purified by the Biomolecular Core Facility at the University of Virginia.

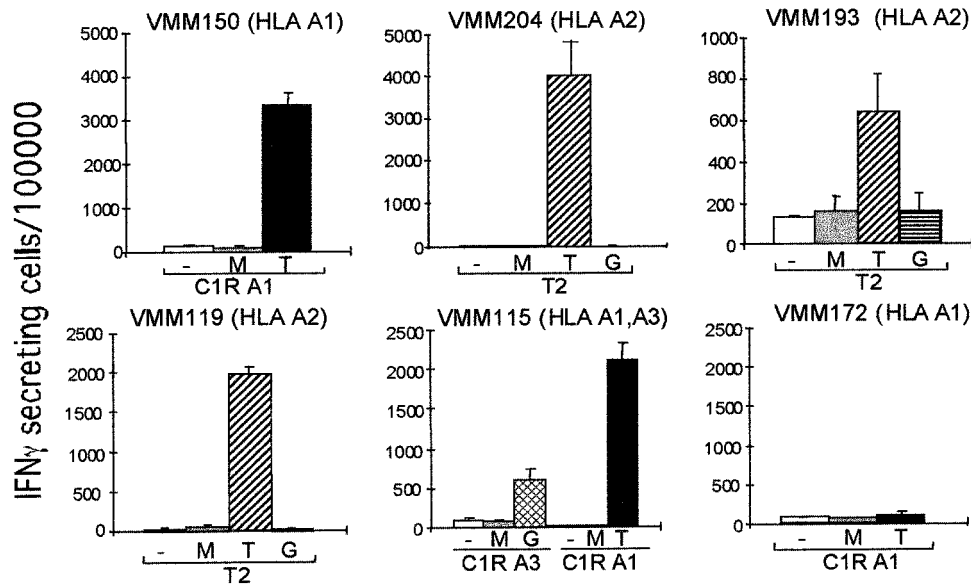
**ELISpot assay.** Lymphocytes were cultured in complete RPMI with 10% heat-inactivated human AB serum (Sigma, St. Louis, MO), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Pen-Strept; GIBCO, Grand Island, NY). They were assayed 2 weeks after a single sensitization *in vitro* with peptide. Briefly,  $2 \times 10^6$  lymphocytes/ml in complete medium were incubated with the mixture of melanoma peptides used for immunization (40 µg/ml of each) for 2 hr at 37°C, 5% CO<sub>2</sub>. Cells were pelleted, resuspended in complete medium containing IL-2 (20 U/ml) and cultured for 14 days. Complete medium was replaced as needed.

Immulon 2 flat-bottomed plates (Dynatech, Chantilly, VA) were coated with anti-IFN-γ monoclonal antibodies (MAbs) (M-700A; Endogen, Woburn, MA). Lymphocytes were mixed with equal numbers of antigen-presenting cells (APCs) alone or cells pulsed with peptide (40 µg/ml) in the first row of the plate. Serial dilutions were made such that responder cell numbers ranged from 100,000 to 5,000/well. Plates were incubated at 37°C, 5% CO<sub>2</sub> for 18 hr. After extensive washing with 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 BCIP substrate in 1% low melting agarose (Sigma). The number of blue spots corresponding to the number of cells secreting IFN-γ was calculated for each well. Each sample was tested in triplicate at each of several dilutions of lymphocytes. The number of T cells responding to the peptide was calculated as the difference between the number of cells secreting IFN-γ in response to APCs (C1RA1, C1RA3 or T2) loaded with that peptide and the highest negative control result (APCs alone or loaded with irrelevant malaria peptide).

**IFN-γ release assay based on intracellular staining.** CTLs were incubated with stimulator cells at a responder:stimulator ratio of 2:1 ( $2 \times 10^5$  CTLs and  $1 \times 10^5$  target cells per well in a 96-well plate). We determined the number of IFN-γ-releasing cells using a kit for intracellular cytokine staining (Pharmingen). FACS analysis using FACScan with CELLQuest software (Becton Dickinson, San Jose, CA) was performed for enumeration of CD8<sup>+</sup> IFN-γ<sup>+</sup> cells.



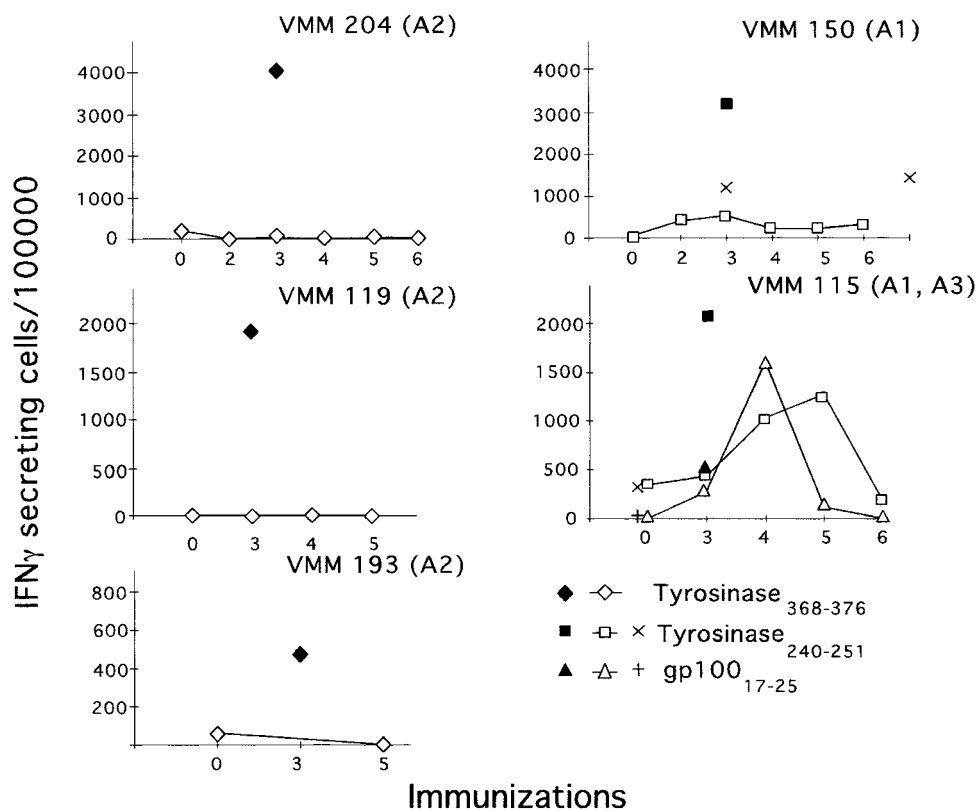
**FIGURE 1** – Cryopreserved cells from the SIN of patient VMM119 and from a tumor-negative lymph node (BRC2421, HLA-A2) of a breast-cancer patient were thawed and enriched for CD8<sup>+</sup> cells. Cells were triple-stained with the tetramers, anti-CD8 antibody and anti-TCR- $\alpha/\beta$ -1 antibody. We used FACScan with CELLQuest software to enumerate tetramer<sup>+</sup>CD8<sup>+</sup> cells. The population of tetramer<sup>+</sup>CD8<sup>+</sup> cells is designated with a small rectangle on each plot. The percentage of CD8<sup>+</sup> cells that are tetramer<sup>+</sup> is shown. The difference between SIN and negative node binding corresponds to 0.17% of CD8<sup>+</sup> lymphocytes bearing TCR specific for YMDGTMSQV peptide and the absence of CD8<sup>+</sup> cells with YLEPGPVTA-specific TCR.



**FIGURE 2** – Mononuclear cells from the SIN of 5 patients were cryopreserved at the time of resection. Cells were thawed and sensitized with the mixture of 4 immunizing peptides, cultured for 14 days, then evaluated in an ELISPOT assay for reactivity to the immunizing peptides pulsed on C1RA1, T2 or C1RA3. Negative controls included C1RA1, T2 and C1RA3 alone or pulsed with the irrelevant peptide YLKKIKNSL (CSP<sub>334-342</sub>). Error bars represent 1 SD. Mononuclear cells from the SIN of patient VMM172, receiving a different vaccine, were similarly stimulated and evaluated. x axis labels represent target cells (C1RA1, T2 or C1RA3) and the peptide pulsed on that target cell (-, no peptide; M, irrelevant malaria peptide; G, gp100 peptide; T, tyrosinase peptide). □ (-)C1RA1, C1RA3, or T2 corresponding to patient HLA type; ▨ (M)C1RA1, C1RA3 or T2 + Malaria CSP<sub>334-342</sub>; ■ (T) C1RA1+Tyrosinase<sub>240-251</sub>; ▩ (T) T2 + Tyrosinase<sub>368-376</sub>; ▤ (G) C1RA3 + gp100<sub>17-25</sub>; ▥ (G) T2+ gp100<sub>280-288</sub>.

*Peptide-MHC tetramer staining.* HLA-A\*0201/YMDGTMSQV, HLA-A\*0201/YLEPGPVTA and HLA-A\*0301/ALLAVGATK tetramers were provided by the NIAID MHC Tetramer Core Facility (Atlanta, GA). The specificity of tetramers was confirmed by titration using polyclonal CTL lines. A PBL sample of a normal donor and a tumor-free lymph node sample from a breast-cancer patient were used as negative controls. Patient

samples were evaluated after enrichment for CD8<sup>+</sup> cells using negative selection (Stem Cell Technologies, Vancouver, Canada). Cells were stained with the tetramers (1:100 dilution; phycoerythrin), anti-CD8 antibody (APC, MHCD 0804; Caltag) and anti-TCR- $\alpha/\beta$ -1 antibody (FITC, 347773; Becton Dickinson). We used FACScan with CELLQuest software to enumerate tetramer<sup>+</sup>CD8<sup>+</sup> cells.



**FIGURE 3** – Patient lymphocytes from PBLs, SINs, TINs or metastatic tumor deposits (TILs) were cryopreserved at the time of collection. They were thawed, sensitized once *in vitro* with the vaccinating peptides, washed, cultured for 14 days with IL-2 and evaluated by ELISpot assay. The number of T cells responding to the peptide was calculated as the difference between the number of cells secreting IFN- $\gamma$  in response to APCs (C1RA1, C1RA3 or T2) loaded with that peptide and the highest negative control result. Negative controls included lymphocytes alone, lymphocytes + APC alone and lymphocytes + APC + irrelevant peptide. Results were obtained with lymphocytes prior to vaccination (0), 1 week after the second (2), 2 weeks after the third (3), 1 week after the fourth (4), 1 week after the fifth (5) or 1 month after the sixth (6) vaccine. The SIN was harvested 8 to 10 days after vaccine 3. Responses to tyrosinase<sub>368–376D</sub> pulsed on T2 cells are shown for PBLs (open diamonds) and SIN (solid diamonds) from patients VMM204, VMM119 and VMM193. Responses by patient VMM150 are shown to tyrosinase<sub>240–251S</sub> pulsed on C1RA1 in PBLs (open squares) and in the SIN (solid squares). The 2 data points represented by an X are from lymphocytes infiltrating cutaneous metastatic tumor deposits, 1 harvested after the third vaccine and the other 8 weeks after the sixth vaccine. Also shown are responses by patient VMM115 to tyrosinase<sub>240–251S</sub> pulsed on C1RA1, in the PBLs (open squares) or in the SIN (solid squares) and to gp100<sub>17–25</sub> pulsed on C1RA3 cells, in PBLs (open triangles) or in the SIN (solid triangles). The isolated data points before vaccination represent responses to tyrosinase<sub>240–251S</sub> (X) and gp100<sub>17–25</sub> (+) measured in lymphocytes from a tumor-involved node (TIN) resected prior to vaccination.

**TABLE I** – LEVEL OF PEPTIDE-SPECIFIC CTL IN THE DIFFERENT COMPARTMENTS IN THE COURSE OF IMMUNIZATION

Patient	HLA type	Immunizing peptide	Peptide-specific CTL/10 <sup>5</sup> (SD)							SIN	TIL
			PBL, week								
			0	2	3	4	5	6			
VMM204	HLA-A2	YLEPGPVTA	0	0	6 (11)	1 (3)	20 (14)	2 (5)	0	NA	
		YMDGTMSQV	195 (107)	2 (33)	18 (2)	3 (7)	34 (28)	23 (9)	4,040 (851)	NA	
VMM119	HLA-A2	YLEPGPVTA	0	NT	0	0	0	NT	0	NA	
		YMDGTMSQV	0	NT	0	0	0	NT	1,902 (96)	NA	
VMM193	HLA-A2	YLEPGPVTA	72 (766)	NT	NT	NT	62 (82)	NT	0	NA	
		YMDGTMSQV	72 (845)	NT	NT	NT	27 (104)	NT	477 (185)	NA	
VMM150	HLA-A1	DAEKSDICTDEY	58 (100)	404 (190)	530 (138)	221 (117)	220 (149)	311 (192)	3,258 (272)	1,217 (243) <sup>2</sup> 1,433 (174) <sup>3</sup>	
										322 (71) <sup>1</sup>	
VMM115	HLA-A1	DAEKSDICTDEY	338 (225)	NT	432 (139)	1,029 (270)	1,272 (401)	189 (109)	2,093 (221)	322 (71) <sup>1</sup>	
		HLA-A3	ALLAVGATK	3 (29)	NT	273 (188)	1,602 (352)	111 (29)	0 (145)	512 (145)	0 <sup>1</sup>

NA, sample is not available; NT, not tested; 0, negative values, which are below background. <sup>1</sup>Before vaccination. <sup>2</sup>After 3 vaccinations. <sup>3</sup>After 6 vaccinations.

**T-cell expansion.** For T-cell expansion, we used a protocol modified from Crossland *et al.*<sup>22</sup> T cells were expanded *in vitro* without additional antigen stimulation by culturing with anti-CD3 antibody (OKT3, 10 ng/ml; Pharmingen) in the presence of several groups of feeder cells. T cells (n = 50,000) were co-cultured in a T-25 flask with 5 × 10<sup>6</sup> irradiated allogeneic EBV-B cells and 25 × 10<sup>6</sup> irradiated mixed PBLs for 14 days in complete medium with 25 U/ml IL-2. This usually yielded 10 to 50 × 10<sup>6</sup> T cells with specificity comparable to that of the original T cells.

**Cytotoxicity assays.** Cell-mediated lysis of target cells was determined using a standard 4 hr <sup>51</sup>Cr-release assay. Briefly, <sup>51</sup>Cr-labeled target cells were plated at 1 to 2 × 10<sup>3</sup> cells/well in triplicate on 96-well V-bottomed plates (Costar, Cambridge, MA) with the indicated ratio of target to effector cells in a final volume of 200 μl. Wells containing either culture medium or 1 M HCl in place of the effector cells served as spontaneous and maximum <sup>51</sup>Cr-release controls, respectively.

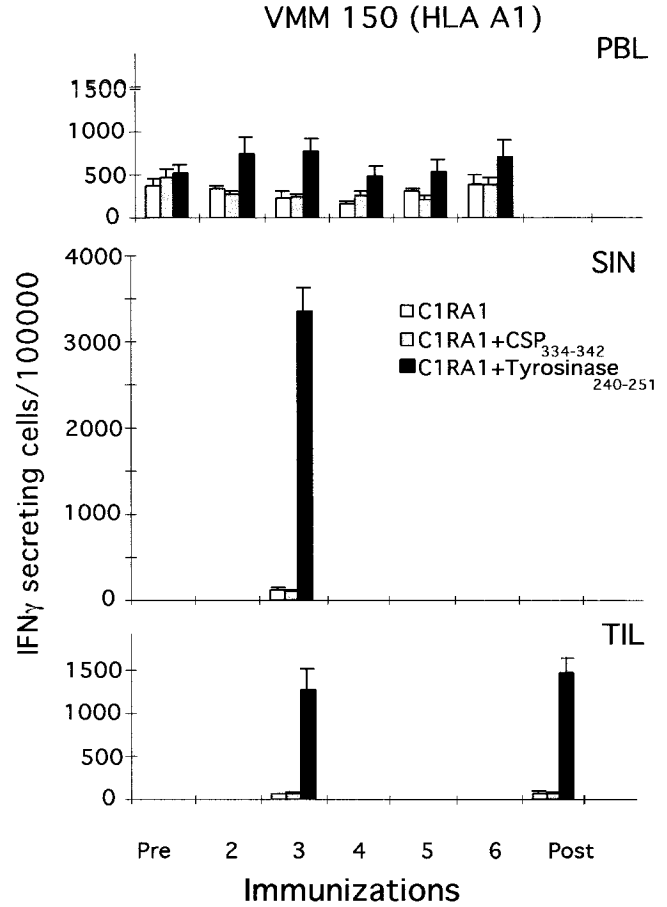
RESULTS

Peptide-specific responses in the SIN

We tested immune responses in 5 patients vaccinated with 4 melanoma peptides in an emulsion of GM-CSF-in-adjuvant, followed by systemic, low-dose IL-2. These peptides included DAEKSDICTDEY, YMDGTMSQV, YLEPGPVTA and ALLAVGATK. The vaccine also contained a tetanus helper peptide, with the intent of stimulating a simultaneous T-helper response; but the focus of these studies was on the CTL response. We collected the lymph node (SIN) draining a vaccine site approximately 1 week (8–10 days) after the third vaccination and evaluated the immune response by ELISpot assays, IFN-γ release assays, peptide-MHC tetramer staining and cytotoxicity assays.

T cells specific for melanoma peptides used for immunization could be detected in SIN *ex vivo* by tetramer staining as well as by ELISpot assay. Results of MHC-tetramer staining for the uncultured VMM119 SIN sample are shown in Figure 1, where the difference between SIN and negative node binding corresponds to 0.17% of CD8<sup>+</sup> lymphocytes bearing TCR specific for YMDGTMSQV peptide and the absence of CD8<sup>+</sup> cells with YLEPGPVTA-specific T-cell receptor (TCR). ELISpot assay of the same SIN sample *ex vivo* gave similar results, with 0.14% of CD8<sup>+</sup> cells secreting IFN-γ in response to YMDGTMSQV peptide and no response to YLEPGPVTA peptide (data not shown). We also found by ELISpot *ex vivo* that 0.24% of CD8<sup>+</sup> cells in SIN of patient VMM204 were reactive to YMDGTMSQV and none to YLEPGPVTA (data not shown). These results confirm that peptide-specific CTLs induced in SIN are not anergic and suggest that the majority of them can produce IFN-γ in response to antigen.

To further investigate the ability of lymphocytes to recognize antigen and to proliferate in response to that antigen, we tested SIN lymphocytes by ELISpot assay at 14 days after sensitization *in vitro* with the 4 melanoma peptides used for vaccination. CTLs in the SIN responded to at least 1 of the immunizing peptides in all vaccinated patients (Fig. 2). Patients VMM119, VMM193 and VMM204 demonstrated prominent responses to the HLA-A2–restricted tyrosinase peptide YMDGTMSQV, while responses to the HLA-A2–restricted gp100 peptide YLEPGPVTA were not observed (Fig. 2). In a parallel experiment, the SIN of VMM119 was also evaluated after sensitization *in vitro* with only the YLEPGPVTA peptide, and no reactivity was observed by ELISpot (data not shown). Patient VMM150 demonstrated a strong response to the HLA-A1–restricted peptide DAEKSDICTDEY in the SIN (Fig. 2), and patient VMM115 exhibited a response both to the HLA-A1–restricted tyrosinase peptide DAEKSDICTDEY and to the HLA-A3–restricted gp100 peptide ALLAVGATK (Fig. 2). As a negative control, SINS of a patient vaccinated in a different manner (VMM172) were evaluated. Despite the same *in vitro* stimulation and culture, we could not detect CTL responses to the relevant immunizing peptides in SINS of this patient (Fig. 2). This negative response was obtained in a patient vaccinated s.c.

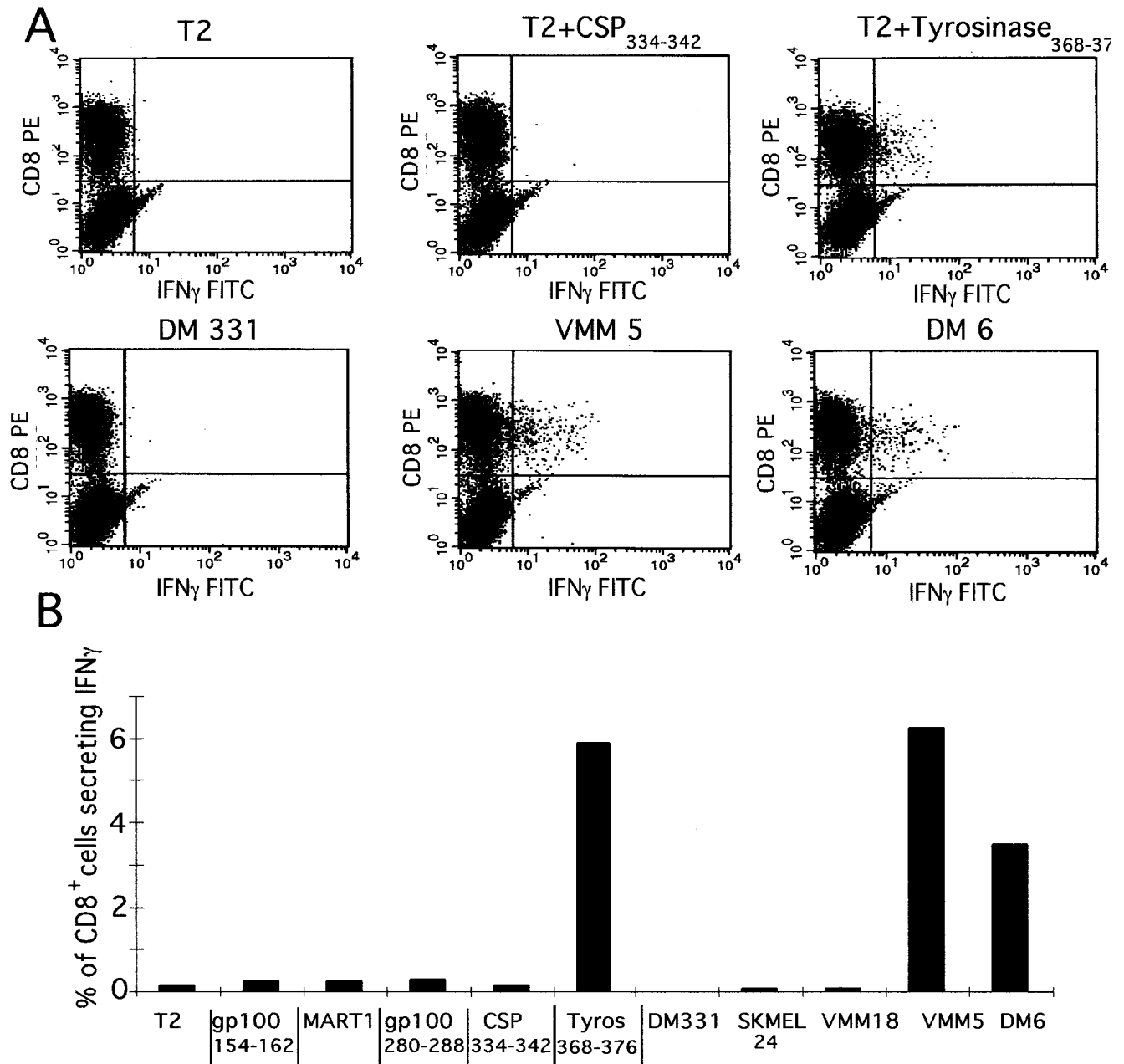


**FIGURE 4**—Mononuclear cells from PBLs of 2 metastatic tumor deposits (TILs) and from the SIN of patient VMM150 were cryopreserved at the time of collection. Cells were thawed and sensitized with the mixture of the 4 immunizing peptides in parallel cultures. After 14 days, they were evaluated in an ELISpot assay for reactivity to the immunizing peptide tyrosinase<sub>240-251</sub> pulsed on C1RA1. Negative controls included C1RA1 alone or pulsed with the irrelevant malaria peptide CSP<sub>334-342</sub>.

with immature autologous dendritic cells pulsed with the same 4 melanoma peptides. Results with this patient served as a negative control, demonstrating that prominent immune responses seen in the SIN (Fig. 2) can be attributed to the *in vivo* effects of immunization rather than to *in vitro* stimulation. The specificity in this assay system is further demonstrated by the failure of gp100<sub>280-288</sub> (YLEPGPVTA) to stimulate a detectable response (Fig. 2). Thus, peptides YMDGTMSQV, ALLAVGATK and DAEKSDICTDEY were immunogenic in melanoma patients, as measured at the SIN.

CTL responses in PBLs under-estimate immunogenicity

To compare the immune response at the immunized node to the systemic immune response in PBLs, we collected blood after each vaccination and tested samples in parallel with the SIN. In only 2/5 patients did we detect CTLs specific for vaccinating peptides in peripheral blood (Fig. 3, Table I). For patient VMM150, immune reactivity to DAEKSDICTDEY in PBLs was evident after the second vaccination, persisted for up to 6 weeks after the last vaccination and was <20% the magnitude of the response in the SIN (Fig. 3). The most prominent reactivity to immunizing peptides in PBLs was detected in patient VMM115, but it was transient and fell to undetectable levels 6 weeks after completion of the vaccination protocol (Fig. 3). In these 5 patients, evaluation of PBLs alone markedly under-estimated the T-cell response compared to evaluation of the SIN.



**FIGURE 5** – VMM204 T cells from the SIN were sensitized once *in vitro* with the 4 immunizing peptides and then expanded with anti-CD3 antibody. They were added to T2 cells pulsed with 1 of 5 peptides or to 1 of 5 different tumor cells. The number of CD8<sup>+</sup> cells secreting IFN- $\gamma$  was determined by FACS after staining for CD8 and intracellular staining for IFN- $\gamma$ . (a) Raw FACS data are shown for 6 different stimulators. (b) Results represent the percentage of CD8<sup>+</sup> cells secreting IFN- $\gamma$  as measured in this assay.

#### Baseline immunity in tumor-involved nodes (TINs) before immunization

We had access to TINs resected prior to vaccination in 2 patients. Lymphocytes from a TIN of patient VMM115 were sensitized with 4 melanoma peptides *in vitro* and cultured in parallel with the SIN and PBLs, then assayed by ELISpot. CTLs reactive to the DAEKSDICTDEY peptide were detected in that node at a level nearly identical to that found in PBLs prior to vaccination (Fig. 3). Similarly, CTLs reactive to ALLAVGATK were not detected in the TIN and were equally undetectable in PBLs (Fig. 3). For patient VMM119, TIN lymphocytes were cultured *in vitro* with autologous tumor cells and evaluated at 14 days by ELISpot: no reactivity to YMDGTMSQV and YLEPGPVTA peptides was observed prior to vaccination (data not shown). Thus,

in both of these patients, reactivity to tumor antigens in PBLs prior to vaccination correlated well with pre-vaccination reactivity in lymph nodes exposed to tumor, serving as a useful baseline measure of reactivity to defined antigens prior to vaccination.

#### Peptide-specific immune responses detected in distant metastases

It has been postulated that the number of CTLs in PBLs may be affected by trafficking of tumor-reactive lymphocytes from PBLs to sites of metastasis.<sup>23</sup> While the present study was not formally designed to test that hypothesis, we did evaluate peptide-specific reactivity of CTLs isolated from metastatic tumor deposits during vaccination. Patient VMM150 had multiple cutaneous metastases, most of which regressed markedly during vaccination. One metastasis was removed the same day the SIN was harvested. Another

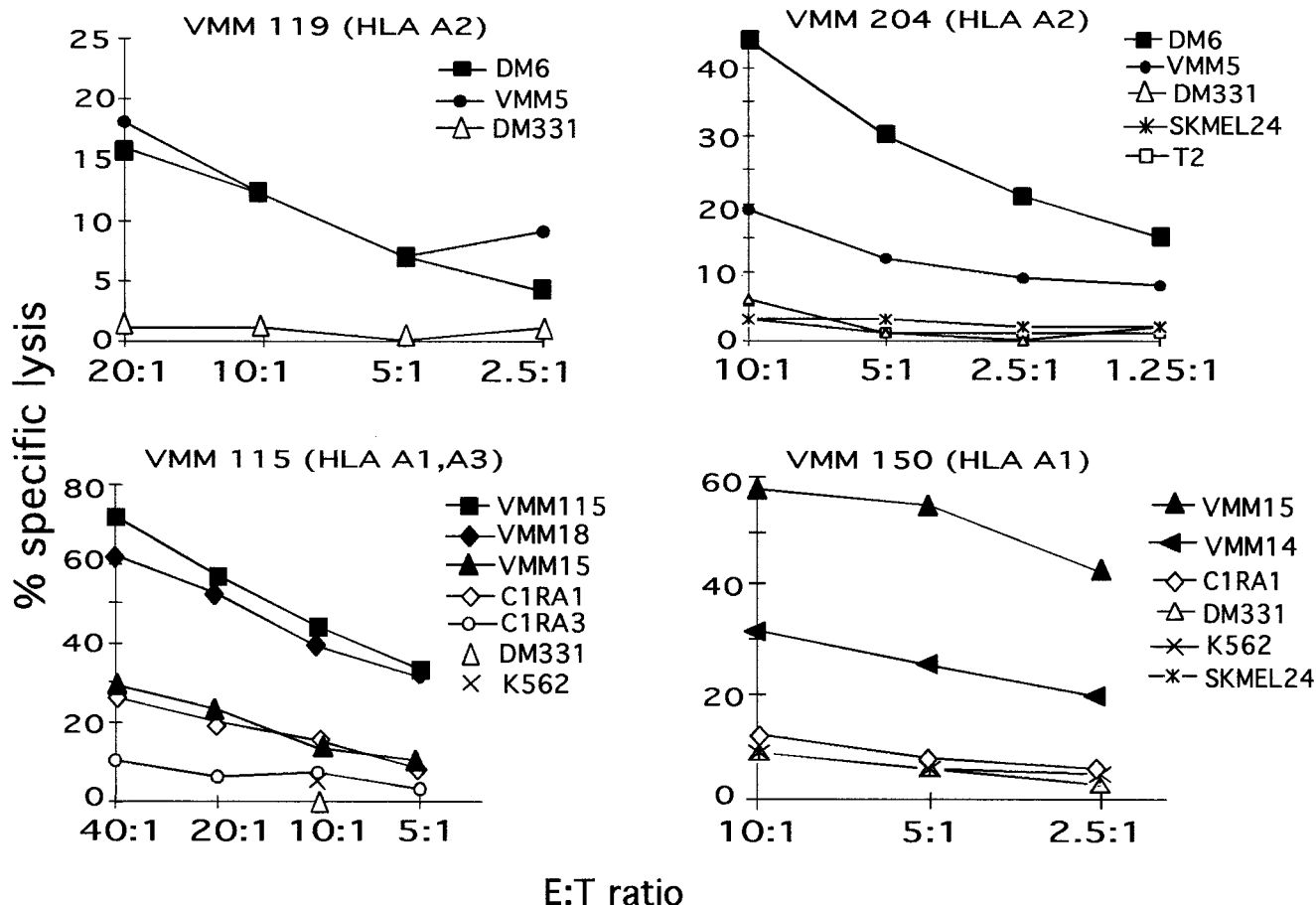
was removed 8 weeks after the end of the vaccination protocol. Both tumors were tyrosinase-positive, but the latter lesion had decreased tyrosinase expression, by immunohistochemistry and Western analysis of freshly cryopreserved tumor cells (data not shown). In both metastases, there were CTLs reactive to the DAEKSDICTDEY peptide (Figs. 3, 4). After 3 vaccinations, maximal reactivity was detected in the SIN, intermediate reactivity in the metastatic tumor and the lowest reactivity in PBLs (Fig. 3). Our results are consistent with the hypothesis that CTLs induced by vaccination may accumulate at sites of metastatic tumor and may thus be depleted from PBLs. These data further demonstrate that the CTL response to vaccinating peptide differs markedly in different lymphoid populations within the same individual.

*CTLs induced by peptide vaccine recognize and lyse melanoma cells*

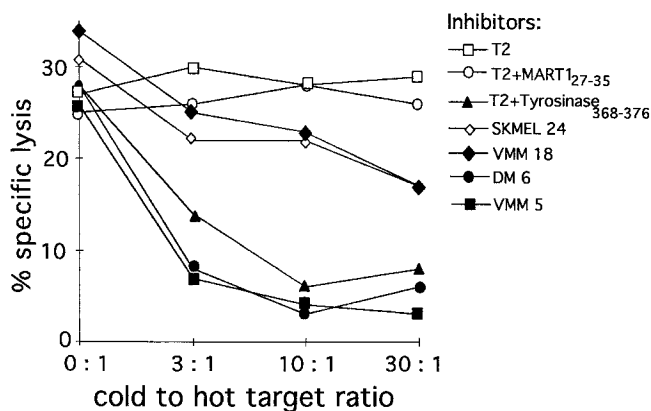
To determine whether CTLs from vaccinated patients can recognize tumor cells naturally expressing the peptide epitopes, lymphocytes from the SIN were evaluated for IFN- $\gamma$  production in response to peptides pulsed onto appropriate APCs or to tumor cells naturally expressing those peptides. Representative data are shown for VMM204, with significant IFN- $\gamma$  release upon stimulation with tyrosinase peptide-pulsed T2 cells, as well as with 2

HLA-A2<sup>+</sup>tyrosinase<sup>+</sup> melanoma cell lines, VMM5 and DM6 (Fig. 5). Most of the peptide-reactive CD8<sup>+</sup> cells expanded from the SIN of VMM204 were of high enough affinity to recognize tumor cells.

Furthermore, we assessed the cytolytic activity of peptide-reactive CTLs from the SIN. T cells expanded from these SINs lysed APCs pulsed with the peptides YMDGTMSQV, ALLAVGATK and DAEKSDICTDEY (data not shown). T cells expanded from the SIN after 1 or 2 rounds of sensitization with peptide were able to lyse melanoma cells (Fig. 6). T cells from the SIN of VMM119 and VMM204 lysed HLA-A2<sup>+</sup>tyrosinase<sup>+</sup> melanomas DM6 and VMM5 without lysing the HLA-A1<sup>+</sup>/-A2<sup>+</sup>tyrosinase<sup>-</sup> melanomas DM331 and SkMel24. VMM115 SIN T cells lysed the HLA-A3<sup>+</sup> melanoma VMM18 and the HLA-A1<sup>+</sup>A3<sup>+</sup> autologous tumor line VMM115. There was low-level lysis also of the A1<sup>+</sup>tyrosinase<sup>+</sup> melanoma VMM15 but no lysis of the A1<sup>+</sup>tyrosinase<sup>-</sup> melanoma DM331 (Fig. 6). VMM150 CTLs recognized and lysed VMM15 and VMM14 tumor cells, both of which are A1<sup>+</sup>tyrosinase<sup>+</sup> melanomas, but not DM331, SkMel24, K562 or C1RA1 (Fig. 6). In another assay, VMM150 CTLs also lysed autologous VMM150 tumor cells (data not shown). Collectively, these data establish that peptides YMDGTMSQV, DAEKSDICTDEY and ALLAVGATK



**FIGURE 6** – T cells from the SIN of patients VMM119 and VMM204 were stimulated once *in vitro* with the 4 immunizing melanoma peptides at 40  $\mu$ g/ml, then expanded with anti-CD3 antibodies at 14 days. T cells from VMM115 and VMM150 were stimulated once with the peptides, then restimulated once with the peptides pulsed on autologous irradiated PBLs prior to anti-CD3 expansion. These cultured T cells were then assayed for cytotoxicity against several melanoma and non-melanoma target cells in 4 hr Cr-release assays. DM6 (solid squares) and VMM5 (solid circles) are HLA-A2<sup>+</sup>tyrosinase<sup>+</sup>gp100<sup>+</sup>. VMM15 (solid upward triangles) and VMM14 (solid left triangles) are HLA-A1<sup>+</sup>tyrosinase<sup>+</sup>. VMM18 (solid diamonds) is HLA-A3<sup>+</sup>tyrosinase<sup>+</sup>gp100<sup>+</sup>. VMM115 (solid squares) is an autologous tumor line from the VMM115 nodal metastases, which is HLA-A1<sup>+</sup>HLA-A3<sup>+</sup>tyrosinase<sup>+</sup>gp100<sup>+</sup>. Negative control melanoma cells included DM331 (open triangles) and SkMel24 (asterisks), which are both HLA-A1<sup>+</sup>A2<sup>+</sup> and tyrosinase<sup>-</sup>gp100<sup>-</sup>. Non-melanoma targets included C1R-A1 (open diamonds), C1R-A3 (open circles), T2 (open squares) and K562 (X). All are negative for tyrosinase and gp100 and express HLA-A1, -A3, -A2 or no class I MHC, respectively.



**FIGURE 7**—Chromium-labeled (hot) DM6 melanoma cells were incubated with unlabeled (cold) target cells at cold:hot ratios of 0:1 to 30:1 for 1 hr prior to addition of cultured VMM204 SIN T cells at a 20:1 E:T ratio.  $^{51}\text{Cr}$  release was measured at 4 hr to assess percent lysis of DM6 tumor cells. For the T2+tyrosinase<sub>368-376D</sub> and T2+MART1<sub>27-35</sub>, T2 cells were pulsed with these peptides at 10  $\mu\text{g}/\text{ml}$ , then washed prior to adding as cold targets. DM6 lysis was inhibited only by T2 loaded with tyrosinase<sub>368-376D</sub> peptide and by melanoma cells expressing tyrosinase and HLA-A2, demonstrating that cytolytic activity of VMM204 CTLs is associated with T cells specific for YMDGTMSQV (tyrosinase<sub>368-376D</sub>) peptide.

activate CD8<sup>+</sup> T cells that recognize melanoma tumor cells which naturally express gp100 and/or tyrosinase.

We assumed that the tumor cell lysis observed above was mediated by recognition of the immunizing peptides. To determine this, T cells from VMM204 SIN were evaluated in a cold-target inhibition assay (Fig. 7). Lysis of DM6 tumor (A2<sup>+</sup>tyrosinase<sup>+</sup> melanoma) was completely inhibited by cold DM6, cold VMM5 (another A2<sup>+</sup> tyrosinase<sup>+</sup> melanoma) and cold T2 cells pulsed with YMDGTMSQV peptide but not by T2 alone, T2 pulsed with MART-1 peptide or irrelevant tumor cells SkMel24 and VMM18. Thus, recognition of melanoma cells by these CTLs is mediated by their reactivity to peptide YMDGTMSQV. Similarly, cold-target inhibition studies with VMM150 CTLs demonstrated that lysis of VMM15 tumor is blocked by cold C1RA1 pulsed with DAEKSDICTDEY (data not shown).

#### DISCUSSION

Our results show that at least 3 of the 4 peptides used in the multiple peptide vaccine preparation are immunogenic *in vivo* in humans. These include the HLA-A1 peptide DAEKSDICTDEY, the HLA-A2 peptide YMDGTMSQV and the HLA-A3 peptide ALLAVGATK. This extends the range of patients who may benefit from vaccine strategies using peptide antigens.

The HLA-A2-associated tyrosinase peptide YMDGTMSQV had been reported to stimulate CTL responses in PBLs in a minority of patients,<sup>24</sup> but the present data are more encouraging as a response was observed in all 3 HLA-A2 patients. The gp100<sub>17-25</sub> peptide ALLAVGATK is naturally processed and presented in association with HLA-A3.<sup>4</sup> Reactivity to it has been identified in the blood of melanoma patients and in the TIN of a long-term survivor of melanoma.<sup>25</sup> However, immunogenicity of the synthetic peptide had not been previously evaluated *in vivo*. In our study, CTLs reactive to this peptide were developed both in the SIN and in the PBLs of patient VMM115 after vaccination but could not be detected in a TIN or PBLs prior to vaccination.

The HLA-A1-associated tyrosinase peptide DAEKSDICTDEY was originally identified as a target for tyrosinase-reactive CTLs in our laboratory, but neither it nor its corresponding nonamer (KCDICTDEY) or decamer (EKCDICTDEY) was definitively identified among peptides naturally processed and presented in association with HLA-A1.<sup>1</sup> Furthermore, the cysteine

residue near the N terminus was rapidly modified in biological solutions, so it was necessary to replace it with a serine residue (DAEKSDICTDEY). This modification augmented peptide immune reactivity *in vitro*.<sup>1</sup> The peptide was immunogenic for both HLA-A1<sup>+</sup> patients in our study, with T-cell responses in the SIN and an increase in reactivity in PBLs during vaccination. However, we were concerned that this peptide, because of its modification, might induce T cells that were not cross-reactive with the naturally processed peptide. The encouraging finding is that CTLs reactive to DAEKSDICTDEY in the SIN were capable of lysing VMM15 melanoma cells, which naturally express tyrosinase and HLA-A1 (Fig. 6). Thus, these data confirm that the modified dodecamer peptide DAEKSDICTDEY induces CTLs *in vivo* that are reactive to a naturally processed tyrosinase peptide on melanoma cells.

The HLA-A2-associated gp100<sub>280-288</sub> peptide YLEPGPVTA failed to generate CTL responses in the 3 HLA-A2 patients in our study. The poor immunogenicity of this peptide may be due to intrinsically weak immunogenicity, possibly caused by low affinity for the HLA-A2 molecule.<sup>3</sup> Alternatively, it could be due to competition for binding to HLA-A2 molecules in the presence of the high-affinity peptide YMDGTMSQV. However, our *in vitro* data suggest that competition between these peptides is not adequate to explain non-reactivity.<sup>26</sup> Regardless, the negative results with this peptide demonstrate the ability to discriminate immunogenic peptides from non-immunogenic peptides by evaluation of the responses in the SIN.

The data presented above demonstrate that immune responses to a tumor vaccine detected in PBLs markedly under-estimated the true immunogenicity as T-cell responses were much more readily identified in the SIN than in PBLs. This is consistent with prior results showing that the acute immune response to cutaneous antigen exposure occurs in the first-order draining lymph nodes and peaks approximately 1 week after antigen exposure.<sup>27,28</sup> Subsequently, responding T cells leave the node and establish systemic immunity. Numerous factors in cancer patients may affect the systemic dissemination of the immune response, persistence of the T-cell response and establishment of long-term immunity. Progress in vaccine development will require that these factors be evaluated independently.

Evaluation of the SIN offers a novel approach to defining the immunogenicity of vaccines most directly. As shown for patient VMM150, evaluation of multiple lymphoid compartments or populations over time may permit dissection of the response into immunogenicity (measured in the SIN), systemic dissemination of the immune response (measured in PBLs), trafficking of the lymphocytes to metastatic sites (TILs) and eradication of tumor cells (clinical response). It appears that all 4 events occurred in VMM150 (Fig. 4). For patient VMM115, in contrast, there is evidence of immunogenicity against the HLA-A1- and -A3-associated peptides in the SIN and of responses in PBLs; but the systemic response was transient and associated with prompt clinical progression. Rational progress in vaccine development will depend on the ability to dissect the immune response in this way, and analysis of the SIN permits sensitive and direct evaluation of vaccine immunogenicity.

Evaluation of the SIN, as we have described it, requires a surgical procedure. Thus, it cannot easily be repeated in the same patient over time. Also, it will be difficult to include as a part of large multicenter trials because of the additional effort required for harvesting the node. It may be feasible later to evaluate these nodes by less invasive means, using a needle biopsy. At present, evaluation of the SIN after surgical excision coupled with evaluation of PBLs and TILs can be employed in selected studies to understand vaccine immunogenicity and to compare immune responses to 2 or more different vaccine regimens.

MDPs, including tyrosinase and gp100, are expressed naturally by normal melanocytes, and tolerance to MDP peptides develops in the normal host.<sup>29</sup> It has been presumed that high-affinity CTLs reactive to MDP peptides may be deleted by thymic selection. Thus, there has been concern that CTL responses to MDP peptide vaccines may be limited to low-affinity CTLs capable of recognizing cells pulsed with high concentrations of peptides but non-reactive to tumor cells natu-

rally expressing the same peptides at lower concentration. A significant and most encouraging finding in the present report is that peptide-reactive CTLs from SINs are capable of lysing melanoma cells. Thus, our data provide substantial evidence that CTLs with high affinity for MDP peptides could be induced in melanoma patients. CTL precursors capable of lysing tumor cells at low E:T ratios are present in adult melanoma patients with advanced disease, and they can be expanded *in vivo* by the immunization strategy described here. This finding is consistent with preliminary data from our laboratories that tolerance to MDPs is established and maintained by peripheral, rather than thymic, mechanisms (Colella, Engelhard, data not shown).

By combining an immunogenic vaccine strategy and a sensitive evaluation of immunogenicity, the peptides restricted by HLA-A1, -A2 and -A3 can induce anti-melanoma immune responses in a majority of melanoma patients. Nonetheless, there is substantial need to optimize vaccine strategies. As such optimization is realized, evaluation of immune responses to tumor vaccines should not

be limited to evaluation of responses in PBLs but should, where feasible, include evaluation of responses by lymphocytes in nodes draining the sites of vaccination. This will permit accurate assessment of immunogenicity and of the various components of the immune response to vaccination.

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#### REFERENCES

- Kittlesen DJ, Thompson LW, Gulden PH, Skipper JCA, Colella TA, Shabanowitz JA, et al. Human melanoma patients recognize an HLA-A1-restricted CTL epitope from tyrosinase containing two cysteine residues: implications for tumor vaccine development. *J Immunol* 1998;160:2099-106.
- Skipper JCA, Hendrickson RC, Gulden PH, Brichard V, Vanel A, Chen Y, et al. An HLA-A2-restricted tyrosinase antigen on melanoma cells results from posttranslational modification and suggests a novel pathway for processing of membrane proteins. *J Exp Med* 1996;183:527-34.
- Cox AL, Skipper J, Chen Y, Henderson R, Darrow TL, Shabanowitz J, et al. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. *Science* 1994;264:716-9.
- Skipper JCA, Kittlesen DJ, Hendrickson RC, Deacon DD, Harthun NL, Wagner SN, et al. Shared epitopes for HLA-A3-restricted melanoma-reactive human CTL include a naturally processed epitope from Pmel-17/gp100. *J Immunol* 1996;157:5027-33.
- Jager E, Bernhard H, Romero P, Ringhoffer M, Arand M, Karbach J, et al. Generation of cytotoxic T-cell responses with synthetic melanoma-associated peptides *in vivo*: implications for tumor vaccines with melanoma-associated antigens. *Int J Cancer* 1996;66:162-9.
- Hu X, Chakraborty NG, Sporn JR, Kurtzman SH, Ergin MT, Mukherji B. Enhancement of cytolytic T lymphocyte precursor frequency in melanoma patients following immunization with the MAGE-1 peptide loaded antigen presenting cell-based vaccine. *Cancer Res* 1996;56:2479-83.
- Wang F, Bade E, Kuniyoshi C, Spears L, Jeffery G, Marty V, et al. Phase I trial of a MART-1 peptide vaccine with incomplete Freund's adjuvant for resected high-risk melanoma. *Clin Cancer Res* 1999;5:2756-65.
- Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med* 1998;4:321-7.
- Koch F, Heuffer C, Kampgen E, Schneeweiss D, Bock G, Schuler G. Tumor necrosis factor  $\alpha$  maintains the viability of murine epidermal Langerhans cells in culture, but in contrast to granulocyte/macrophage colony stimulating factor, without inducing their functional maturation. *J Exp Med* 1990;171:159-71.
- Cumberbatch M, Dearman RJ, Kimber I. Langerhans cells require signals from both tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$  for migration. *Immunology* 1997;92:388-95.
- Ahlers JD, Dunlop N, Alling D, Nara P, Berzofsky JA. Cytokine-in-adjuvant steering of the immune response phenotype to HIV-1 vaccine constructs. *J Immunol* 1997;158:3947-58.
- Thurner B, Haendle I, Roder C, Dieckmann D, Keikavoussi P, Jonuleit H, et al. Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J Exp Med* 1999;190:1669-78.
- Nestle FO, Aljagic S, Gilliet M, Sun Y, Grabbe S, Dummer R, et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* 1998;4:328-32.
- Marchand M, van Baren N, Weynants P, Brichard V, Dreno B, Tessier MH, et al. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int J Cancer* 1999;80:219-30.
- Thompson JF, Uren RF, Shaw HM, McCarthy WH, Quinn MJ, O'Brien CJ, et al. Location of sentinel lymph nodes in patients with cutaneous melanoma: new insights into lymphatic anatomy. *J Am Coll Surg* 1999;189:195-204.
- Haddad FF, Shivers SC, Reintgen DS. Historical perspectives and future applications. *Surg Oncol Clin North Am* 1999;8:391-400.
- Panina-Bordignon P, Tan A, Termijtelen A, Demotz S, Corradin G, Lanzavecchia A. Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. *Eur J Immunol* 1989;19:2237-42.
- Farace F, Angevin E, Dietrich P-Y, Leboullaire C, Vanderplancke J, Escudier B, et al. Low-dose IL-2 treatment: activation of discrete T- and NK-cell sub-populations *in vivo*. *Int J Cancer* 1995;62:523-8.
- Stidham KR, Ricci WM, Vervaert C, Abdel-Wahab Z, Seigler HF, Darrow TL. Modulation of specific active immunization against murine melanoma using recombinant cytokines. *Surg Oncol* 1996;5:221-9.
- Slingluff CL Jr, Colella TA, Thompson L, Graham DD, Skipper JCA, Caldwell J, et al. Melanomas with concordant loss of multiple melanocytic differentiation proteins: immune escape that may be overcome by targeting unique or undefined antigens. *Cancer Immunol Immunother* 2000;48:661-72.
- Blum-Tirouanziam UCS, Habluetzel A, Valmori D, Men Y, Esposito F, Del Nero L, et al. Localization of HLA-A2.1 restricted T cell epitopes in the circumsporozoite protein of plasmodium falciparum. *J Immunol* 1995;154:3922-31.
- Crossland KD, Lee VK, Chen W, Riddell SR, Greenberg PD, Cheever MA. T cells from tumor-immune mice nonspecifically expanded *in vitro* with anti-CD3 plus IL-2 retain specific function *in vitro* and can eradicate disseminated leukemia *in vivo*. *J Immunol* 1991;146:4414-20.
- Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, et al. Impact of cytokine administration on the generation of antitumor reactivity in patients with metastatic melanoma receiving a peptide vaccine. *J Immunol* 1999;163:1690-5.
- Jager E, Ringhoffer M, Dienes HP, Arand M, Karbach J, Jager D, et al. Granulocyte-macrophage-colony-stimulating factor enhances immune responses to melanoma-associated peptides *in vivo*. *Int J Cancer* 1996;67:54-62.
- Yamshchikov G, Thompson L, Ross W, Galavotti H, Aquila W, Caldwell J, et al. Analysis of a natural immune response against tumor antigens in a melanoma survivor: lessons applicable to clinical trial evaluations. *Clin Cancer Res* (In press).
- Thompson LW, Brinckerhoff LH, Slingluff CL Jr. Competition for binding between peptides in peptide-based vaccines. *Surg Forum* 1998;49:458-60.
- Rosato A, Zambon A, Macino B, Mandruzzato S, Bronte V, Milan G, et al. Anti-L-selectin monoclonal antibody treatment in mice enhances tumor growth by preventing CTL sensitization in peripheral lymph nodes draining the tumor area. *Int J Cancer* 1996;65:847-51.
- Stephenson KR, Perry-Lalley D, Griffith KD, Shu S, Chang AE. Development of antitumor reactivity in regional draining lymph nodes from tumor-immunized and tumor-bearing murine hosts. *Surgery* 1989;105:523-8.
- Colella TA, Bullock TNJ, Russell LB, Mullins DW, Overwijk WW, Luckey CJ, et al. Self tolerance to the murine homologue of a tyrosinase derived melanoma antigen: implications for tumor immunotherapy. *J Exp Med* 2000;191:1221-32.