

gp100_{209–2M} Peptide Immunization of Human Lymphocyte Antigen-A2⁺ Stage I-III Melanoma Patients Induces Significant Increase in Antigen-Specific Effector and Long-Term Memory CD8⁺ T Cells

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ABSTRACT

Thirty-five HLA-A2⁺ patients with completely resected stage I-III melanoma were vaccinated multiple times over 6 months with a modified melanoma peptide, gp100_{209–2M}, emulsified in Montanide adjuvant. Direct *ex vivo* gp100_{209–2M} tetramer analysis of pre- and postvaccine peripheral blood mononuclear cells (PBMCs) demonstrated significant increases in the frequency of tetramer⁺ CD8⁺ T cells after immunization for 33 of 35 evaluable patients (median, 0.36%; range, 0.05–8.9%). *Ex vivo* IFN- γ cytokine flow cytometry analysis of postvaccine PBMCs after brief gp100_{209–2M} *in vitro* activation showed that for all of the patients studied tetramer⁺ CD8⁺ T cells produced IFN- γ ; however, some patients had significant numbers of tetramer⁺ IFN- γ ⁻ CD8⁺ T cells suggesting functional anergy. Additionally, 8 day gp100_{209–2M} *in vitro* stimulation (IVS) of pre- and postvaccine PBMCs resulted in significant expansion of tetramer⁺ CD8⁺ T cells from postvaccine cells for 34 patients, and these IVS tetramer⁺ CD8⁺ T cells were functionally responsive by IFN- γ cytokine flow cytometry analysis after restimulation with either native or modified gp100 peptide. However, correlated functional and phenotype

analysis of IVS-expanded postvaccine CD8⁺ T cells demonstrated the proliferation of functionally anergic gp100_{209–2M} tetramer⁺ CD8⁺ T cells in several patients and also indicated interpatient variability of gp100_{209–2M} stimulated T-cell proliferation. Flow cytometry analysis of cryopreserved postvaccine PBMCs from representative patients showed that the majority of tetramer⁺ CD8⁺ T cells (78.1 \pm 4.2%) had either an “effector” (CD45 RA⁺/CCR7⁻) or an “effector-memory” phenotype (CD45RA⁻/CCR7⁻). Notably, analysis of PBMCs collected 12–24 months after vaccine therapy demonstrated the durable presence of gp100_{209–2M}-specific memory CD8⁺ T cells with high proliferation potential. Overall, this report demonstrates that after vaccination with a MHC class I-restricted melanoma peptide, resected nonmetastatic melanoma patients can mount a significant antigen-specific CD8⁺ T-cell immune response with a functionally intact memory component. The data further support the combined use of tetramer binding and functional assays in correlated *ex vivo* and IVS settings as a standard for immunomonitoring of cancer vaccine patients.

INTRODUCTION

In recent years the *in vivo* immunogenicity of melanoma peptides has been enhanced by altering the amino acid sequence in the HLA-binding region. This strategy has produced peptides with greater binding affinity for HLA-A2 molecules that have also been more effective when used for *in vitro* stimulation (IVS) of T-cell responses from melanoma patients (1, 2). The substitution of methionine for threonine at position 2 of the native gp100_{209–217} peptide resulted in the modified peptide, gp100_{209–2M} (IMDQVPFSV), which was one of the first modified melanoma peptides tested in clinical trials (3–6). The initial gp100_{209–2M} peptide vaccine trials were conducted in patients with metastatic melanoma. Peptide-specific T-cell responses were usually detected only after extensive IVS of the postvaccine peripheral blood mononuclear cells (PBMCs) of patients, and were rarely detectable by direct *ex vivo* analysis after restimulation of cryopreserved cells with either the native or modified gp100 peptide (3–5). Thus, immunomonitoring of the early clinical trials was influenced by the fact that the patients had advanced disease and potentially higher levels of immunological anergy to peptide vaccination. Immunomonitoring was also hampered by the fact that the IFN- γ -specific ELISA used to monitor patient functional responses did not yield any information about either the phenotype(s) or the number of peptide-specific T cells.

Clinical studies have demonstrated the potential value of sensitive, quantitative functional assays such as cytokine flow cytometry (CFC) and ELISPOT analysis combined with antigen-specific, MHC-I-restricted tetramer analysis to measure

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peptide-specific CD8⁺ T-cell immune responses *in vitro*. The value of this strategy was demonstrated recently in gp100_{209–2M} vaccination studies of metastatic melanoma patients where the combined use of tetramer and CFC(IFN- γ) analysis demonstrated that only ~50% of circulating gp100_{209–2M} tetramer⁺ CD8⁺ T cells in vaccinated patients produced IFN- γ after brief *in vitro* activation with native or modified gp100 peptide (7). Thus, this type of *ex vivo* analysis can produce more complete information on the functional characteristics of circulating tumor antigen-specific CD8⁺ T cells. However, the presence of circulating functionally quiescent tumor antigen-specific T cells may not necessarily reflect a state of terminal functional anergy. Thus, recent work has also demonstrated that limited cognate-gp100_{209–2M} peptide-directed IVS of PBMCs from gp100_{209–2M}-vaccinated metastatic melanoma patients increased the mean frequency of CFC(IFN- γ)⁺ cells from 17% (by direct *ex vivo* analysis) to 84% of all of the gp100_{209–2M} tetramer⁺ CD8⁺ T cells, suggesting that circulating tetramer⁺ CD8⁺ T cells may be similarly responsive to peptide stimulation *in vivo* (8). The utility of such combined phenotype and functional analysis of IVS-expanded CD8⁺ T cells is best realized only when pre-existing “baseline” *ex vivo* postvaccine phenotype and functional data are also available for individual patients. Comparison of *ex vivo* “baseline” tetramer and functional profiles to the IVS responses for each patient can reveal important interpatient differences in *in vitro* proliferation and functional potential, which may be indicative of differences in the strength of the immune response of individual patients to therapeutic melanoma peptide vaccination.

We described previously the results of direct *ex vivo* gp100_{209–2M} tetramer analysis of pre- and postvaccine PBMCs from 29 stage I-III melanoma patients vaccinated with gp100_{209–2M} peptide emulsified in Montanide ISA 51 (9). This preliminary report demonstrated that 28 of 29 patients showed a significant increase in tetramer⁺ CD8⁺ T cells after vaccination (median, 0.34%). Herein we present a comprehensive summary of the *ex vivo* gp100_{209–2M} tetramer analysis of all 35 of the evaluable patients in the clinical trial. Additional experiments were performed to examine interpatient variability of cognate-peptide-specific proliferation and functional responses of CD8⁺ T cells after 8-day gp100_{209–2M} directed IVS of postvaccine PBMCs. Postvaccine gp100_{209–2M} tetramer⁺ CD8⁺ T cells were also interrogated using multiparameter flow cytometry to characterize the phenotype distribution of effector (CD45RA⁺/CCR7⁻), effector-memory (CD45RA⁻/CCR7⁻), and central-memory (CD45RA⁻/CCR7⁺) T cells. In addition to the detailed phenotype and functional analysis of postvaccine cells, PBMCs from long-term (12–24 months after vaccine therapy) blood collections were examined for the durable presence of gp100_{209–2M}-specific CD8⁺ T cells. These studies were performed to determine whether immunization with a MHC class I restricted peptide in Montanide and in the apparent absence of MHC class II restricted help could result in an antigen-specific CD8⁺ memory T-cell response. This study confirms the importance of the integrated use of both T-cell receptor (TCR)-specificity assays (tetramer analysis) and antigen-specific functional assays such as CFC(IFN- γ) for accurate assessment of the immune response in vaccinated cancer patients. The data also support the combined use of direct *ex vivo* analysis of cryopre-

served PBMCs followed by limited cognate-antigen IVS and reanalysis of patient cells to determine more accurately the proliferative and functional potential of circulating tumor antigen-specific T cells.

MATERIALS AND METHODS

Clinical Protocol. HLA-A2⁺ patients with completely resected stage I-III melanoma were vaccinated with the gp100_{209–2M} peptide as described elsewhere (9). Patients were randomized to receive s.c. injections of gp100_{209–2M} peptide and a control “reporter” peptide, human papillomavirus (HPV) 16E7_{12–20}, every 2 weeks (13 vaccinations) or every 3 weeks (9 vaccinations) for 6 months. The HLA-A2-restricted gp100_{209–2M} (NSC #683472) and HPV16E7_{12–20} (NSC #673925) peptides were provided by the Cancer Therapy Evaluation Program under a National Cancer Institute Investigational New Drug Application (BB6123). Peptides were administered s.c. after emulsification in the Montanide ISA 51 (NSC #675756), manufactured by Seppic, Inc. and provided by Cancer Therapy Evaluation Program. A pre- and postvaccine therapy leukapheresis was performed on each patient; PBMCs were harvested using standard Ficoll-Hypaque density gradient centrifuge procedures and frozen in liquid nitrogen at 50×10^6 – 100×10^6 per ampule in 90% fetal bovine serum (Life Technologies, Inc., Rockville, MD) with 10% DMSO (Sigma). The postvaccine therapy leukapheresis was collected 2–4 weeks after the last vaccination. Cryopreserved PBMCs were also available from 50-ml blood draws collected just before each booster vaccination and approximately every 3–4 months out to 24 months after vaccination. The clinical protocol was reviewed by the Cancer Therapy Evaluation Program, National Cancer Institute, and approved by the Providence Health System Institutional Review Board. All of the patients gave their written informed consent before screening for eligibility.

gp100 Peptides and HLA-A2-Restricted Dimers. Both freshly thawed PBMCs and IVS-expanded cells from individual patients were stimulated directly with either native gp100_{209–217} (ITDQVPFSV) or modified gp100_{209–2M} (IMDQVPFSV) peptide; all of the peptides were purchased from ResGen Invitrogen Corp. (Huntsville, AL). IVS and freshly thawed T cells were also stimulated with a gp100 peptide-loaded HLA-A2 dimer molecule produced at our institution (10). The recombinant, “empty,” dimer coexpresses the α_1 , α_2 , and α_3 domains of the HLA-A2 heavy chain combined with a human β_2 microglobulin-IgG1-Fc fusion protein expressed in *Drosophila* S2 cells. Either native or modified gp100 or a control peptide was loaded into the “empty” dimer molecule by combining soluble dimer plus peptide at a weight:weight ratio of 10:1 (dimer to peptide) and incubating at room temperature for 2 h. Thereafter the peptide-loaded dimer could be maintained at 4°C for up to 1 month.

Autologous Dendritic Cells (DCs). DCs were prepared from prevaccine leukapheresis PBMCs from each patient. Fifty to 70×10^6 cryopreserved PBMCs were cultured in X-VIVO 15 medium (BioWhittaker, Walkersville, MD) supplemented with 5% heat-inactivated human AB serum (Irvine Scientific, Irvine, CA) for 2 h at 37°C/5% CO₂ in T-75 tissue culture flasks to

allow for attachment of adherent monocytes. After 2-h nonadherent cells were washed off with prewarmed medium and fresh medium supplemented with 1000 units/ml of granulocyte macrophage colony-stimulating factor and 500 units/ml of interleukin 4 (PeproTech, Rocky Hill, NJ) was added. DC cultures were maintained for 7 days at 37°C/5% CO₂ without feeding, whereupon cells were harvested using cold (4°C) PBS supplemented with 2% human AB serum and then maintained at 4°C on ice in X-VIVO 15 medium plus 5% human AB serum until they were used as antigen presenting cells in CFC assays. Typically DCs were >90% CD11c⁺/HLA-DR⁺/lineage negative.

gp100_{209–2M} IVS T-Cell Cultures. Pre- and postvaccine therapy cryopreserved PBMCs were placed in a single 8-day cycle of gp100_{209–2M}-directed IVS before being tested in CFC functional assays. The IVS expansion culture consisted of PBMCs in a 96-well round-bottomed plate at 2.5×10^5 /well (200 μ l) in X-VIVO 15 medium supplemented with 5% human AB serum, human interleukin 15 at 25 ng/ml (PeproTech) and gp100_{209–2M} peptide (10 μ g/ml) for 2 days. Subsequently 60 IU/ml of interleukin 2 (Chiron, Emeryville, CA) was added to each well, and cultures were maintained for an additional 6 days before effector cells were harvested and used in CFC assays.

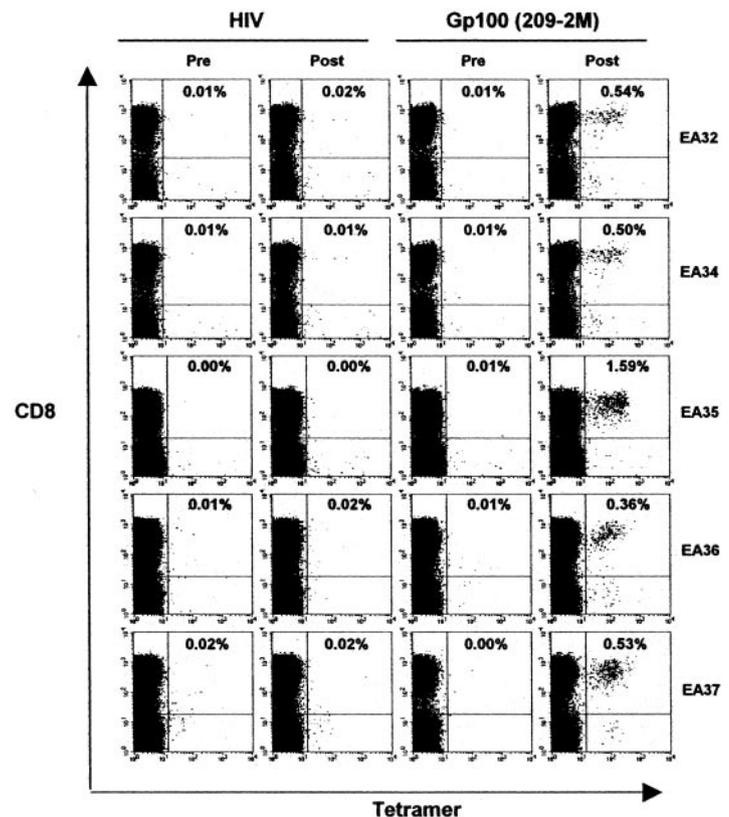
gp100_{209–2M} Tetramer Binding Assay. Phycoerythrin-conjugated, HLA-A2-restricted, gp100_{209–2M} and HIV (pol; ILKEPVHGV) peptide-specific tetramers were produced at the Ludwig Institute (Lausanne, Switzerland). A pretitered optimal concentration of tetramer reagent was added to 10^6 freshly thawed pre- and postvaccine PBMCs or to 10^6 pre- and postvaccine IVS cells from each patient. Cells were incubated at room temperature in 100 μ l of staining buffer (10% BSA and 0.1% NaN₃ in PBS) for 1 h in the dark with gentle mixing every 15 min; staining was performed in 12 \times 75 mm polystyrene Falcon (#352054) tubes. Cells were washed twice in cold (4°C) staining buffer, resuspended in 100 μ l of staining buffer, and stained simultaneously with optimal titrated concentrations of antihuman CD3-PerCP, CD8-allophycocyanin (APC), and CD56-FITC (BD Biosciences, San Jose, CA). Cells were incubated at 4°C for 30 min, washed twice in cold staining buffer, and fixed in 500 μ l of 1% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) made up in staining buffer. Fixed cells were stored in the dark at 4°C for at least 2 h before flow cytometry analysis, which was always performed within 24 h of staining. Cells were analyzed using a four-color acquisition format on a BD Calibur flow cytometry system. Data were collected on 5×10^4 gated CD8⁺/CD3⁺ T cells and analyzed using CellQuest software (BD Biosciences). Negative controls consisted of fluorochrome-matched isotype controls for each test antibody and a phycoerythrin-conjugated HIV (pol), HLA-A2⁺ tetramer, and net tetramer⁺ frequencies were calculated by subtracting HIV (pol) tetramer frequency values from the percentage of gp100_{209–2M} tetramer⁺ cells.

CFC(IFN- γ) Assay Using Cryopreserved PBMCs. Direct *ex vivo* CFC(IFN- γ) analysis was performed using freshly thawed pre- and postvaccine PBMCs. One $\times 10^6$ PBMCs/well were placed in a round-bottomed 96-well plate in 200 μ l of X-VIVO 15 medium supplemented with 5% human AB serum. Cells were rested overnight (37°C/5% CO₂) before stimulation with native gp100_{209–217} peptide or with gp100_{209–2M} peptide

at 2.5 μ g/well (10 μ g/ml). HIV (pol) peptide was added at the same concentration to negative control cultures, and cells were incubated 1 h before 2.5 μ g/well (10 μ g/ml) of brefeldin A (Sigma) was added to each reaction well. Cells were incubated for an additional 4 h before the assay was collected. Twenty five μ l of a 20 mM solution of EDTA (Sigma) was added to each well to stop the IFN- γ response and facilitate the efficient recovery of antigen-stimulated adherent CD8⁺ T cells, and plates were incubated for 10 min at room temperature before collection. Cell samples from replicate wells were pooled, washed twice with 3 ml of staining buffer, and 2×10^6 cells were resuspended in 50 μ l to 100 μ l of staining buffer for fixation, permeabilization, and staining. Cells were fixed in 1–2 ml of 1 \times fluorescence-activated cell sorter (FACS) lysis buffer (BD Biosciences) for 10 min at room temperature. All of the samples were washed 2 \times in cold staining buffer and 500 μ l of FACS permeabilization buffer (BD Biosciences) was added in each sample staining tube, gently mixed, and incubated at room temperature for 15 min. Permeabilized cells were washed twice and resuspended in 50–100 μ l of staining buffer. Cells were stained with anti-IFN- γ (FITC)/CD69(PE)/CD8(peridinin chlorophyll protein-cyanine 5.5)/CD3(APC) monoclonal antibodies (BD Biosciences) and incubated at room temperature for 30 min. Matched isotype control stains consisting of CD3 (APC)/ γ 2a (FITC)/ γ 1(PE)/ γ 2a(peridinin chlorophyll protein-cyanine 5.5) were set up for each PBMC test group. After staining, cells were washed twice in staining buffer, fixed in 500 μ l of 1% paraformaldehyde (Electron Microscopy Sciences), and stored at 4°C in the dark before analysis; analysis was performed within 24 h of staining. Cells were acquired through a standard lymphocyte (90° *versus* forward angle light scatter) light scatter gate, and 5×10^4 to 1×10^5 gated CD8⁺/CD3⁺ T cells were analyzed for IFN- γ staining using a FACS Calibur flow cytometry system and CellQuest software (BD Biosciences). Net frequencies of IFN- γ ⁺ cells were calculated by subtracting background IFN- γ expression values due to HIV (pol) peptide stimulation from IFN- γ ⁺ CD8⁺ frequencies after gp100_{209–2M} stimulation.

CFC(IFN- γ) Assay Using gp100_{209–2M} IVS T Cells. Autologous DCs were placed in a 96-well round-bottomed plate at 12×10^3 /well in 180 μ l of X-VIVO 15 medium supplemented with 5% human AB serum. Dephosphorylated Lipid A (Avanti Polar Lipids, Alabaster, AL) was added to culture wells at 100 ng/ml (20 ng/well) overnight to mature the DCs (11). Lipid A was made up in a 10% ethanol-in-water stock solution at 100 μ g/ml before use. After overnight maturation 2.5 μ g/well of gp100_{209–2M} peptide or native gp100_{209–217} peptide or a negative control HIV (pol) peptide was added (10 μ l aliquot/well) to appropriate wells and incubated for 2 h before the addition of IVS expanded T cells. Eight-day gp100_{209–2M} IVS pre- and postvaccine therapy PBMCs were added at 2.5×10^5 /well to replicate reaction wells containing peptide-pulsed DCs. Eight replicate wells were set up for each test condition (*i.e.*, 2×10^6 effector cells per test group), and cells were cultured for 1 h before the addition of brefeldin A at 5 μ g/ml (1.25 μ g/well) and additional overnight (12–14 h) incubation. Cells from replicate wells were pooled and interrogated for IFN- γ cytokine expression. Parallel stimulation cultures were also set up using IVS PBMCs at 2.5×10^5 /well except

Fig. 1 *Ex vivo* detection of circulating gp100_{209-2M}-specific CD8⁺ T cells. Cryopreserved pre- and postvaccine therapy peripheral blood mononuclear cells from 5 patients were thawed and stained with a phycoerythrin-conjugated HLA-A2⁺/gp100_{209-2M} tetramer reagent in combination with antihuman CD8 (allophycocyanin) and CD3 (PerCP) monoclonal antibodies. Negative control staining was assessed using an HLA-A2⁺-restricted HIV (pol) peptide tetramer. Two-parameter dot plots of CD8 (Y-axis) versus gp100_{209-2M} or HIV (pol) tetramer (X-axis) show the quantitation of HIV (pol) and gp100_{209-2M} tetramer-binding cells expressed as a percentage of gated CD8⁺/CD3⁺ T cells.



gp100₂₀₉₋₂₁₇, gp100_{209-2M}, or HIV (pol) peptide-loaded dimers were added to each reaction well at 0.25 μ g/well. Similarly, dimer-stimulated cultures were incubated for 1 h before adding 5 μ g/ml of brefeldin A and subsequently cultured overnight (12–14 h) before CFC(IFN- γ) analysis. Cells were harvested by adding 25 μ l of a 20 mM solution of EDTA to each well for 10 min at room temperature before collection and pooling. CFC (IFN- γ) analysis was carried out exactly as described for cryopreserved cells.

Four-Color Flow Cytometry Phenotype Analysis.

Freshly thawed PBMCs from the postvaccine leukapheresis were stained with several different combinations of monoclonal antibodies that have been used to delineate subsets of human memory/effector CD8⁺ T cells. Each sample (2×10^6 PBMCs) was stained with phycoerythrin-conjugated gp100_{209-2M} tetramer and a custom-conjugated (clone 2ST8.5H7; Beckman Coulter, Brea, CA) antihuman CD8 β chain-specific monoclonal antibody linked to the tandem dye, phycoerythrin-cyanine 7. The CD8 $\alpha\beta$ heterodimer is expressed by TCR $\alpha\beta^+$ CD8⁺ T cells, whereas human TCR $\gamma\delta^+$ CD8⁺ T cells and CD8⁺ natural killer cells express only the CD8 $\alpha\alpha$ homodimer (12). A series of different two-antibody staining combinations were added to this basic 2-color reagent set. Thus, CD45RA(FITC) and CD45RO(APC) were added simultaneously to define the CD45 isoform subset profile on gp100_{209-2M} tetramer⁺ CD8⁺ T cells. Additionally, CD45RA(APC) plus FITC-conjugated CD27, CD28, CD57, or CCR7, were combined to quantitate the number of tetramer⁺ CD8⁺CD45RA⁺ T cells expressing each of the

four effector/memory T-cell markers. After the addition of the monoclonal antibodies, cells were incubated at 4°C for 30 min, washed twice in staining buffer, and fixed in 500 μ l of 1% paraformaldehyde. Cells were stored in the dark at 4°C until analysis, which occurred within 24 h of staining. Flow cytometry data were collected on 10^4 gated CD8 β^+ /gp100_{209-2M} tetramer⁺ T cells. All of the analysis was performed on a four-color FACS Calibur instrument using CellQuest software. Appropriate compensation and isotype controls were run for all of the samples.

Statistical Methods. Data in this study were evaluated using standard descriptive techniques, graphical methods, correlation and regression analyses, independent and paired tests, and paired nonparametric tests. We performed various tests on distributions of immune parameter values to test assumptions underlying the procedures. For ease in interpretation of within-subject analyses (*e.g.*, pre- versus postresults, tetramer versus CFC assay results, ratios, and so forth) we report two-sided probability values obtained from Wilcoxon signed ranks tests.

RESULTS

Ex Vivo gp100_{209-2M} Tetramer Staining of Pre- and Postvaccine PBMCs. Direct *ex vivo* gp100_{209-2M} tetramer staining and flow cytometry analysis were performed on cryopreserved pre- and postvaccine therapy PBMCs from 35 patients. The results for the first 29 patients were reported previously (9). The results for 5 of the final 6 patients examined are

shown in Fig. 1; patient EA33 had no gp100_{209-2M}-specific response detected by *ex vivo* tetramer analysis. Similar to the results reported previously, EA32 and EA34-EA37 demonstrated no gp100_{209-2M}-specific tetramer staining on prevaccine PBMCs, but did show bright, well-resolved tetramer staining of postvaccine cells (range, 0.36%–1.59%). Pre- and postvaccine PBMCs were comparably negative for the HIV (pol) negative control tetramer stain.

A comprehensive summary analysis of the *ex vivo* tetramer data from the first 29 patients (9) and the data in Fig. 1 demonstrated that in 33 of 35 patients vaccination resulted in an increase in the percentage of brightly stained circulating gp100_{209-2M} peptide-specific CD8⁺ T cells; patients EA03 and EA33 were negative. The median postvaccine increase (0.36%) was highly significant ($P < 0.0001$). Patients could be stratified into four groups based on the percentage of tetramer-binding cells. Thirteen patients had positive staining frequencies $\leq 0.25\%$ of all of the gated CD8⁺ T cells (range, 0.05%–0.25%), whereas in another 11 patients $>0.25\%$ to $\leq 0.75\%$ of CD8⁺ T lymphocytes were tetramer⁺. Two additional clusters of patients displayed tetramer⁺ CD8⁺ T-cell percentages of $>0.75\%$ to $<1.5\%$ (5 patients), or tetramer staining in excess of 1.5% of circulating CD8⁺ T cells (4 patients); 2 patients in the latter group, EA08 and EA14, had tetramer⁺ CD8⁺ values of 8.86% and 4.96%, respectively. All of the patients were also immunized with a nonself A2-restricted peptide, HPV16E7, to assess overall T cell response to a reporter antigen. A cumulative HPV tetramer staining analysis of all of the patients in the initial pilot study (9) and the HPV data from the final patients enrolled in the trial (EA32-EA37; data not shown) demonstrated that there was no overall difference between the median response to the gp100_{209-2M} peptide and the HPV16E7 peptide ($P = 0.92$). Unexpectedly, several patients showed significantly higher gp100_{209-2M} staining than HPV tetramer binding re-

sponses (9), suggesting that a low T cell response to an HPV16E7 reporter antigen does not necessarily predict responses to gp100_{209-2M} immunization.

Ex Vivo CFC(IFN- γ) Analysis of Pre- and Postvaccine PBMCs. Fig. 2 shows the results of multiple direct *ex vivo* CFC(IFN- γ) assays of postvaccine cryopreserved cells from 12 representative patients and the matched *ex vivo* gp100_{209-2M} tetramer-staining results for the same patients. Tetramer staining and CFC(IFN- γ) analysis were performed 3–10 times on individual patients. The tetramer staining and CFC(IFN- γ) results were reproducible for all of the patients; all of the coefficients of variation were $\leq 22\%$. Statistical analysis demonstrated that mean tetramer percentages were significantly higher than mean CFC(IFN- γ) percentages ($P < 0.05$) for 9 of the 12 patients (*i.e.*, EA02, EA08, EA11, EA12, EA14, EA16, EA24, EA28, and EA30). The overall difference between the tetramer analysis and the functional response was most noticeable for EA08 and EA12 where the mean CFC(IFN- γ) frequencies were 35% and 32% of the tetramer-binding frequencies, respectively.

Comparison of Tetramer Staining to CFC(IFN- γ) Responses of Pre- and Postvaccine IVS CD8⁺ T Cells. Because we had *ex vivo* tetramer⁺ CD8⁺ frequencies for 33 of 35 patients (based on the direct analysis of unstimulated cryopreserved postvaccine PBMCs) we were subsequently able to investigate the potential interpatient differences in the proliferation of functionally responsive CD8⁺ T cells after IVS with cognate gp100_{209-2M} peptide. Fig. 3 depicts gp100_{209-2M} tetramer staining and the matched DC and/or dimer-mediated gp100_{209-2M} peptide-specific CFC(IFN- γ) response of IVS CD8⁺ T cells for all 35 patients. All of the data are from a single experimental analysis of IVS-expanded postvaccine PBMCs from each patient, and show the net values (background values have been subtracted) for tetramer and CFC(IFN- γ) expression. The cells used to initiate the IVS cultures were from the same

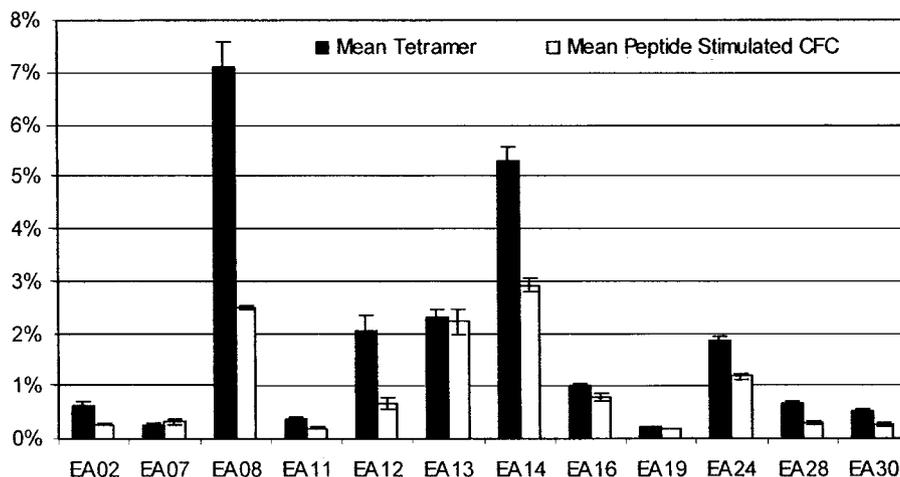


Fig. 2 Mean *ex vivo* gp100_{209-2M} tetramer⁺ and cytokine flow cytometry (CFC; IFN- γ)⁺ frequencies of cryopreserved CD8⁺ T cells from 12 representative patients. CFC(IFN- γ) analysis and correlated gp100_{209-2M} tetramer staining were performed on multiple samples (3–10/patient) of cryopreserved postvaccine peripheral blood mononuclear cells. CFC(IFN- γ) assays were performed using 10 $\mu\text{g/ml}$ of gp100_{209-2M} peptide or HIV (pol) peptide (negative control) to stimulate *in vitro* cultures of cryopreserved cells. Bar graphs represent the mean \pm SE tetramer response (■) and the mean \pm SE CFC (IFN- γ) response (□) for each patient. Data are expressed as the net mean frequency of gated CD8⁺/CD3⁺ T cells that bind tetramers or produce IFN- γ .

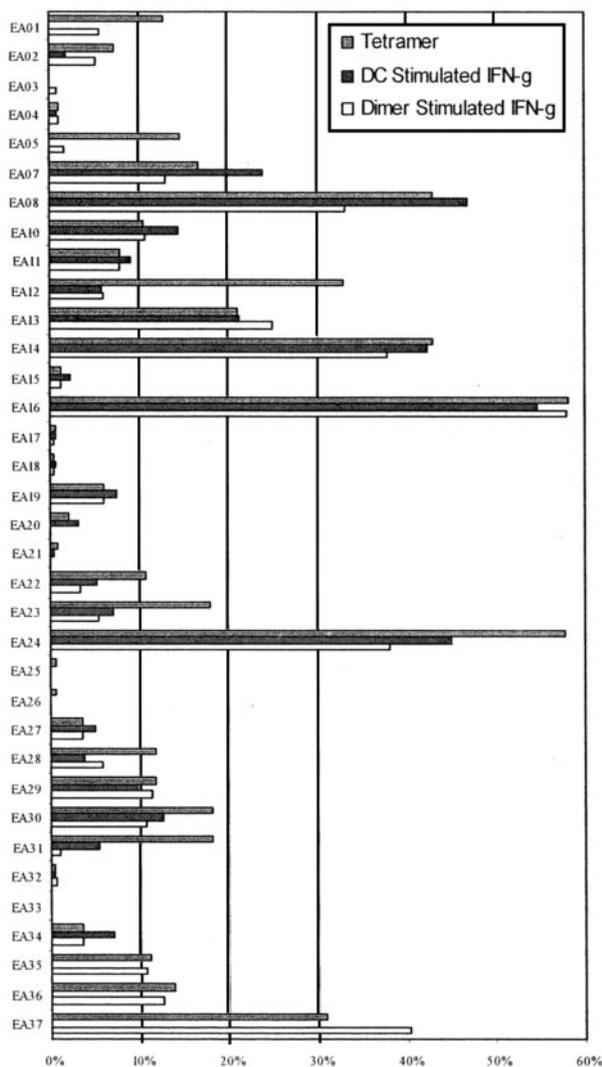


Fig. 3 Summary of correlated gp100_{209-2M} tetramer binding and cytokine flow cytometry (CFC; IFN- γ) responses by *in vitro* stimulation (IVS) T cells. Data represent a comparative summary of the frequency of gp100_{209-2M} tetramer⁺ CD8⁺ cells after IVS expansion of postvaccine peripheral blood mononuclear cells (▨) with corresponding dendritic cell (DC)-stimulated (■), and/or dimer-stimulated CFC(IFN- γ ; □) responses for each vaccinated patient. Data are expressed as a net percentage of CD8⁺/CD3⁺ T cells, and were obtained from a single set of *in vitro* assays for each patient. DC-stimulated CFC(IFN- γ) responses were not measured in every patient.

cryopreserved sample used to perform the baseline *ex vivo* tetramer assay on each patient. Matched DC-mediated and dimer-mediated gp100_{209-2M} stimulation were performed on 24 patients; the Pearson correlation between DC and dimer-stimulated values for these 24 patients was high ($r = 0.97$), demonstrating excellent agreement between these two methods of antigen-specific T-cell stimulation (10). We elected to compare both the DC and dimer-stimulated CFC(IFN- γ) assays because we anticipated that DCs may not always be available for some patients (e.g., EA01, EA05, EA35, EA36, and EA37; Fig. 3); and if there was statistical equivalence between the two assays

the dimer stimulation method would be logistically easier to use in future studies. The net tetramer and CFC(IFN- γ) frequencies for prevaccine IVS cells were negative in all of the patients. Many patients, such as EA07, EA08, EA10, EA11, EA13, EA14, EA16, EA19, EA27, EA29, EA35, EA36, and EA37 had comparable tetramer staining and correlated CFC(IFN- γ) frequencies. However, other patients exemplified by EA12, EA22, EA23, EA 24, EA28, and EA31 showed lower percentages of functionally active cells (IFN- γ expression) compared with the frequency of tetramer⁺ T cells even after IVS.

We reported previously that patients EA02, EA12, EA16, and EA20 had essentially identical frequencies of *ex vivo* gp100_{209-2M} tetramer⁺ CD8⁺ T cells at 1.2% (9); however, cells from these patients demonstrated dramatically different proliferative increases in the frequency of tetramer⁺ CD8⁺ T cells after identical 8-day gp100_{209-2M} IVS. EA12 and EA16 expanded to very high levels of gp100_{209-2M} tetramer⁺ T-cell expression (32.8% and 58.2%, respectively), whereas EA02 and EA20 responded with significantly lower frequencies of cognate-antigen stimulated tetramer⁺ CD8⁺ T cells (7.2% and 2%, respectively; Fig. 3). With the exception of EA33, all of the patients in the study showed increased frequencies of gp100_{209-2M} tetramer⁺ CD8⁺ T cells after IVS compared with their *ex vivo* tetramer staining values. IVS tetramer-staining frequencies, although greater than *ex vivo* percentages, were very low in 9 patients; EA03, EA04, EA15, EA17, EA18, EA21, EA25, EA26, and EA32 had tetramer-binding responses after IVS expansion ranging from a low of 0.30% to a high of 1.2%.

Fig. 4 shows the mean (\pm SE) for multiple (3–10) CFC(IFN- γ) and correlated tetramer-binding assays performed on IVS-expanded CD8⁺ T cells from 6 patients. CFC(IFN- γ) analysis was performed after stimulation with gp100_{209-2M}

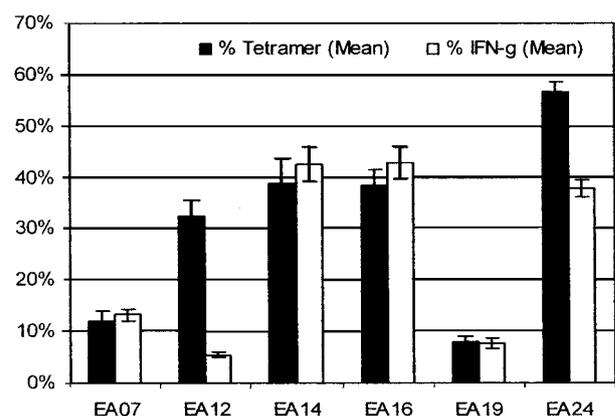


Fig. 4 Mean gp100_{209-2M} tetramer staining and cytokine flow cytometry (CFC; IFN- γ) frequencies for postvaccine *in vitro* stimulation (IVS) CD8⁺ T cells. CFC(IFN- γ) analysis and correlated gp100_{209-2M} tetramer staining were performed on multiple samples (3–10/patient) of IVS-expanded postvaccine peripheral blood mononuclear cells from six representative patients. IVS cells were stimulated *in vitro* with gp100_{209-2M}-loaded dimers. Bar graphs represent the mean \pm SE tetramer response (■) and the mean \pm SE CFC(IFN- γ) response (□) for each patient. Data are presented as the net mean frequency of gated IVS CD8⁺/CD3⁺ T cells that were positive for tetramer binding or for IFN- γ expression.

loaded HLA-A2⁺ dimers. The data demonstrate that similar to *ex vivo* results (Fig. 2) both assays were also reproducible using IVS T cells (coefficients of variation $\leq 25\%$). Four patients, EA07, EA14, EA16, and EA19, had mean CFC(IFN- γ) responses comparable with mean tetramer-binding responses ($P < 0.05$). By contrast, patients EA12 and EA24 showed statistically higher ($P < 0.05$) mean tetramer-binding values compared with their mean CFC(IFN- γ) responses, with CFC(IFN- γ):tetramer ratios of 0.14 and 0.69, respectively.

IVS CD8⁺ T Cells Recognized Native gp100₂₀₉₋₂₁₇ Peptide. Modified gp100_{209-2M} peptide was used as the immunogen to stimulate a more potent *in vivo* immune response, which was expected to cross-react with the native gp100₂₀₉₋₂₁₇ melanoma peptide. Thus, it was important to test the functional immune response of postvaccine CD8⁺ T cells to native gp100 peptide and to assess patient-to-patient variation in response to the native antigen. Modified gp100_{209-2M} IVS CD8⁺ T cells from 3 representative patients were restimulated *in vitro* with gp100_{209-2M} peptide and with the native gp100₂₀₉₋₂₁₇ peptide across a five-log titration range of 5 $\mu\text{g/ml}$ –0.0005 $\mu\text{g/ml}$. All 3 of the patients differed in their response to the native *versus* the modified gp100 peptide (Fig. 5A). EA08 sustained a very comparable CFC(IFN- γ) response for both peptides at the high-dose range of the peptide titration (5.0–0.5 $\mu\text{g/ml}$), but at concentrations < 0.5 $\mu\text{g/ml}$ the native peptide was less effective. In EA08, at the highest concentration (5 $\mu\text{g/ml}$), the native peptide stimulated 92.4% of the CFC(IFN- γ) response triggered by the modified gp100 peptide (Fig. 5A). Patient EA14 similarly showed a sustained IFN- γ functional response over a one-log titration range (5.0–0.5 $\mu\text{g/ml}$) for both peptides, but the response was uniformly lower for the native peptide; at the highest concentration the gp100₂₀₉₋₂₁₇ stimulated IFN- γ response was still 76% of the response triggered by the modified peptide. EA30 displayed a third response pattern in which the gp100₂₀₉₋₂₁₇ stimulated CFC(IFN- γ) response was very low (42% of the gp100_{209-2M} effect) at the highest antigen concentration, and the functional response was lost rapidly at lower peptide concentrations compared with that induced by the modified peptide. Differences in functional avidity for different peptide antigens by a given effector T-cell population will commonly be reflected by quantitatively significant differences in the EC₅₀ values for each peptide over their titration range, and by whether or not the different peptide ligands achieve equivalent optimal stimulation effects at saturating concentrations. Using these commonly accepted functional parameters the data in Fig. 5A demonstrate that gp100_{209-2M}-stimulated IVS CD8⁺ T cells recognize the native peptide with overall lower functional avidity than the modified peptide, and T cells may vary significantly between patients with regard to their functional avidity for the native peptide. Fig. 5B shows the CFC(IFN- γ) responses of postvaccine IVS cells from 15 patients to both direct gp100_{209-2M} peptide stimulation and to stimulation by gp100₂₀₉₋₂₁₇ at 5 $\mu\text{g/ml}$. The ratio of the CFC(IFN- γ)⁺CD8⁺ frequency stimulated by native gp100 peptide divided by the frequency induced by the modified peptide was calculated for each patient from the data in Fig. 5B. The mean (\pm SE) of these ratio values for all 15 of the patients was $69.8 \pm 4.4\%$, indicating that at high antigen concentration the native peptide stimu-

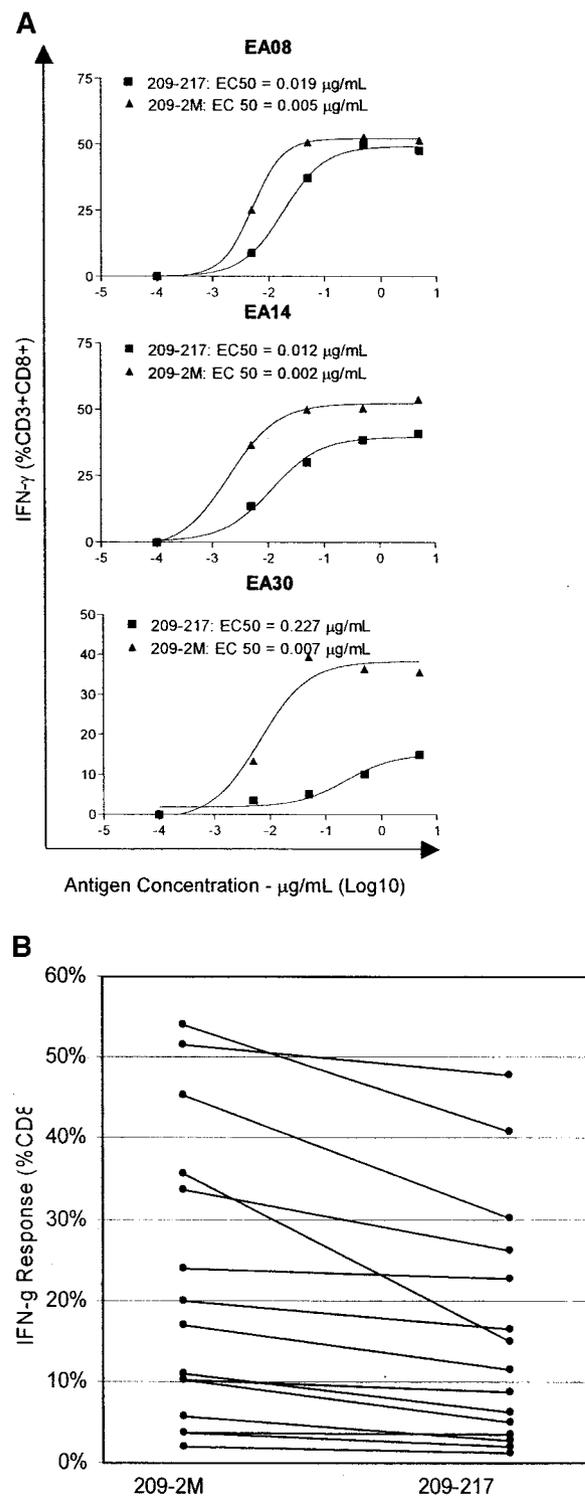


Fig. 5 Postvaccine gp100_{209-2M} *in vitro* stimulation (IVS) CD8⁺ T cells recognize native gp100₂₀₉₋₂₁₇ peptide. A, data show the cytokine flow cytometry (CFC;IFN- γ) response of postvaccine IVS CD8⁺ T cells from 3 patients after stimulation over a five-log titration (5 $\mu\text{g/ml}$ to 0.0005 $\mu\text{g/ml}$) of native gp100₂₀₉₋₂₁₇ or modified gp100_{209-2M} peptide. Nonlinear curve fitting analysis and EC₅₀ calculations were performed using Prism Graphics software; "r" values for all curves shown were > 0.95 . B, also shown are the comparative CFC(IFN- γ) responses of IVS CD8⁺ T cells from 15 patients stimulated with the native and modified peptide at 5 $\mu\text{g/ml}$.

Table 1 Memory/effector phenotype analysis of postvaccine cryopreserved gp100_{209-2M} tetramer⁺ CD8⁺ T cells

CD8 Phenotype	EA08	EA12	EA13	EA14	EA16	EA24
CD45RA ⁺ /CCR7 ⁺	8.0	23.2	12.7	9.9	14.6	18.7
CD45RA ⁺ /CCR7 ⁻	57.2	32.5	64.0	70.4	57.5	70.8
CD45RA ⁻ /CCR7 ⁺	3.7	15.4	3.2	2.7	7.45	2.5
CD45RA ⁻ /CCR7 ⁻	30.3	27.2	20.1	14.9	18.2	5.4
CD45RA ⁺ /CD27 ⁺	13.8	28.9	16.6	40.6	17.6	32.9
CD45RA ⁺ /CD27 ⁻	54.8	26.7	58.4	39.0	55.8	55.9
CD45RA ⁻ /CD27 ⁺	5.4	17.0	10.9	9.6	5.1	2.7
CD45RA ⁻ /CD27 ⁻	24.9	27.5	14.1	8.7	20.4	4.3
CD45RA ⁺ /CD28 ⁺	8.0	48.7	19.5	27.4	32.3	28.2
CD45RA ⁺ /CD28 ⁻	57.8	4.3	58.0	52.1	39.5	61.4
CD45RA ⁻ /CD28 ⁺	4.7	43.1	19.8	14.1	25.3	9.5
CD45RA ⁻ /CD28 ⁻	29.5	0.83	2.7	6.3	2.9	0.9
CD45RA ⁺ /CD57 ⁺	48.1	9.3	58.3	48.4	34.0	71.7
CD45RA ⁺ /CD57 ⁻	16.5	47.4	16.3	30.0	36.0	16.5
CD45RA ⁻ /CD57 ⁺	21.1	3.9	4.3	5.2	5.0	2.63
CD45RA ⁻ /CD57 ⁻	3.3	39.4	17.9	13.3	21.4	9.1

Cells from each patient were analyzed using four different reagent staining combinations with CD8 β , gp100_{209-2M} tetramer, and CD45RA in combination with either anti-human CCR7, CD27, CD28, or CD57 monoclonal antibodies. Data was collected on 10⁴ gated CD8⁺/tetramer⁺ T cells, and is shown as the percentage of antigen-specific CD8⁺ T cells for each patient that had the cell surface phenotype listed in the left hand column of the table.

lated on average ~70% of the functional response triggered by cognate modified gp100_{209-2M} peptide.

gp100_{209-2M} Tetramer⁺ CD8⁺ T Cells Have Effector and Effector-Memory Phenotypes. Table 1 shows the phenotype profile of gp100_{209-2M} tetramer⁺ CD8⁺ cells from the postvaccine cryopreserved PBMCs of 6 patients with high frequencies of tetramer⁺ cells. Cells were stained with a series of four epitope-specific reagents as described in "Materials and Methods." The staining combinations were based on reports that the reciprocal expression of the CD45RA/CD45RO isoforms on CD8⁺ T cells can, in combination with CCR7, CD27, CD28, and CD57, distinguish functionally discrete subsets of memory/effector CD8⁺ T cells (13–20). The CD45RA staining frequency for each patient (expressed as a percentage of gp100_{209-2M} tetramer⁺ CD8⁺ T cells) was EA08 = 62.7%, EA12 = 57.7%, EA 13 = 75.3%, EA14 = 79%, EA16 = 73%, and EA24 = 89.1%. Results of the *ex vivo* four-color phenotype analysis of postvaccine PBMCs indicated that, by CD45RA and CCR7 staining criteria (13), a majority of tetramer⁺ CD8⁺ T cells from all 6 of the patients (78.1 \pm 4.2%) were CCR7⁻ and were either in the terminal-effector, CD45RA⁺/CCR7⁻ (58.7 \pm 5.8%), or the effector-memory compartment, CD45RA⁻/CCR7⁻ (19.4 \pm 3.6%). A smaller but significant component of tetramer⁺ CD8⁺ T cells from all of the patients was CCR7⁺ (20.3 \pm 3.7%), and most of these cells expressed the so-called "naïve" (13) phenotype, CD45RA⁺/CCR7⁺ (14.5 \pm 2.1%). In all of the patients except EA12 only minor subpopulations of antigen-specific cells showed the central-memory (CD45RA⁻/CCR7⁺) phenotype.

Using a phenotype model based on combined CD45RA and CD27 staining (8, 16, 17), a comparable majority of antigen-specific CD8⁺ T cells were CD27⁻ (65.1 \pm 5.3%) in all 6 of the patients; these were also partitioned between the effector compartment, CD45RA⁺/CD27⁻ (48.4 \pm 4.7%), and the effector-memory subset, CD45RA⁻/CD27⁻ (16.7 \pm 3.4%). Minor subpopulations of tetramer⁺ CD8⁺ T cells exhibited a central-memory phenotype (CD45RA⁻/CD27⁺) with EA12

showing the highest percentage (17%). There were notably higher percentages of tetramer⁺ T cells showing the naïve phenotype (CD45RA⁺/CD27⁺) for patients EA12 (28.9%), EA14 (40.6%), and EA24 (32.9%). Combined CD45RA and CD28 staining analysis generally recapitulated the CD27 and CCR7 staining patterns for 4 of 6 patients and indicated that a majority (67.2 \pm 5.8%) of the antigen-specific CD8⁺ T cells from 4 patients were CD28⁻, and seemed to be partitioned between effector (CD45RA⁺/CD28⁻) and effector-memory (CD45RA⁻/CD28⁻) subpopulations. By contrast, EA12 and EA16 had 91.8% and 57.6% CD28⁺ antigen-specific CD8⁺ T cells, respectively, and these were equally divided into naïve (CD45RA⁺/CD28⁺) and memory (CD45RA⁻/CD28⁺) subsets. Notably 4 of 6 patients demonstrated a high mean (\pm SE) percentage of CD57⁺/CD8⁺ T cells (64.9 \pm 3.9%); by contrast a majority of tetramer⁺ cells in EA12 (86.8%) and EA16 (57.4%) were CD57⁻. The expression of CD57 has been associated with antigen-activated effector T-cell function (16, 19–20). Overall, the phenotypes of tetramer⁺ T cells in these 6 patients suggested that gp100_{209-2M} vaccination induced predominantly effector and effector-memory CD8⁺ T cells.

Vaccinated Patients Have Long-Term Memory (LTM) CD8⁺ T Cells. Cryopreserved PBMCs collected 12–24 months after the last vaccine were directly stained *ex vivo* with gp100_{209-2M} tetramers and interrogated by flow cytometry (Fig. 6A). The frequencies of circulating LTM gp100_{209-2M}-tetramer⁺ CD8⁺ T cells for 2 representative patients were significantly diminished at 1.1% (EA08) and 0.10% (EA13) compared with the matched frequencies of tetramer⁺ T cells in PBMCs obtained 2–4 weeks postvaccine therapy, 8.86% (EA08) and 2.42% (EA13). To determine the proliferative potential of LTM cells, they were exposed to 8-day IVS expansion with gp100_{209-2M} peptide. The bottom two rows of Fig. 6A show gp100_{209-2M} tetramer staining of IVS-expanded prevaccine, postvaccine, and LTM T cells. EA08 shows a 4.8-fold increase (8.86% to 42.9%) of gp100_{209-2M}-specific CD8⁺ T cells after IVS expansion of the postvaccine PBMCs as compared with a 16.3-fold increase (1.1% to 17.9%) of LTM

PBMCs. A similar comparison for patient EA13 indicates the number of gp100_{209-2M} tetramer⁺ CD8⁺ T cells increased from 0.10% (*ex vivo*) to 2.59% after IVS (25.9-fold increase). By contrast, the IVS expansion of PBMCs collected only 2 weeks after vaccine therapy increased from 2.42% to 21% (8.7-fold increase). The observed difference in the expanded percentage of antigen-specific T cells after IVS suggests the PBMCs at ≥ 12 months contain circulating gp100_{209-2M}-specific memory T

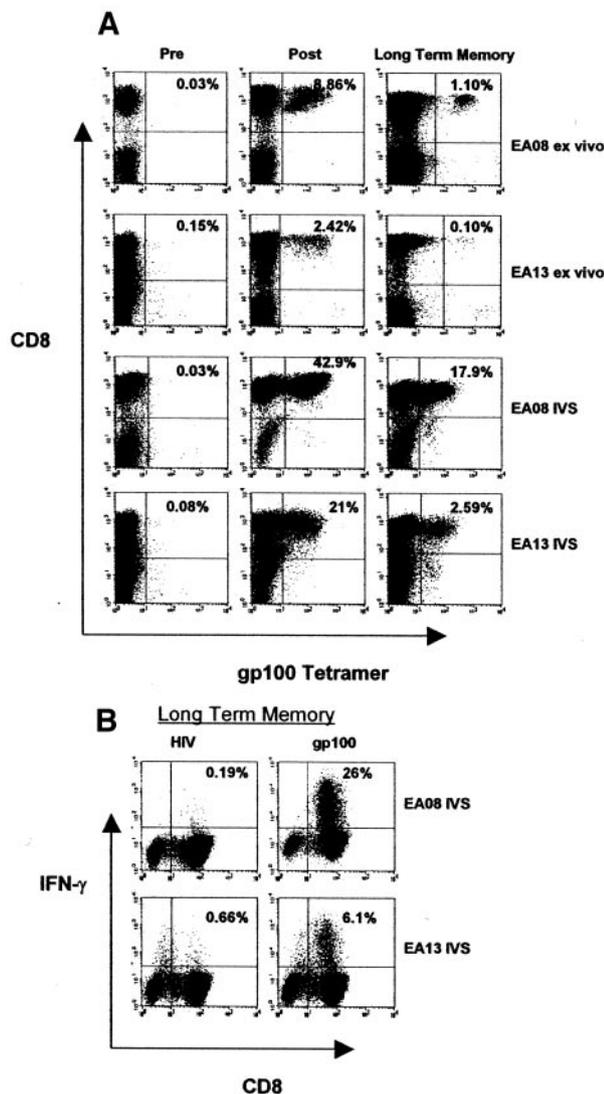


Fig. 6 Flow cytometry analysis of gp100_{209-2M} tetramer binding and cytokine flow cytometry (CFC:IFN- γ) expression by pre- and postvaccine, and long-term memory CD8⁺ T cells. **A**, data are presented for 2 patients as the percentage of gated CD8⁺/CD3⁺ T cells that were gp100_{209-2M} tetramer⁺ for freshly thawed prevaccine and postvaccine (2–4 weeks postvaccine therapy) peripheral blood mononuclear cells (PBMCs), and for long-term memory (15 months postvaccine therapy) PBMCs. The *bottom two rows* show the results of the same analysis using 8-day gp100_{209-2M} IVS CD8⁺ T cells for both patients. **B**, also shown are the CFC(IFN- γ) responses of 8-day IVS long-term memory CD8⁺ T cells after *in vitro* restimulation with HIV (pol)-loaded dimer or gp100_{209-2M}-loaded dimer for both patients.

Table 2 Fold-increase^a of gp100_{209-2M} tetramer⁺ CD8⁺ T cells after *in vitro* stimulation (IVS) expansion of postvaccine therapy^b versus long-term memory (LTM)^c peripheral blood mononuclear cells (PBMCs)

Patient	Post-treatment	LTM
EA02	6.4	22.0
EA07	68	181.6
EA08	4.8	16.2
EA11	23.7	14.2
EA12	29.4	107.9
EA13	8.5	25.9
EA14	8.7	113.1
EA17	8.1	96.0
EA19	35.6	228.3
EA23	84.5	108.3
EA24	31	106.1

^a Fold-increase values indicate the ratio of the frequency of tetramer⁺ CD8⁺ T cells after IVS expansion of postvaccine and LTM PBMCs divided by the baseline *ex vivo* frequency of tetramer⁺ CD8⁺ T cells in the cryopreserved postvaccine and LTM sample, respectively.

^b PBMC harvested within 2 to 4 weeks after the last vaccine administration.

^c LTM refers to PBMCs harvested 12 to 24 months after the last vaccine administration.

cells, and these circulating long-term memory T cells are capable of a heightened proliferative response. Fig. 6B shows the matched CFC(IFN- γ) functional response of CD8⁺ T cells from the same IVS cultures of LTM PBMCs from patients EA08 and EA13. Both patients demonstrated elevated levels of functionally active cells after restimulation with gp100_{209-2M} peptide-loaded dimers. Both CFC(IFN- γ) responses were greater than the matched tetramer values for the same IVS effector/memory cells for each patient. This observation may be attributable to the down-regulation of gp100 TCR expression to below levels detectable by flow cytometry (*i.e.*, ~ 1000 copies/cell) after gp100_{209-2M} IVS. Although cells may have diminished gp100 peptide TCR expression the remaining TCR complexes were sufficient to allow for activation of detectable IFN- γ production by cognate gp100_{209-2M} peptide. Table 2 shows the antigen-specific CD8⁺ T cell fold-increase values for 2–4 week postvaccine therapy PBMCs and for the paired LTM PBMCs after gp100_{209-2M} directed IVS. Ten of 11 patients showed amplified gp100_{209-2M}-specific proliferation by the LTM PBMCs compared with PBMCs collected shortly after vaccine therapy.

DISCUSSION

Unlike many previous vaccine studies of melanoma patients with metastatic disease, the direct *ex vivo* analysis of cryopreserved cells from patients vaccinated in the adjuvant setting demonstrated significant increases in postvaccine circulating gp100_{209-2M} tetramer⁺ CD8⁺ T cells in 94% of vaccinated patients. Notably, 14 patients (40%) had percentages of tetramer⁺ CD8⁺ T cells $\geq 0.5\%$ (range, 0.58–8.86%), and 9 of these patients (25.7%) displayed tetramer⁺ CD8⁺ T-cell percentages $> 1.0\%$, indicating the presence of circulating frequencies of gp100_{209-2M}-specific memory/effector T cells similar to those reported for patients in infectious disease settings (21–23). Importantly, correlated *ex vivo* CFC(IFN- γ) assays indicated interpatient variability with regard to the functional responsive-

ness of peptide-specific CD8⁺ T cells suggesting that a subset of melanoma peptide-specific T cells was functionally anergic in some patients. Presently the average time postvaccine therapy for all of the patients is <2 years. Thus, it is too early to assess the possible clinical correlation with the observed interpatient differences in circulating frequencies of functionally responsive (IFN- γ ⁺) tetramer⁺CD8⁺ T cells.

At present there is general agreement that antigen-directed IVS of PBMCs may alter the phenotype and function of circulating antigen-specific T cells in vaccinated patients, and thereby yield an inaccurate characterization of the *in vivo* immune response (24). This conclusion seems intuitively correct in the context of any immunomonitoring situation where the immune response of patients is so weak that direct *ex vivo* analysis cannot detect recall-antigen-specific functional responses, or even demonstrate the presence of antigen-specific T cells. However, if initial *ex vivo* assays readily detect both the frequency and function of antigen-specific T cells, as in this study, the follow-up analysis of IVS-expanded cells could then be compared to the *ex vivo* results for each patient, and thus be used to assess antigen-specific immune response differences between patients. Once baseline postvaccine *ex vivo* numbers of antigen-specific T cells and their functional status are determined, recall-antigen IVS may then reveal potentially important interpatient differences in the proliferation and functional responsiveness of antigen-specific T cells, as well as the functional avidity for cognate antigen, which may empirically prove to be valuable immune status index parameters that could potentially correlate with clinical outcome. More extensive data from larger clinical trials will be required to confirm this type of clinical correlation.

To study the effect of limited gp100_{209–2M} IVS on the rate of proliferation and functional activation of antigen-specific CD8⁺ T cells in each patient, we performed 8-day gp100_{209–2M}-directed IVS for all 35 of the patients. A single cycle of IVS induced significant increases in the percentage of tetramer⁺ CD8⁺ T cells above baseline *ex vivo* levels for 34 of 35 patients (Fig. 3). The combined use of the CFC(IFN- γ) assay and tetramer-binding analysis on IVS cells showed that many patients, *e.g.* EA13 and EA16 (Fig. 3), had comparable responses in both assays, indicating that the IVS protocol used resulted in the expansion of functionally competent, antigen-specific cells. Other patients, exemplified by EA12 and EA31 (Fig. 3), showed significantly lower percentages of functionally responsive cells compared with the net percentage of tetramer⁺ CD8⁺ T cells after IVS suggesting the presence of a subpopulation of anergic CD8⁺ T cells, even after IVS. Theoretically, IVS could result in the down-modulation of functional activity of proliferating, antigen-specific cells. Our observations to date suggest that this is not the case, because IVS usually results in either the retention of observed *ex vivo* patterns of functional anergy or in an increase in the frequency of functionally responsive tetramer⁺ CD8⁺ T cells (Figs. 2 and 4). Thus, if IVS perturbs the *ex vivo* functional response profile it appears to do so by favoring proliferation of functionally responsive antigen-specific CD8⁺ T cells or by inducing functional activity in cells that were quiescent by *ex vivo* interrogation. Either response could indicate interpatient differences predictive of clinical response. The data also suggest these anergic, antigen-

specific CD8⁺ T cells had a high proliferative response to cognate-antigen stimulation. Comparison of IVS T-cell tetramer-binding data to the matched *ex vivo* tetramer-binding values for each patient also revealed important interpatient differences in the overall proliferation potential of gp100_{209–2M}-specific CD8⁺ T cells. This was most apparent in patients with essentially equivalent baseline *ex vivo* tetramer⁺CD8⁺ frequencies, such as EA02, EA12, EA16, and EA20, who subsequently demonstrated significantly different tetramer⁺ T-cell frequencies after IVS (Fig. 5). This observation of interpatient heterogeneity with regard to proliferation potential after cognate-antigen stimulation would not have been made if our immunomonitoring strategy had relied only on direct *ex vivo* interrogation; and it may reflect potentially important *in vivo* differences in patient responses to a melanoma tumor antigen vaccine.

There was heterogeneity among patient *in vitro* response to native gp100_{209–217} versus gp100_{209–2M} stimulation. Rosenberg *et al.* (3) documented differences in IFN- γ response by IVS-expanded cells from immunized patients after restimulation *in vitro* with T2 cells pulsed with native gp100_{209–217} peptide or with gp100_{209–2M}. Herein we also demonstrated patient-to-patient variation in the *in vitro* response of IVS cells to native gp100_{209–217} peptide versus gp100_{209–2M}. Fig. 5B shows the comparative CFC(IFN- γ) response of IVS cells from 15 patients to both peptides at an optimal antigen concentration of 5 μ g/ml. The mean ratio (\pm SE) of native peptide over modified peptide CFC(IFN- γ) response for all 15 of the patients was $69.8 \pm 4.4\%$ (range, 42–94%). This mean ratio value for IVS cells is comparable with the corresponding *ex vivo* ratio value of 71.5% we reported previously for 10 of the same patients analyzed in Fig. 5B (9), and suggests that gp100_{209–2M} IVS expansion of postvaccine PBMCs did not alter the functional avidity of CD8⁺ T cells for the native peptide compared with the modified peptide antigen. It is axiomatic that the levels of melanoma-associated native gp100 peptide “seen” by memory/effector CTLs in melanoma patients *in vivo* may be substantially lower than the levels on dimers or stimulator cells used *in vitro*. In this regard, it is noteworthy that of 19 patients selected from this clinical trial, gp100_{209–2M} IVS T cells produced IFN- γ after restimulation by HLA-A2⁺/gp100⁺ melanoma tumor cell lines in 13 patients (68%),⁶ suggesting the functional avidity of IVS-expanded CD8⁺ T cells from immunized patients was strong enough to recognize the levels of native peptide expressed on melanoma cells. This observation was also reinforced by the correlated data from Meijer *et al.*,⁶ which demonstrated that cryopreserved postvaccine PBMCs from 6 of 9 patients studied (67%) also produced IFN- γ after direct *ex vivo* stimulation by HLA-A2⁺/gp100⁺ melanoma cell lines.

In our detailed four-color flow cytometry analysis of postvaccine cryopreserved PBMCs from 6 patients the majority of tetramer⁺ CD8⁺ T cells (65%–78%) were in effector and ef-

⁶ S. L. Meijer, A. Dols, S. Jensen, H. M. Hu, W. Miller, E. B. Walker, P. Romero, B. A. Fox, and W. J. Urba. Antimelanoma activity of circulating CD8⁺ T cells following adjuvant vaccination of melanoma patients with gp100_{209–2M} peptide, submitted for publication.

factor-memory subpopulations as delineated by the differential expression of the CD45RA, CD27, CD28, CD57, and CCR7 molecules (Table 1). Similar subpopulations of CD8⁺ T cells have been described in melanoma patients vaccinated with Melan-A/MART-1, tyrosinase, and gp100 peptides, as well as in unvaccinated melanoma patients. Pittet *et al.* (25) showed that the frequency of circulating Melan-A/MART-1_{26–35}-specific CD8⁺ T cells in a single patient increased >20-fold after multiple immunizations. Multiparameter flow cytometry analysis indicated that the increase in functionally responsive Melan-A tetramer⁺ CD8⁺ T cells was associated with a concomitant increase in the percentage of tetramer⁺ CD8⁺ T cells that expressed an “activated/memory” phenotype characterized by simultaneous CD45RA^{low}/CD27⁻/CD28⁻/CD57⁺ staining. Notably, the percent of Melan-A tetramer⁺ cells with this phenotype increased over time with repetitive immunization (25). In a related study, Valmori *et al.* (15) characterized *ex vivo* the functional properties and cell surface phenotype of constitutively expressed tyrosinase_{368–376} tetramer⁺ CD8⁺ T cells in a single unimmunized melanoma patient. The tyrosinase tetramer⁺ cells comprised >5% of the circulating CD8⁺ T cells in this patient and were detectable over a 3-year period. The tyrosinase-specific CD8⁺ T cells were directly lytic *ex vivo* for tyrosinase⁺ tumor lines, and were predominantly CD45RA⁺/CD27⁻CD28⁻/CD57⁺ expressing the so-called terminal effector phenotype (15). Fourteen-day IVS expansion down-regulated CD45RA expression completely, but longer (21-day) IVS-cultured CD8⁺ T cells re-expressed CD45RA concomitantly with low cell surface expression of CD45RO; the fidelity of the CD27⁻/CD28⁻/CD57⁺ staining was maintained even over long-term IVS. Importantly, after sorting, these “terminally differentiated” effector CD8⁺ T cells could proliferate after mitogen stimulation. The *in vitro* ability to proliferate and modulate CD45 isoform expression suggested that they might have the same differentiation/proliferation properties *in vivo* (15). In another report Monsurro *et al.* (8) demonstrated that 13 melanoma patients vaccinated multiple times with the gp100_{209–2M} peptide had an average increase in circulating gp100_{209–2M} tetramer⁺ cells equal to 1.2% of CD8⁺ T cells. These antigen-specific CD8⁺ T cells were predominantly effector, CD45RA⁺/CD27⁻ (43% ± 6%), or effector-memory, CD45RA⁻/CD27⁻ (30 ± 4.1%), phenotype. However, *ex vivo* functional analysis using a CFC (perforin) assay indicated that the tetramer⁺ cells were poorly responsive after stimulation with gp100_{209–2M}-pulsed melanoma cell lines (17% perforin positive). Although gp100_{209–2M}-specific IVS followed by 10-day expansion in interleukin 2 dramatically increased the frequency of perforin⁺ cells to 84 ± 3.6%, these functionally active cells now unexpectedly displayed increased expression of CD27⁺; *i.e.*, 67% of all of the tetramer⁺ CD8⁺ T cells were CD27⁺ regardless of CD45RA expression. The predominate phenotypes after IVS were 23% CD45RA⁺/CD27⁺ (naïve) and 44% CD45RA⁻/CD27⁺ (memory; Ref. 8). The plasticity of these predominantly CD27⁻ effector and effector-memory T cells after cognate-antigen IVS suggested that the cells were not terminally differentiated CD8⁺ T lymphocytes and that CD27 expression may be a marker of their activation status rather than end-stage differentiation as suggested by Valmori *et al.* (15). Similarly the subsequent increased expression of the CD45RA⁺/CD27⁺ na-

ive phenotype on 23% of all of the tetramer⁺ CD8⁺ T cells after IVS also suggested that this so-called naïve staining profile was not uniquely characteristic of antigen-inexperienced cells. Thus, our description of the predominant effector and effector-memory phenotypes of the gp100_{209–2M}-specific CD8⁺ T cells in this report agrees with the phenotype descriptions of melanoma peptide-specific T cells in previous studies. However, a comparative analysis of the data from previous studies also suggests that the description of the natural history of the CD8⁺ T cell effector-memory differentiation pathway remains unresolved due to the proliferation potential and phenotype plasticity after activation of presumably end-stage memory/effector CD8⁺ T-cell subpopulations (8, 15–18). Additional work involving the detailed *ex vivo* six to eight-color flow cytometry analysis of antigen-specific CD8⁺ T cells to assess the overlapping expression of markers like CD27, CD28, CCR7, and CD57 on the CD45RA or CD45RO background is required, as well as similar detailed phenotype and functional interrogation of sorted subsets of tetramer⁺ CD8⁺ T cells after IVS expansion with cognate-recall antigen to determine the phenotype and functional stability of CD8⁺ T-cell subpopulations.

To date, there have been few descriptions of the detailed functional and/or phenotype characterization of long-term memory/effector CD8⁺ T cells (*i.e.*, ≥12 months postvaccine therapy) from vaccinated nonmetastatic melanoma patients. In this study *ex vivo* tetramer analysis demonstrated the sustained presence of low frequencies of tetramer⁺ CD8⁺ T cells (range, 0.08–1.1%) in peptide-vaccinated patients at time points out to 1 and 2 years after vaccination. We also demonstrated that these circulating long-term tetramer⁺ CD8⁺ T cells had elevated proliferation responses after IVS, a trait characteristic of memory T cells (26). Thus, gp100_{209–2M} immunization resulted in the maintenance of long-term memory T cells that were capable of accelerated proliferation response and were functionally active by CFC(IFN-γ) analysis after IVS expansion. Antigen-specific effector and memory CD8⁺ T-cell proliferation and activation is governed by many factors, including the amount of antigen available *in vivo* (27, 28) and the presence of CD4 helper T-cell function at the time of CD8 T-cell priming (29, 30). Presently we have no mechanistic explanation for the durable CD8 memory T-cell response observed in the absence of an exogenous source of CD4 MHC class II-restricted antigen. However, the chemical structure of the adjuvant, Montanide ISA 51, may provide some insight into the immune stimulation of a memory CD8⁺ T-cell response by the gp100_{209–2M} peptide. The principal surfactant in Montanide is a Mannide mono oleate lipid complex (31). Montanide adjuvant formulations and related lipid-complexed adjuvants made up of palmitic acid groups admixed with or conjugated to class I-restricted peptides have been shown to stimulate weak memory CD8 T-cell responses (32, 33). Recent data suggests that DCs copresenting class I restricted peptides and the CD1d-binding glycolipid α-galactosyl-ceramide can prime CTL responses in a class II independent fashion by causing the activation of natural killer T cells. Natural killer T cells, in turn, provide help to facilitate antigen-specific CD8 T-cell priming by as-yet poorly understood mechanisms (34, 35), which may involve the reciprocal maturation of DCs (36). It is unknown whether a similar CD1d-mediated DC-natural killer T nexus is facilitated by the Mannide

mono oleate lipid surfactant found in Montanide. However, the absence of any known CD4 antigenic stimulus in Montanide suggests that some component of the lipid chemistry of the adjuvant is supporting long-term CD8⁺ memory to the MHC class I-restricted peptide antigen perhaps by a mechanism similar to that of glycolipid α -galactosyl-ceramide.

Key objectives of this clinical study were to determine whether the gp100_{209-2M} vaccine regimen used resulted in significant immune stimulation in this high-risk patient population, and to characterize interpatient differences in immune response using *ex vivo* analysis and correlated analysis of IVS T cells. Additionally we were interested in determining whether the MHC class I-restricted peptide antigen, gp100_{209-2M}, generated a long-term memory response. Overall, the experimental results demonstrate that resected nonmetastatic melanoma patients can mount a strong antigen-specific immune response when immunized multiple times with the gp100_{209-2M} peptide over a 6-month period, and this response appears to include the induction of long-lived antigen-specific memory CD8⁺ T cells. The resulting data suggest the most accurate method to perform such immunomonitoring analysis in the future should include the combined use of functional and (where possible) assays of TCR specificity, such as tetramer binding, to quantitate antigen-specific immune responses. Additionally, the data also support the general concept of combining direct *ex vivo* functional and tetramer analysis with follow-up duplicate assays of cognate-antigen IVS CD8⁺ T cells, because there may be patient variation in antigen-specific proliferative response by both functionally active and functionally anergic T cells after antigen reactivation. Thus, patient differences in T-cell functional and phenotype changes after IVS compared with baseline *ex vivo* quantitation of antigen-specific, functionally responsive T cells may yet empirically prove to be important index parameters predictive of clinical response. The utility of this approach will only be validated after more extensive clinical studies involving combined *ex vivo* and IVS analysis of antigen-specific T cells have been completed.

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gp100₂₀₉₋₂₁₁ Peptide Immunization of Human Lymphocyte Antigen-A2⁺ Stage I-III Melanoma Patients Induces Significant Increase in Antigen-Specific Effector and Long-Term Memory CD8⁺ T Cells

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