

## Characterization of the Class I-Restricted gp100 Melanoma Peptide-stimulated Primary Immune Response in Tumor-Free Vaccine-draining Lymph Nodes and Peripheral Blood

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**Abstract Purpose:** The aim of this study was to characterize the primary gp100<sub>209-2M</sub>-specific T-cell response in vaccine-draining, metastases-free lymph nodes and peripheral blood of peptide-vaccinated stage I to III melanoma patients.

**Experimental Design:** After two or three gp100<sub>209-2M</sub> vaccinations, sentinel lymph nodes that drained both the primary tumor and adjacent vaccine sites were excised concomitant with wide excision of the tumor. Comparative 7-color flow cytometry phenotype analysis was done on gp100 tetramer-positive CD8<sup>+</sup> T cells from sentinel lymph nodes, closely proximate time-related peripheral blood mononuclear cells (PBMC) collected 2 to 4 weeks after sentinel lymph node excision, and on PBMC collected 6 months later after 7 or 11 more immunizations. Lymph node and peripheral blood T cells were tested for proliferative response, functional avidity, and tumor cell – induced CD107 mobilization.

**Results:** The frequencies of gp100-specific CD8<sup>+</sup> T cells from time-related PBMC and sentinel lymph nodes were comparable and were similar to those reported for virus-specific memory T cells. Their respective *in vitro* proliferation responses were also equivalent but statistically higher than proliferation responses of peripheral blood T cells collected after completion of the entire vaccine regimen. By contrast, functional avidity and CD107 responses were significantly higher in circulating T cells. Sentinel lymph node – derived, gp100-specific CD8<sup>+</sup> T cells predominantly expressed central and effector memory phenotype signatures, whereas there were higher frequencies of effector T cells in the peripheral blood.

**Conclusion:** Priming immunization with gp100<sub>209-2M</sub> without coadministration of CD4<sup>+</sup> helper T cell – restricted antigens induced the effective expansion of peptide-specific central and effector memory CD8<sup>+</sup> T cells with high proliferation potential in vaccine-draining lymph nodes of stage I to III melanoma patients. Lymph node memory T cells gave rise to circulating gp100-specific effector T cells exhibiting increased functional maturation.

Melanoma vaccines have been given primarily to patients with metastatic disease and potentially compromised immunity secondary to large tumor burdens (1–4). Immunomonitoring in these studies indicated that immunization with class I-restricted peptides from melanoma-associated proteins, such as gp100 and MART-1, without antigen-stimulated co-activation of CD4<sup>+</sup> helper T cells resulted in the *in vivo* proliferation of functionally attenuated or senescent peptide-specific CD8<sup>+</sup> T cells

that did not induce long-lasting tumor regression (reviewed in refs. 5, 6). In addition to the analysis of peripheral blood T-cell responses, the priming effects of s.c./intradermal administration of class I-restricted melanoma peptide vaccines on sentinel immunization nodes have also been examined in such patients. Vaccine-draining sentinel immunization nodes were contralateral to the sentinel lymph nodes draining the primary melanoma tumor because these studies sought to test the direct effects of melanoma peptide vaccination under conditions that avoided or minimized potential T-cell activation or suppression caused by endogenous tumor antigens or infiltrating tumor cells, respectively (3, 7, 8). This more comprehensive immunomonitoring strategy provided an opportunity to characterize the induction of local antitumor immunity, and to compare the initial priming response in the vaccine-draining lymph node to the systemic antitumor response of circulating T cells. Overall, lower frequencies of melanoma peptide-specific CD8<sup>+</sup> T cells with diminished proliferative and functional responses were observed in the peripheral blood compared with the lymph node – derived T-cell responses in these sentinel immunization node studies. These data suggested there was attenuation of effector T-cell functional maturation in the peripheral blood after antigen priming in the vaccine-draining lymph node.

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### Translational Relevance

Data presented herein show that class I-restricted melanoma peptide (gp100<sub>209-2M</sub>) immunization in the absence of CD4<sup>+</sup> helper T-cell costimulation can drive effective priming of central and effector memory CD8<sup>+</sup> T cells in vaccine-draining lymph nodes of melanoma patients with early-stage disease. Priming immunization also resulted in the induction of comparable frequencies of peptide-specific T cells in the peripheral blood with predominantly effector phenotype and functional signatures. However, as reported in our recent CCR paper [August 15, 2008;14(16):5270-5283], the long-term memory CD8<sup>+</sup> T cells induced in these patients, after priming and subsequent repetitive immunizations, were functionally attenuated, and did not produce robust anamnestic responses to boosting immunization with cognate peptide administered 1 to 2 years after the initial vaccine regimen. Our data suggest that although class I-restricted peptide immunization stimulated a seemingly normal primary CD8<sup>+</sup> T-cell response, it was not predictive for the maintenance of functionally competent long-term memory T cells. Future melanoma vaccine strategies should not be based on class-I peptide administration only, but should also include concomitant CD4<sup>+</sup> helper T-cell activation.

However, this may have been attributable to the coadministration of interleukin 2 (IL-2) with the vaccine, and the fact that T-cell analysis was done on peripheral blood mononuclear cells (PBMC) collected after five to six additional vaccines (7, 8). Repetitive vaccination over several weeks combined with IL-2 administration could have increased regulatory T cell suppression of peripheral blood T cells, and also induced trafficking of melanoma-specific T cells to tumor sites and other tissues. Consequently, we sought to determine if vaccination of patients with less advanced, nonmetastatic disease, and without any systemic cytokine therapy would provide a better opportunity to characterize the direct effects of class I-restricted melanoma-peptide stimulation on primary T-cell activation in vaccine-draining lymph nodes, and determine if priming immunization also resulted in the effective expansion of circulating tumor-reactive CD8<sup>+</sup> effector T cells exhibiting increased functional maturation.

Herein we report the results of a clinical study in which the class I-restricted melanoma peptide gp100<sub>209-2M</sub> was administered in incomplete Freund's adjuvant adjacent to patients' primary melanoma lesions prior to wide excision and sentinel lymph node biopsy. Patients subsequently received 7 or 11 additional s.c. vaccines over 6 months, and proliferation, functional avidity, and CD107 expression assays of *in vitro* stimulated, sentinel lymph node-derived tumor-specific T cells were done, and compared with responses of gp100-specific T cells in time-related PBMC collected within 2 to 4 weeks of the sentinel lymph node excision, and to T cells collected 6 months later, after the completion of the vaccine regimen. Seven-color flow cytometry phenotype analysis was done on sentinel lymph node- and PBMC-derived T cells. The majority of patients had uninvolved sentinel lymph nodes (i.e. stage I or II disease); to

reduce the possibility of concomitant tumor-induced stimulation or suppression, only such patients with metastases-negative nodes were studied. In contrast to previous reports, our data suggest that class I-restricted melanoma-peptide immunization primed the effective expansion of tumor-reactive central- and effector-memory sentinel lymph node CD8<sup>+</sup> T cells that exhibited heightened proliferation potential, and also gave rise to comparable frequencies of circulating T cells exhibiting increased effector T-cell differentiation and functional maturation. The frequencies of peptide-specific T cells in sentinel lymph nodes and peripheral blood after priming immunization were also comparable with those reported for virus-induced long-term memory T cells (3). The implications of these data for the generation and maintenance of long-term functionally competent self-tumor antigen-specific memory T cells are discussed.

### Materials and Methods

**Patient samples.** Thirty-five HLA-A2<sup>+</sup> patients with resected stage I to III melanoma were randomized to receive gp100<sub>209-2M</sub> peptide in Montanide ISA adjuvant every 2 or 3 wk for 6 mo (9). PBMC collections were made just prior to each vaccine and a leukapheresis was done before treatment and 1 to 2 wk post completion of the initial vaccine regimen (PIVR). The HLA-A2-restricted gp100<sub>209-2M</sub> peptide (NSC# 683472) was provided by the Cancer Therapy Evaluation Program (CTEP) under a National Cancer Institute (NCI) Investigational New Drug Application (BB6123). The protocol was reviewed by CTEP-NCI and approved by the Providence Health System Institutional Review Board. All patients gave their written informed consent before screening for eligibility. Fifteen of the 35 patients who received the first two or three immunizations adjacent to the primary tumor site were scheduled for sentinel lymph node excision, which occurred approximately two weeks after the second or third vaccine. Thereafter vaccinations were rotated among all extremities except the limb in which the lymph node surgery had been done. Lymph nodes from 10 patients contained enough viable cells for cryopreservation and analysis – 3 patients had lymph node metastases and 7 patients did not. Comparative functional and phenotype studies were done using sentinel lymph node T cells, T cells from time-related PBMC collected shortly after (2-4 weeks) sentinel lymph node excision, and PIVR T cells from each patient.

**In vitro stimulation.** Cells from cryopreserved PIVR, sentinel lymph node, and closely proximate time-related interval blood collections (on the day of vaccine 3 or 5) were stimulated *in vitro* with gp100<sub>209-2M</sub> in the presence of IL-15 and low-dose IL-2 for 7 d as described previously (10). Cells were thawed and washed twice in Dulbecco's PBS with 2% human AB serum (Irvine Scientific) before being cultured in X-vivo-15 medium (BioWhittaker) with 5% human AB serum. Cells were plated at  $12.5 \times 10^5$ /mL in 200uL/well in a round-bottom polystyrene 96-well plate in medium containing gp100<sub>209-2M</sub> peptide (0.01 µg/mL) and human IL-15 (50 ng/mL; Peprotech), and cultured for 2 d at 37°C in a 5% CO<sub>2</sub> humidified incubator. On day 2 rhIL-2 (PROLEUKIN; Chiron Corporation) was added to provide a final concentration of 60 IU/mL in each culture well. On day 7, 2 mmol/L EDTA was added to each well for 5 min; cells were harvested, transferred to 5 mL polystyrene tubes, and washed three times in PBS prior to functional avidity or CD107 analysis.

**Seven-color flow cytometry analysis.** Fluorescent antibodies were purchased or fluorescenciated in-house with the use of commercially available QDot antibody conjugation kits (Invitrogen, Molecular Probes). The staining panel consisted of the following: CCR7-FITC (R&D Systems), HLA-A2-restricted gp100<sub>209-2M</sub> (IMDQVPFSV), and gp100<sub>209-217</sub> (ITDQVPFSV) tetramer-PE (iTag; Beckman Coulter), CD8β-PE-TR, CD28-PE-CY7 (Beckman Coulter), CD14 PE-Cy5

(Beckman Coulter), CD19-PE-Cy5 (eBioscience), CD45RA-APC, and affinity-purified CD27 (BD Bioscience, Pharmingen) conjugated to Quantum Dot 605. The use of a "dump cocktail" of CD19- and CD14-PE-Cy5 in combination with 5 µg/mL of 7-aminoactinomycin D (7-AAD; Invitrogen, Molecular Probes) in 1× PBS was employed to stain cells with high cell surface Fc receptor (FcR)-mediated nonspecific binding of antibodies, and to discriminate between live and dead cells (11). All data were acquired on a 9-color Dako Cyan ADP flow cytometer equipped with three diode lasers (488 nm, 635 nm, and 407 nm), and modified with optimal bandpass and dichroic filters (Dako). PBMC and lymph node mononuclear cells were examined by direct *ex vivo* interrogation of unmanipulated freshly thawed cells or 7 d *in vitro*-stimulated cells using the staining panel described above. Viable CD14<sup>+</sup>/CD19<sup>+</sup> lymphocytes were gated for positive CD8β and gp100 tetramer staining, and gp100-specific CD8β<sup>+</sup> T cells were further interrogated for expression of the remaining four cell surface markers (CCR7, CD45RA, CD27, CD28). All data were acquired in FCS format (Summit 4.2) and analyzed using Winlist 5.0 Software (Verity House Software). Computer-assisted digital compensation was done using single-color staining controls via the Hyperlog transform algorithm (Verity House Software; refs. 12, 13). Fluorescence minus one controls were used to set hinged-gating and define histogram regions that distinguished positive from negative events for each fluorescent parameter (11, 13, 14). Fidelity controls ensured that there was no loss of staining frequency and intensity between lower order panels and the corresponding fluorescence for each monoclonal antibody (mAb) in the 7-color panel.

**Data reduction and FCOM analysis.** FCOM (which stands for "combination function") is a parameter in Winlist that can be used to categorize and bin a fluorescent cellular event based on all the combinations of predefined (pregated) fluorescence regions that contain the event. This function uses gated positive regions of multiple fluorescence parameters to enumerate cells in all of the possible subphenotypes as defined by the number of gating parameters (13). The absolute number of gated cells of each defined subphenotype can be assessed and displayed as a discrete peak on the FCOM histogram array. The relative percentage of all cells in each of the subphenotypes was calculated using FCOM analysis. Because the number of subphenotypes for any given sample population is a power function ( $2^n$ , where  $n$  = number of phenotype gates), there were 16 subphenotypes detected with the four antibodies in this study.

**Carboxyfluorescein succinimidyl ester analysis.** PBMC and lymph node cells were thawed, washed in Dulbecco's PBS, and pelleted by centrifugation. Cells were resuspended in prewarmed (37°C) Dulbecco's PBS at  $50 \times 10^6$  cells/mL and loaded with 7 µL of a 0.05 µmol/L working stock of carboxyfluorescein succinimidyl ester (CFSE) in DMSO (CellTrace; Invitrogen) per 1 mL of cell suspension. After CFSE incubation for 10 min at 37°C, staining was quenched by adding 5 volumes of ice-cold culture medium containing 10% human AB serum and incubating on ice for 5 min. Cells were washed three times in Dulbecco's PBS, resuspended at  $1 \times 10^6$ /mL in X-vivo-15 medium supplemented with IL-15 (50 ng/mL), IL-2 (60 IU/mL), and 5% human AB serum, and plated at  $2.5 \times 10^5$ /well (250 µL) in a 96-well plate. Cells were stimulated with 0.0025 µg/well (0.01 µg/mL) of gp100<sub>209-2M</sub> peptide for 2 to 7 d. Cells were collected at different time points and stained with anti-CD3 PE-Cy7 (BD Bioscience), CD8 APC-H7 (BD Bioscience, Pharmingen), and gp100<sub>209-217</sub> tetramer APC (iTag, Beckman-Coulter). CFSE (FITC) staining of pregated viable, gp100 tetramer-positive CD8<sup>+</sup> T cells was analyzed on a Cyan ADP flow cytometer, and the number of cell divisions was calculated from CFSE fluorescence decay data using ModFit software analysis (Verity House Software).

**Functional avidity assay: IFN-γ flow cytometry analysis.** IFN-γ cytokine flow cytometry functional avidity analysis was done using freshly harvested *in vitro*-stimulated T cells.  $1 \times 10^6$  T cells/well were placed in a round-bottomed 96-well plate in 200 µL of X-Vivo-15 medium supplemented with 5% human AB serum (complete medium). Cells were restimulated with gp100<sub>209-2M</sub> or gp100<sub>209-217</sub> peptide at

various concentrations covering a 7-log range (0.00001 µg/mL to 10 µg/mL). The cells were incubated 1 h before brefeldin A (Sigma Chemical) was added at 1.25 µg/well (5 µg/mL). Cells were incubated an additional 14 h before they were collected and stained. Twenty-five microliters of 20 mmol/L EDTA were added to each well for 10 min at room temperature to stop the IFN-γ response before collection. Cells were washed twice with 3 mL of staining buffer and suspended in 50 to 100 µL of staining buffer for subsequent fixation, permeabilization, and staining. Cells were fixed in 1 mL of BD FACS-lysis solution (BD Biosciences) for 10 min at room temperature. All samples were washed in staining buffer, and 500 µL of BD FACS permeabilization solution (BD Biosciences) were added to each tube and incubated at room temperature for 15 min. Permeabilized cells were washed twice, suspended in 50 to 100 µL of staining buffer, and stained with CD3 (FITC)/anti-IFN-γ (phycoerythrin)/CD8 (PerCP-Cy5.5) mAbs (BD Biosciences) prior to incubation at room temperature for 30 min. After staining, cells were washed twice in staining buffer, fixed in 250 µL of 1% paraformaldehyde (Electron Microscopy Sciences), and stored at 4°C in the dark before analysis. Analysis was done within 24 h of staining. Cells were acquired through a standard lymphocyte light scatter gate (90° versus forward angle), and  $5 \times 10^4$  to  $1 \times 10^5$  gated CD8<sup>+</sup>/CD3<sup>+</sup> T cells were analyzed for IFN-γ staining using a FACS Calibur flow cytometer and CellQuest software (BD Biosciences). Net frequencies of IFN-γ-positive cells were calculated by subtracting background antigen negative (media only) values from the frequency of IFN-γ-positive CD8<sup>+</sup> T cells after gp100<sub>209-2M</sub> peptide stimulation. Data were analyzed with Prism 4.0 (GraphPad Software, Inc.). The half maximal effective concentration (EC<sub>50</sub>) values were obtained from nonlinear sigmoidal dose response curves (variable slope, 4-parameter).

**CD107a/b functional assay.** CD107a/b functional analysis was done using a previously described protocol (15).  $1 \times 10^6$  *in vitro*-stimulated T cells were added to 1 mL cultures in complete medium in a 6-well plate. They were stimulated with  $0.25 \times 10^6$  Mel 118 (A2<sup>+</sup>/gp100<sup>+</sup>) or the negative control, Mel 103 (A2<sup>+</sup>/gp100<sup>-</sup>), melanoma tumor cells per well in the presence of 2 µL of CD107 a/b mAb (phycoerythrin) and 1 µL (1,000×) monensin (eBioscience) in each well. Cultures were incubated at 37°C for 6 h after which cells were incubated with EDTA for 10 min at room temperature before they were transferred to staining tubes. Cells were washed twice with 3 mL of staining buffer before staining with gp100<sub>209-2M</sub> tetramer (allophycocyanin) for 1 h at room temperature in the dark. Cells were again washed twice with staining buffer and subsequently stained with CD3 (FITC), and CD8 (PerCP-CY5.5) mAbs for 30 min at 4°C. Cells were washed twice in staining buffer, fixed in 250 µL of 1% paraformaldehyde, and stored at 4°C prior to analysis within 24 h. Cells were analyzed on a FACS Calibur using CellQuest software. Gating and acquisition were as described for the functional avidity assay –  $1 \times 10^4$  to  $2 \times 10^4$  CD8<sup>+</sup>/CD3<sup>+</sup>/tetramer<sup>+</sup> T cells were analyzed for the frequency of CD107a/b expression. Background CD107a/b expression stimulated by the negative control cell line Mel 103 was subtracted from the response induced by Mel 118.

**Statistical analysis.** Data in this study were evaluated using standard descriptive and graphical methods, nonlinear regression analyses, and paired parametric and nonparametric tests. Tests of significance, and 4-parameter logistic nonlinear regression comparative analyses were done using Prism 4.0 (GraphPad Software, Inc.). Two-sided probability values are reported for all tests of significance.

## Results

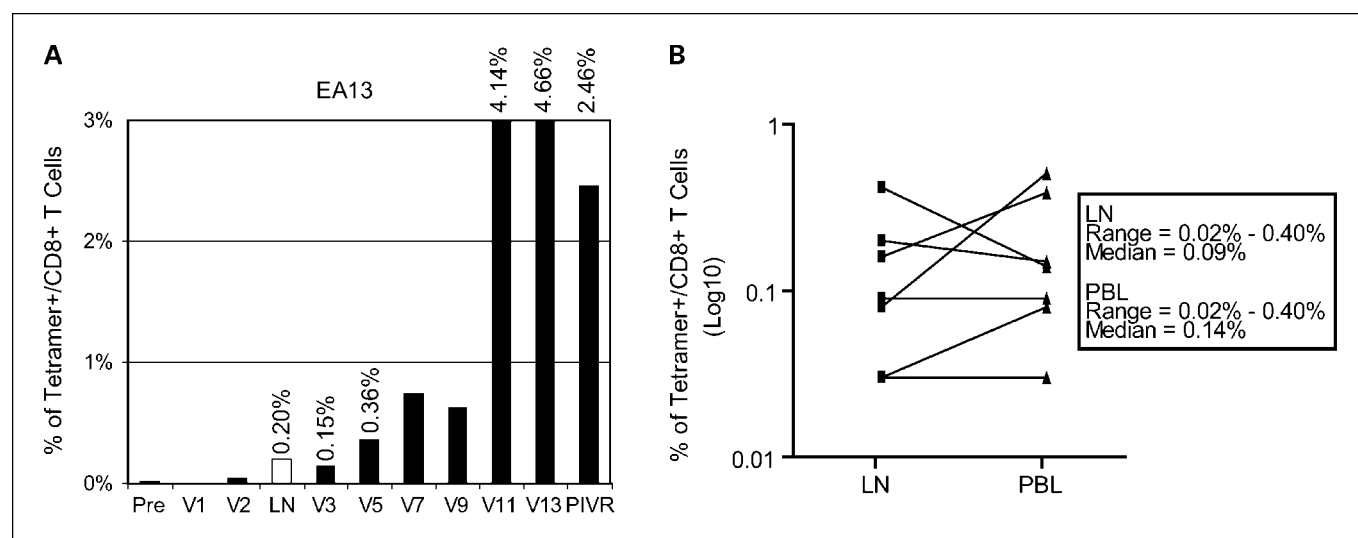
**Gp100<sub>209-2M</sub> vaccination induced comparable frequencies of peptide-specific CD8<sup>+</sup> T cells in sentinel lymph nodes and peripheral blood.** Lymph node excision and cell collections were done two weeks after the second or third vaccine was administered. Sufficient PBMC were not collected at some interval vaccine time points to permit tetramer analysis for

some patients. Gp100-specific CD8<sup>+</sup> T-cell frequencies were also measured for prevaccine and postvaccine regimen (PIVR) leukapheresis PBMC. Tetramer<sup>+</sup> CD8<sup>+</sup> T cells were detected in lymph node samples from 10 of 15 patients studied. Figure 1A shows the *ex vivo* frequency of gp100<sub>209-2M</sub> tetramer<sup>+</sup> CD8<sup>+</sup> T cells from the vaccine-draining sentinel lymph node of a representative patient graphed along with the frequency of tetramer-positive PBMC collected at multiple time points, including a time-related one collected within 2 to 4 weeks of the sentinel lymph node excision. The frequencies of tetramer-positive lymph node T cells were equal to or less than the frequencies of peptide-specific T cells from the time-related samples collected just prior to the third (V3) or fifth (V5) interval vaccine for most patients (Fig. 1B). Patient EA13 had a comparable, but slightly lower peripheral blood peptide-specific T-cell response (Fig. 1A). Only one patient (EA02) had a much lower frequency of gp100-specific T cells in the peripheral blood, and it was still equal to the median value (0.14%) for all seven time-related peripheral blood samples. Thus, within as few as 2 to 3 weeks of the second gp100<sub>209-2M</sub> vaccine the frequencies of circulating peptide-specific CD8<sup>+</sup> T cells equaled or exceeded those of lymph node T cells. The frequencies of lymph node-derived peptide-specific CD8<sup>+</sup> T cells in patients with tumor-free sentinel lymph nodes were equal to or greater than those detected in sentinel immunization nodes from melanoma patients in previous studies (3, 7). This suggested there was minimal or no tumor-directed suppression of vaccine-induced gp100-specific T-cell proliferation in tumor-free nodes in our patients. Additional vaccines were administered at 2- to 3-week intervals, and successive vaccines generally produced increased frequencies of circulating gp100-specific CD8<sup>+</sup> T cells. The highest level of peptide-specific T cells in most patients occurred after the last 1 or 2 vaccines in the immunization regimen or in PIVR leukapheresis (Fig. 1A).

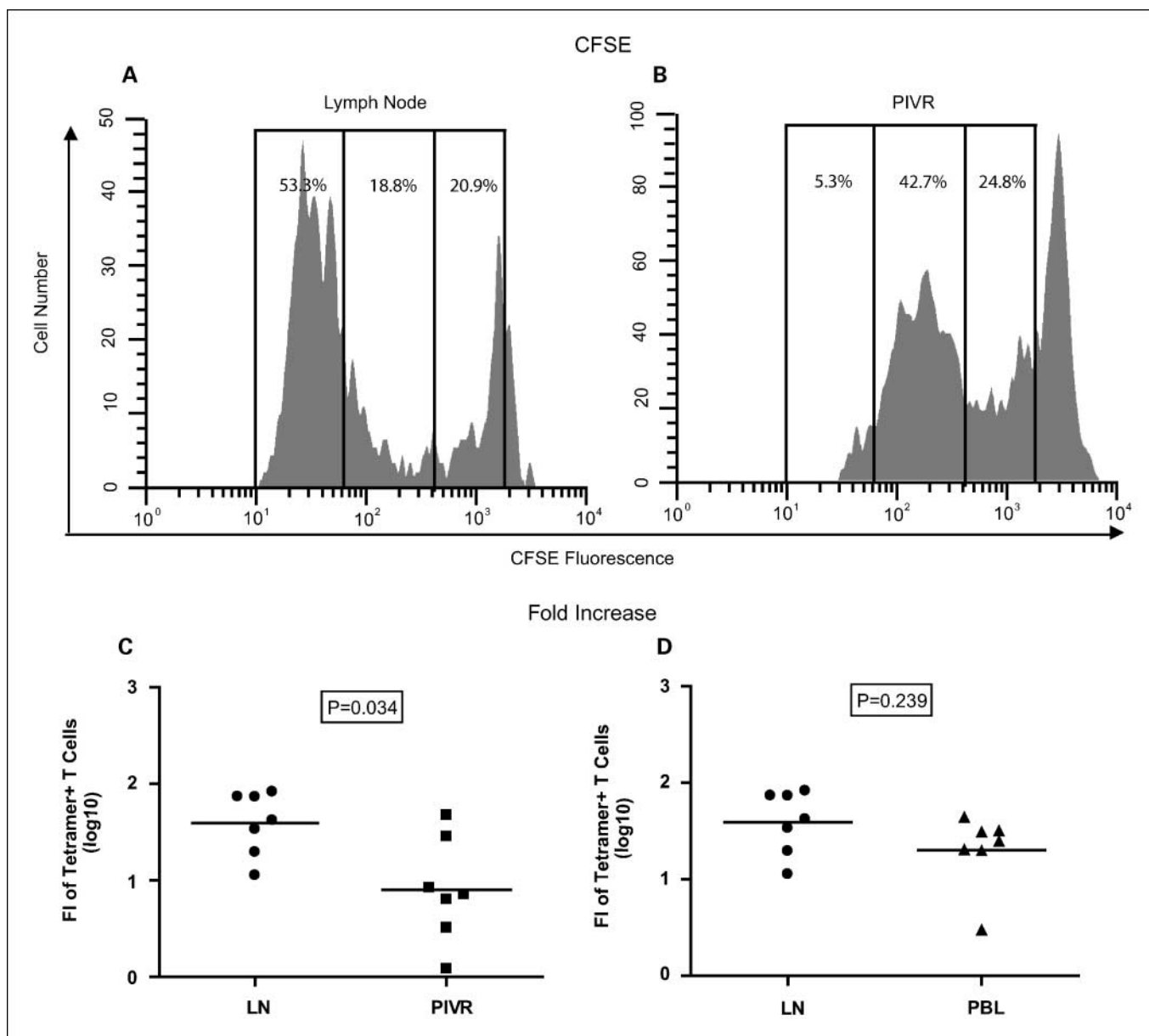
**Gp100 peptide stimulates rapid *in vitro* proliferation of lymph node-derived CD8<sup>+</sup> T cells.** Modfit analysis of the loss of CFSE

fluorescence by lymph node-derived tetramer-positive T cells compared with that of PIVR tetramer-positive T cells after gp100 peptide *in vitro* stimulation showed a more rapid proliferation kinetics of lymph node-derived T cells in most patients. As shown in Fig. 2A, after 3 days in culture 10 times (53.8%) as many lymph node-derived tetramer-positive T cells had divided >6 times compared with peptide-specific PIVR (5.3%) T cells (Fig. 2B). The more rapid proliferation of lymph node-derived T cells was accompanied by an increase in the mean proliferation response (mean fold increase) of *in vitro*-stimulated, peptide-specific lymph node T cells from all 7 patients with tumor-free sentinel lymph nodes. The mean fold increase for lymph node T cells (50-fold) was significantly higher ( $P = 0.034$ ) than the mean fold increase (15-fold) of *in vitro*-stimulated postvaccine regimen T cells (Fig. 2C). By contrast, the mean fold increase of gp100-specific T cells from *in vitro*-stimulated cultures of time-related (vaccine 3/ vaccine 5) PBMC (26-fold) for all 7 patients was not significantly different than the lymph node T-cell response ( $P = 0.239$ ) by paired *t* test analysis (Fig. 2D).

**Functional avidity of peripheral blood tumor antigen-specific CD8<sup>+</sup> T cells is higher than that of lymph node-derived T cells.** Functional maturation of effector T cells as well as long-term memory T cells is characterized by increased functional avidity and cytokine secretion (16–19). PIVR- and lymph node-derived cells were stimulated with low-dose gp100<sub>209-2M</sub> peptide (0.01 μg/mL) and cultured in IL-15 plus low-dose IL-2 for 7 days. The functional avidity of the two *in vitro*-stimulated T cell populations was compared by measuring IFN-γ production after re-exposure to increasing concentrations of the modified and native gp100 peptide in a standard cytokine flow cytometry assay. Nonlinear regression analysis of the IFN-γ response curves was done, and log EC<sub>50</sub> values were calculated to compare the functional avidity of lymph node and PIVR T cells from individual patients. Figure 3A and B show the IFN-γ response curves for lymph node and PIVR *in vitro*-stimulated T cells from a representative patient stimulated with the native (gp100<sub>209-217</sub>)



**Fig. 1.** The frequency of gp100<sub>209-2M</sub> tetramer<sup>+</sup> CD8<sup>+</sup> T cells in vaccine-draining lymph nodes and time-related PBMC. The frequencies of tetramer<sup>+</sup> CD8<sup>+</sup> T cells are shown for the sentinel lymph node and peripheral blood collected prior to sequential vaccines (V3–V13), and PIVR for a representative patient (A). Peptide-specific T cell frequencies for lymph nodes from 6 of 7 patients studied were comparable to or lower than those detected in PBMC samples collected 2 to 6 wk later (B).

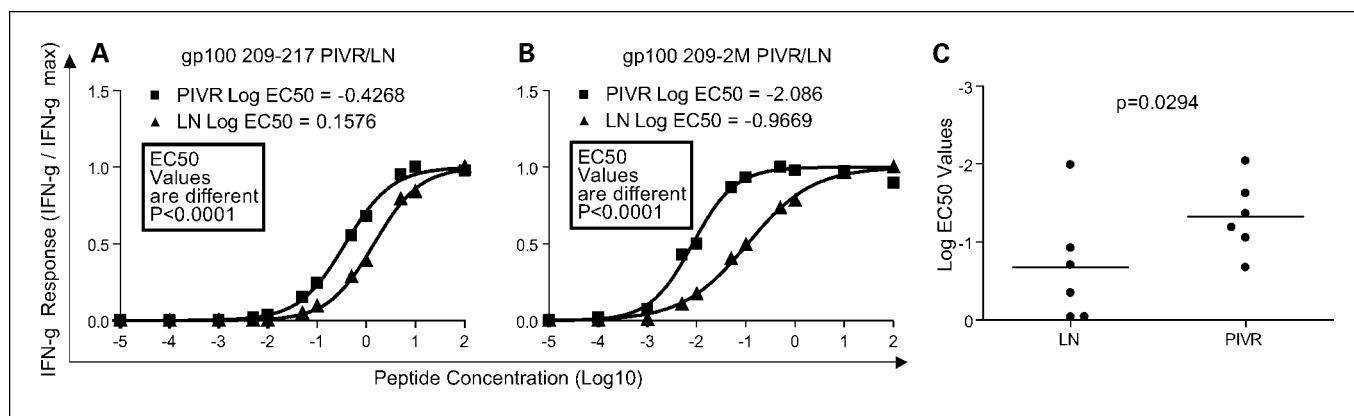


**Fig. 2.** *In vitro* proliferation of lymph node – and PIVR-derived gp100-specific CD8<sup>+</sup> T cells after peptide stimulation. CFSE fluorescence decay of gp100 tetramer<sup>+</sup> CD8<sup>+</sup> T cells from a representative patient after 72 h *in vitro* stimulation with gp100<sub>209-2M</sub> shows that lymph node cells exhibited very rapid proliferation resulting in a 10-fold higher frequency of T cells that had divided >6 times (A) compared with the PIVR T-cell proliferation response (B). Seven-day *in vitro* stimulation with gp100 peptide induced higher proliferation responses by lymph node T cells compared with PIVR cells (C), but these were not higher than the proliferation response of time-related PBMC-derived T cells (D).

and modified (gp100<sub>209-2M</sub>) peptides, respectively. The functional avidity of *in vitro*-stimulated T cells from both PIVR and lymph node cell cultures was higher after stimulation with the modified peptide; PIVR T cells had higher functional avidity (lower log EC<sub>50</sub> values) than lymph node-derived T cells with either peptide stimulus. Six of the seven patients with tumor-free nodes had adequate lymph node cells to do the functional avidity assay. The mean log EC<sub>50</sub> value for PIVR T cells from these patients was significantly lower than the mean log EC<sub>50</sub> of the lymph node-derived T cells ( $P = 0.0294$ ) by paired *t* test analysis (Fig. 3C). This suggested lymph node-derived gp100-specific T cells had lower average functional avidity, but the avidity increased in circulating PIVR gp100-specific T cells after repeated

immunization over 6 months. Similar studies of T cells from time-related, peripheral blood samples, collected shortly after sentinel lymph node excision, were limited by the fact that only 2 of 6 patients had enough vaccine 3 or vaccine 5 PBMC to do the assay. However, for these two samples, the PIVR T-cell and time-related peripheral blood T-cell functional avidity responses were not significantly different in either patient (data not shown) – suggesting that peptide-specific CD8<sup>+</sup> T cells entering the peripheral blood early (following only 2 or 3 vaccines) already exhibited increased functional avidity. Although the functional avidity of circulating tumor-specific T cells for cognate gp100 peptide was higher than that of lymph node-derived T cells, additional analysis was needed to determine if this also





**Fig. 3.** Comparison of functional avidity for PIVR and lymph node gp100-specific CD8<sup>+</sup> T cells. Cytokine flow cytometry analysis of IFN- $\gamma$  expression by *in vitro* – stimulated CD8<sup>+</sup> T cells was done after restimulation with gp100<sub>209-2M</sub> or gp100<sub>209-217</sub> peptide. Log EC<sub>50</sub> values were calculated as an index of functional avidity. Cytokine flow cytometry IFN- $\gamma$  response curves and log EC<sub>50</sub> values for a representative patient show that lymph node T cells exhibited significantly lower functional avidity than PIVR cells when restimulated with either the native (A) or modified gp100 peptide (B). The cumulative mean log EC<sub>50</sub> value for all patient lymph node T-cell responses was significantly higher than the mean log EC<sub>50</sub> value for PIVR T cells (C).

resulted in increased T-cell activation by melanoma tumor-associated gp100 antigen.

**CD107 mobilization following stimulation with melanoma cells is higher in circulating T cells compared with lymph node-derived T cells.** CD107 cell surface expression has been correlated with the secretion of cytolytic molecules such as perforin and granzyme B following CTL activation (20). *In vitro* – stimulated gp100-specific T-cell cultures of tumor-free lymph node cells and PBMC collected shortly after sentinel lymph node excision from 7 patients were re-stimulated *in vitro* with an A2<sup>+</sup>/gp100<sup>+</sup> melanoma cell line, Mel 118, and an A2<sup>+</sup>/gp100<sup>-</sup> negative control melanoma cell line, Mel 103. CD107 expression was measured in a 6-hour assay, and the net frequency of gp100 tetramer<sup>+</sup> CD8<sup>+</sup> T cells that were CD107<sup>+</sup> was calculated for each sample by subtracting the background CD107 fluorescence stimulated by Mel 103. Mel 118-induced activation of T cells resulted in no down-regulation of T cell receptor-specific tetramer staining in this assay (data not shown). As shown in Fig. 4A the mean frequency of CD107<sup>+</sup> tetramer<sup>+</sup> CD8<sup>+</sup> T cells following tumor cell stimulation of PBMC was significantly higher than that of lymph node-derived T cells ( $P = 0.013$ ). There was no significant difference between the mean CD107 mobilization response of PIVR T cells compared with that of time-related T cells for all patients (data not shown). These data showed that the binding affinity for tumor-associated gp100 antigen by T cells from sentinel lymph nodes, as well as from early time-related (vaccine 3/ vaccine 5) and late PIVR PBMC was sufficiently strong to drive a substantial CD107 mobilization response. Moreover, the higher functional avidity of gp100-specific peripheral blood CD8<sup>+</sup> T cells correlated with higher frequencies of CD107<sup>+</sup> tetramer<sup>+</sup> CD8<sup>+</sup> T cells in peripheral blood compared with lymph node-derived T cells. The significantly higher CD107 response of peripheral blood T cells was also associated with much higher frequencies of more fully differentiated effector T cells in the 7-day *in vitro* – stimulated cultures of PBMC compared with those of sentinel lymph node T cells. As shown for a representative patient (Fig. 4B), the dominant subpopulations of CD107<sup>+</sup>/tetramer<sup>+</sup>/CD8<sup>+</sup> T cells were CD45RA<sup>-</sup>/CCR7<sup>-</sup> in *in vitro* – stimulated cultures of both sentinel lymph node (86.4%) and

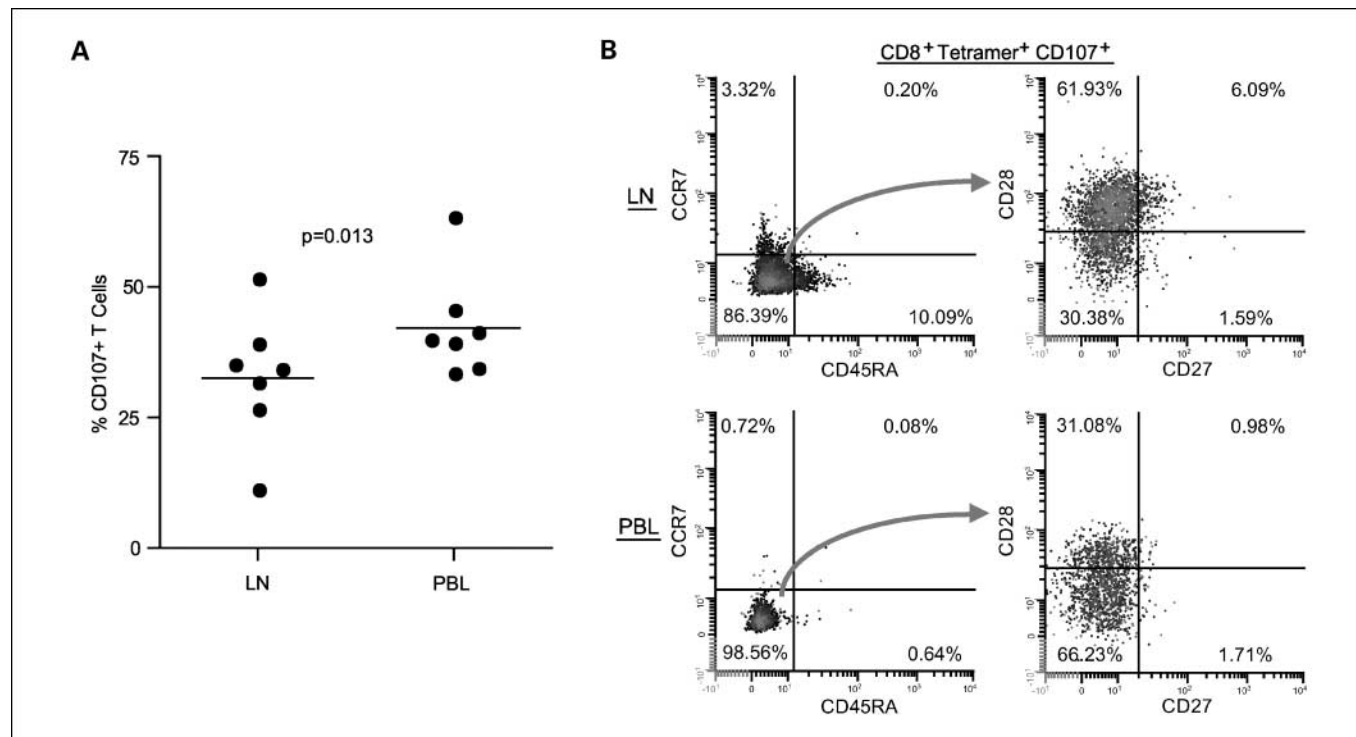
PBMC (98.6%). However, a much higher percentage of CD45RA<sup>-</sup>/CCR7<sup>-</sup> peripheral blood T cells (66.2%) exhibited the more fully differentiated CD45RA<sup>-</sup>/CCR7<sup>-</sup>/CD27<sup>-</sup>/CD28<sup>-</sup> effector T cell phenotype than did sentinel lymph node T cells (30.4%). By comparison, sentinel lymph node CD45RA<sup>-</sup>/CCR7<sup>-</sup> T cells exhibited a significantly higher frequency of cells with a less differentiated late effector-memory phenotype (61.9%), in which CD28 expression was retained (e.g. CD45RA<sup>-</sup>/CCR7<sup>-</sup>/CD27<sup>-</sup>/CD28<sup>+</sup>).

**Gp100-specific lymph node and peripheral blood CD8<sup>+</sup> T cells have different phenotypes.** Only two patients had sufficient numbers of lymph node-derived gp100 tetramer<sup>+</sup> CD8<sup>+</sup> T cells to do 7-color *ex vivo* flow cytometry phenotype analysis. Sentinel lymph node T cells from both patients had similar *ex vivo* phenotype signatures. Figure 5A shows the analysis for the patient with the highest number of tetramer<sup>+</sup> CD8<sup>+</sup> T cells using the following mAb panel: CD8 $\beta$ /gp100 tetramer/CCR7/CD45RA/CD27/CD28/CD14 plus CD19 cocktail. *Ex vivo* analysis of PIVR PBMC from the same patient (Fig. 5B) showed phenotypic differences between lymph node- and PBMC-derived gp100-specific CD8<sup>+</sup> T cells. Gated viable gp100 tetramer<sup>+</sup> CD8<sup>+</sup> T cells were first analyzed for CD45RA and CCR7 expression; subsequently each of the four subpopulations delineated on a CD45RA versus CCR7 dot plot were analyzed for CD27 and CD28 cell surface expression. Lymph node-derived gp100-specific T cells expressed predominantly memory subphenotypes. Although the CCR7<sup>+</sup>/CD45RA<sup>+</sup>/CD27<sup>+</sup>/CD28<sup>+</sup> phenotype has been described as being unique to naïve T cells, we have previously shown it was only detectable in melanoma patients after they received multiple gp100<sub>209-2M</sub> vaccines, and was maintained in the peripheral blood 12 to 24 months after gp100 immunization (15). Thus, this phenotype signature may also characterize a subpopulation of peripheral blood gp100-specific, long-term “early” central memory T cells before the loss of CD45RA expression – a population we have referred to as T<sub>CMRA</sub> (15). The high frequency (~25%) of gp100-specific CD8<sup>+</sup> T cells with this phenotype in the draining lymph node (Fig. 5A) further suggests this phenotype signature is not unique to naïve T cells. Moreover, between 1% and 10% of all peptide-specific

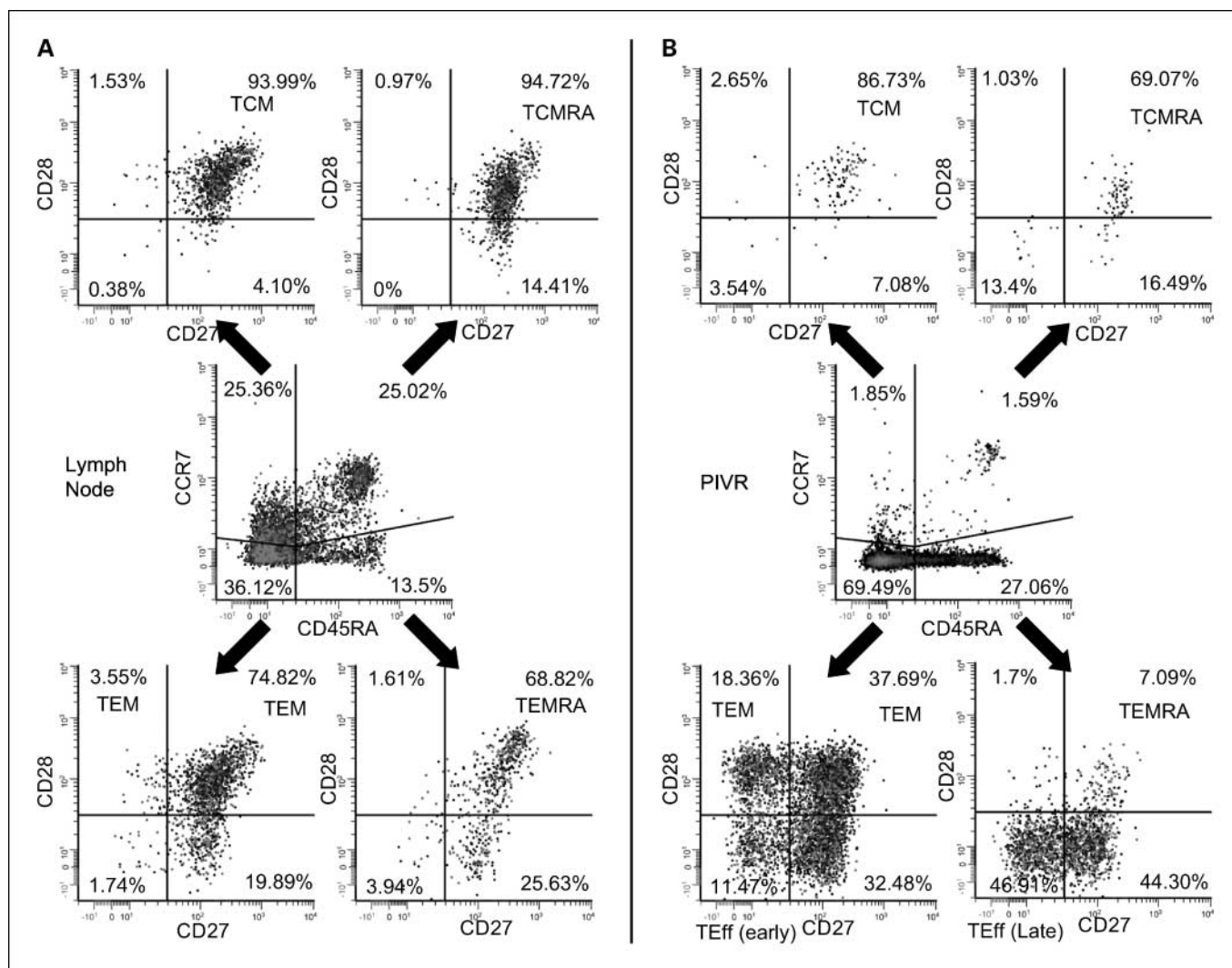
T cells from several 7-day *in vitro*-stimulated expansion cultures exhibited the T<sub>CMRA</sub> phenotype (Fig. 6A) – indicating it can also be expressed by rapidly dividing antigen-educated T cells. A later central-memory (T<sub>CM</sub>) subphenotype, CCR7<sup>+</sup>/CD45RA<sup>-</sup>/CD27<sup>+</sup>/CD28<sup>+</sup>, was predicted by 3- to 4-color consensus models of CD8<sup>+</sup> memory T-cell differentiation (21–24), and previously shown by us to identify a subpopulation of long-term, peripheral blood-derived, gp100-specific memory CD8<sup>+</sup> T cells (15). Similarly, the other dominant lymph node-derived peptide-specific T cells (Fig. 5A) were either early effector-memory (T<sub>EMRA</sub>) characterized by the phenotype CCR7<sup>-</sup>/CD45RA<sup>+</sup>/CD27<sup>+</sup>/CD28<sup>+</sup> (15), or effector-memory (T<sub>EM</sub>) cells expressing the phenotype CCR7<sup>-</sup>/CD45RA<sup>-</sup>/CD27<sup>+</sup>/CD28<sup>+</sup> (15, 25, 26). By contrast, there were very low frequencies of gp100-specific T<sub>CM</sub> or T<sub>CMRA</sub> cells as well as low frequencies of early T<sub>EMRA</sub> CD8<sup>+</sup> T cells in *ex vivo* PIVR PBMC (Fig. 5B). There were, however, comparable T<sub>EM</sub> frequencies in peripheral blood, and higher frequencies of early effector T<sub>EFF</sub> (CCR7<sup>-</sup>/CD45RA<sup>-</sup>/CD27<sup>+</sup>/CD28<sup>-</sup>), and a subpopulation referred to as “terminal-effector” (described herein as T<sub>EFF(LATE)</sub>) because it recapitulates the expression of CD45RA on the effector subphenotype, i.e. CCR7<sup>-</sup>/CD45RA<sup>+</sup>/CD27<sup>+</sup>/CD28<sup>-</sup> (21, 24). Thus, *ex vivo* analysis indicated higher frequencies of effector and effector-memory T cells in the peripheral blood compared with lymph node-derived T cells.

**Gp100-specific effector-memory and central-memory T cells are more prevalent in lymph nodes than peripheral blood.** The low frequencies of gp100-specific T cells in lymph nodes and time-

related blood collections in most patients made it difficult to compare the *ex vivo* subphenotype signatures of lymph node- and PBMC-derived tumor antigen-specific T cells in all but a few patients. However, *in vitro* stimulation with gp100<sub>209-2M</sub> peptide for 7 days in IL-15 and low-dose IL-2 produced sufficient peptide-specific T cells from both lymph node and PBMC cell cultures to compare the dominant subpopulations. The same mAb panel used for *ex vivo* analysis (Fig. 5) was used to phenotype *in vitro*-stimulated T cells. Figure 6A shows that the mean frequency of lymph node-derived early central-memory (T<sub>CMRA</sub>) peptide-specific T cells for all 7 patients with tumor-free nodes was significantly higher than the mean frequency of cells with this phenotype in time-related PBMC ( $P = 0.0149$ ). Similarly, the mean of the sums of the frequencies of T<sub>EMRA</sub> and T<sub>EM</sub> peptide-specific T-cell subpopulations for all 7 patients was also higher ( $P = 0.0236$ ) in lymph node-derived *in vitro*-stimulated cultures compared with peripheral blood *in vitro*-stimulated cells (Fig. 6B). By comparison, the mean frequency of gp100-specific T cells with a more fully activated effector phenotype (Fig. 6C) was higher in peripheral blood compared with lymph node-derived T cells ( $P = 0.0324$ ). Thus, even after cognate peptide *in vitro* stimulation, gp100-specific lymph node-derived CD8<sup>+</sup> T cells with central- and effector-memory phenotypes proliferated and were maintained at higher frequencies than were observed in cultures of time-related PBMC. Conversely, the significantly higher mean frequency of peptide-specific effector T cells in PBMC cultures suggested circulating gp100-specific T cells were



**Fig. 4.** Comparison of CD107 mobilization by lymph node and time-related peripheral blood gp100-specific T cells after *in vitro* challenge with an A2<sup>+</sup>/gp100<sup>+</sup> melanoma cell line, Mel 118. The mean frequency of lymph node-derived tetramer<sup>+</sup> CD8<sup>+</sup> *in vitro*-stimulated T cells expressing CD107 was significantly lower than the frequency of peripheral blood-derived CD107<sup>+</sup> T cells (A). Background CD107 staining induced by the A2<sup>-</sup>/gp100<sup>-</sup> negative control melanoma cell line, Mel 103, was subtracted from Mel 118 stimulated values. The majority of CD107<sup>+</sup> peripheral blood T cells had a CD45RA<sup>-</sup>/CCR7<sup>+</sup>/CD27<sup>+</sup>/CD28<sup>+</sup> effector T-cell phenotype, whereas sentinel lymph node CD107<sup>+</sup> T cells predominately expressed a less differentiated CD45RA<sup>+</sup>/CCR7<sup>-</sup>/CD27<sup>+</sup>/CD28<sup>+</sup> late effector-memory phenotype signature (B).



**Fig. 5.** *Ex vivo* 7-color flow cytometry phenotype analysis of lymph node- and PIVR-derived gp100 peptide-specific CD8<sup>+</sup> T cells from a representative patient. The staining panel consisted of reagents specific for CD8 $\beta$ /gp100 tetramer/CCR7/CD45RA/CD27/CD28/CD14, CD19, 7AAD cocktail. Only viable gp100<sub>209-217</sub> tetramer<sup>+</sup> CD8<sup>+</sup> T cells were selected for analysis from lymph node cells (A), or from PIVR PBMC (B). CCR7 versus CD45RA dot plots divided all tetramer<sup>+</sup> CD8<sup>+</sup> T cells in the lymph node and PIVR sample into four subpopulations. Each of the CCR7 versus CD45RA dot plot quadrants were then analyzed for CD27 versus CD28 staining. Sixteen subpopulations including T<sub>EM</sub>, T<sub>EMRA</sub>, T<sub>CM</sub>, T<sub>CMRA</sub>, and early and late effector (T<sub>EFF</sub>) subphenotypes were delineated for lymph node- and PIVR peptide-specific CD8<sup>+</sup> T cells. Lymph node T cells had higher frequencies of T<sub>EM</sub>/T<sub>EMRA</sub> and T<sub>CM</sub>/T<sub>CMRA</sub> subphenotypes whereas the PIVR PBMC exhibited higher frequencies of early and late effector T cells.

more readily activated *in vitro* to differentiate to an effector phenotype than lymph node-derived T cells.

## Discussion

Tumor-draining sentinel lymph nodes are the first lymphoid organ encountered by micrometastatic tumor cells or particulate/soluble tumor antigen released from a primary tumor. Tumor antigen capture, processing, and presentation by lymph node dendritic cells to naïve, resident T cells and direct T-cell activation by tumor cells may play a central role in priming tumor-specific immunity (reviewed in ref. 27) – similar to the localized lymph node T-cell response to viral infections (28, 29). Clinical studies have also shown that tumor cell infiltration can establish a tolerogenic environment within tumor-draining lymph nodes by several mechanisms including tumor-induced activation of regulatory T cells (30), trans-

forming growth factor- $\beta$ 1-stimulated dendritic cell apoptosis (31), and IL-10-mediated inhibition of T-cell proliferation (32; reviewed in ref. 27). Sentinel lymph nodes are thus potentially critical sites for primary tumor-antigen specific T-cell activation or tolerance induction, which may occur as a consequence of immunotherapy designed to enhance the mobilization of autologous tumor antigen and increase the uptake, processing, and cross-presentation by antigen presenting cells. Similarly, the effectiveness of s.c./intradermal tumor antigen vaccines may depend on the strength of the primary immune response, and the resulting expansion of central-memory T cells (T<sub>CM</sub>) that occur in vaccine-draining lymph nodes (33, 34). More comprehensive immunomonitoring strategies that include the comparison of tumor-specific immune function and phenotype signatures of lymph node-derived T cells, PBMC, and tumor-infiltrating T cells in individual patients may improve our understanding of the

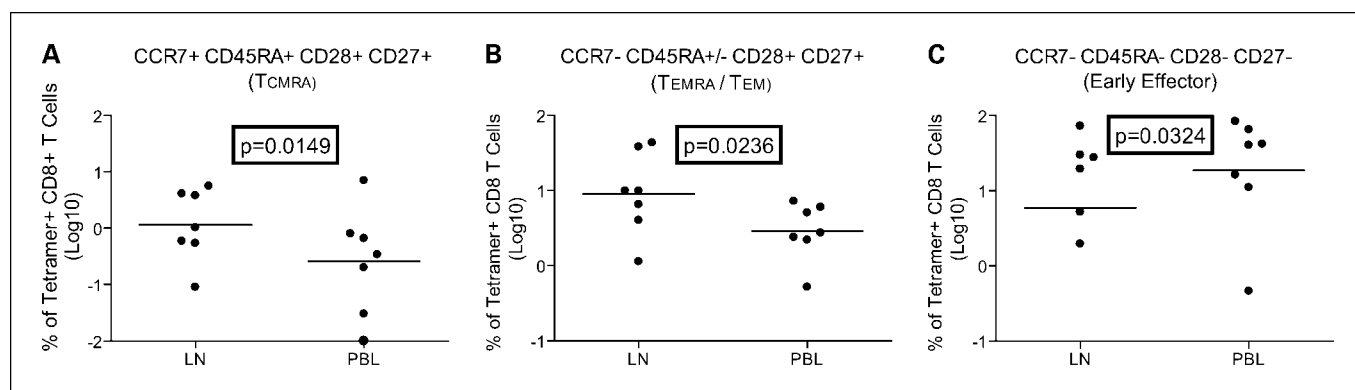


positive and/or negative modulation of antitumor immunity induced by a particular immunotherapy strategy.

Previous melanoma vaccine studies have attempted to examine the priming effect of s.c./intra-dermal immunization with class I-restricted melanoma peptides on T cells from sentinel immunization nodes in patients with advanced disease (3, 7, 8). Results indicated that stage III melanoma patients immunized with a Melan-A peptide in QS21 adjuvant (3), or stage IV patients immunized with gp100 and tyrosinase peptides in an emulsion of granulocyte monocyte colony-stimulating factor and Montanide ISA51 (7, 8) exhibited elevated levels of peptide-specific CD8<sup>+</sup> T cells in vaccine-draining lymph nodes after 2 or 3 vaccines compared with their frequencies in the peripheral blood, or in tumor-draining metastases-positive contralateral sentinel lymph nodes. In these clinical studies sentinel immunization node-derived T cells proliferated better and produced significantly more IFN- $\gamma$  than peripheral blood or tumor-draining sentinel lymph node T cells after *in vitro* stimulation with cognate peptide. The lower frequency of circulating peptide-specific CD8<sup>+</sup> T cells and their lower proliferative and functional responses could have resulted from the coadministration of IL-2 in these trials because IL-2 may have increased regulatory T-cell suppression of peripheral blood T cells collected several weeks after sentinel immunization nodes were excised and following five to six additional vaccines. The presence of advanced disease in these patients may have also induced suppression of tumor antigen-specific T cells. In contrast, our analysis of gp100<sub>209-2M</sub> priming of T cells in tumor-free vaccine-draining sentinel lymph nodes showed the frequencies of gp100-specific CD8<sup>+</sup> T cells in time-related PBMC collected within 2 to 4 weeks of the sentinel lymph node excision were comparable with those detected in sentinel lymph nodes from the same patients. Our data further indicated that in melanoma patients with no evidence of metastatic disease, class I-restricted melanoma peptide immunization primed the expansion of tumor-reactive CD8<sup>+</sup> T cells with central and effector memory phenotypes in sentinel lymph nodes that also exhibited heightened *in vitro* proliferation responses characteristic of memory T cells (16), and, in the absence of IL-2, gave rise to comparable frequencies of circulating effector T cells exhibiting increased functional maturation. Practical limitations prevented any direct compar-

ison of the gp100 peptide-specific primary immune response with the primary immune response stimulated by viral infections such as cytomegalovirus and influenza. However, the frequency of gp100-specific sentinel lymph node and peripheral blood CD8<sup>+</sup> T cells was comparable with that reported for lymph node-derived influenza-specific memory T cells (3), and for circulating memory T cells specific for hantavirus, hepatitis C virus, EBV, and cytomegalovirus (35–37). This suggested gp100<sub>209-2M</sub> primary immunization was as effective as viral antigen-activation of memory T-cell expansion.

Several animal studies have shown that, although primary CTL responses to viral and bacterial antigens can occur in the absence of CD4<sup>+</sup> helper T cell activation, the resulting memory CD8<sup>+</sup> T cells were defective in their ability to produce anamnestic responses after secondary stimulation (38–40). A recent study from our group also showed that repetitive immunization with a class I-restricted melanoma peptide similarly resulted in the development of long-term memory T cells with attenuated anamnestic responses upon secondary tumor antigen challenge (15). In order to characterize the anamnestic response of long-term gp100 peptide-specific memory T cells, 16 patients who had remained disease free for >12 months after completion of the initial vaccine regimen (9, 10) were given multiple peptide-boosting immunizations (15). The low frequencies of circulating long-term peptide-specific memory CD8<sup>+</sup> T cells observed in most patients in the trial prior to boosting were comparable with the frequencies of cytomegalovirus-specific and influenza-specific memory T-cells in the same patients. We observed that although circulating gp100-specific long-term memory T cells expressed some properties of competent memory T cells, such as heightened *in vitro* peptide-stimulated proliferation and an increase in central memory (T<sub>CM</sub>) differentiation, when compared with PIVR T-cell responses measured after the initial vaccine regimen, they did not exhibit the enhanced functional avidity which is usually associated with competent memory T-cell maturation (17, 19). Notably, although boosting immunization did drive a modest anamnestic response that resulted in increased frequencies of circulating gp100-specific T cells that were higher than those of long-term memory T cells in most patients, they were nonetheless attenuated in that they did not equal or exceed the frequencies of peptide-specific



**Fig. 6.** Phenotype analysis of gp100-specific CD8<sup>+</sup> T cells from *in vitro* – stimulated cultures of lymph node cells and time-related PBMC from 7 patients with tumor-free nodes. Gp100 peptide-specific CD8<sup>+</sup> T cells from lymph node cultures had significantly higher mean frequencies of subpopulations with early central-memory (A) and effector-memory (B) phenotypes compared with PBMC-derived, *in vitro* – stimulated T cells. By comparison, the mean frequency of PBMC-derived early effector CD8<sup>+</sup> T cells was significantly elevated compared with the mean frequency of early effector T cells in lymph node *in vitro* – stimulated cultures (C).

CD8<sup>+</sup> T cells measured at the end of the initial vaccine regimen in the majority (75%) of patients. Four of the seven patients with metastases-negative lymph nodes analyzed in this study were also included in our previous analysis of the anamnestic response of long-term gp100 peptide-specific CD8<sup>+</sup> T cells after boosting immunization (15). All four patients (EA02, EA29, EA28, and EA35) exhibited the maintenance of peripheral blood memory T cells 12 to 24 months after the initial vaccine regimen (range, 0.05-0.2%). However, as with all patients in the study, boosting immunization did not increase the frequency of circulating peptide-specific T cells above the PIVR levels. There was also no statistical difference in functional avidity, CD107 expression, or memory phenotype signatures between long-term memory and post-boosting immunization peripheral blood T cells (15).

Several factors may work in concert to contribute to the failure of class-I-restricted peptide vaccines to elicit long-term memory T cells capable of exhibiting strong anamnestic responses. The repetitive vaccination required to break tolerance to self-tumor antigens may also induce suppressive mechanisms similar to those that impair the normal development of memory CD8<sup>+</sup> T-cell differentiation and function observed following persistent chronic viral infection (41–43), or in settings of persistent tumor antigen challenge (44–46). Thus, other, as yet poorly understood, suppressive mechanisms resulting from chronic antigen exposure may “exhaust” both the proliferative and functional potential of tumor-specific T cells (reviewed in ref. 21). Of major significance is the fact that class I melanoma peptide vaccines have been administered without the coadministration of melanoma-associated antigens capable of stimulating CD4<sup>+</sup> helper T-cell function. Recent data from several groups have clearly established the critical requirement for CD4<sup>+</sup> helper T cells for the induction and maintenance of memory CD8<sup>+</sup> T cells. Although class I-restricted antigen activation alone can stimulate CD8<sup>+</sup> T-cell proliferation and functional activation, the absence of helper T cells results in CD8<sup>+</sup> memory T cells with diminished functionality, antigen-independent homeostasis-driven maintenance, and, most importantly, the inability to exhibit a strong anamnestic response to secondary pathogen or tumor antigen challenge (38–40; reviewed in ref. 47). Disagreement exists as to whether helper T cells are required only at priming to “program” normal memory CD8<sup>+</sup> T cell expansion (38), or are also required for the long-term maintenance of competent memory CD8<sup>+</sup> T cells after priming immunization (48, 49). Thus, the lack of helper T cell stimulation at priming in our clinical trial could have resulted in the inadequate programming of long-term memory CD8<sup>+</sup> T cells capable of robust

anamnestic response. However, the data presented herein suggest the attenuated anamnestic response of class I-restricted melanoma peptide-induced memory T cells observed in our initial study (15) cannot be directly deduced or in any way predicted from the phenotype and functional characterization of the primary gp100-specific T cell immune response. This is true because primary immunization induced the effective expansion of predominately T<sub>CM</sub>/T<sub>EM</sub> subpopulations of gp100-specific memory T cells in sentinel lymph nodes with high proliferation potential, and comparable frequencies of peptide-specific T cells with enhanced functional maturation in time-related peripheral blood samples. Moreover, the size of the primary response, reflected by the frequencies of sentinel lymph node and peripheral blood gp100-specific T cells, was comparable with those of virus-specific memory T cells.

In conclusion, our data suggest that modified class I-restricted melanoma peptide antigens such as gp100<sub>209-2M</sub> in the absence of CD4<sup>+</sup> helper T cell coactivation are fully capable of driving primary expansion of tumor-reactive memory T cells in tumor-free draining lymph nodes and in peripheral blood. However, this seemingly effective primary response does not otherwise result in the maintenance of fully competent long-term memory T cells capable of strong anamnestic response to secondary antigen challenge, and is thus not predictive for the induction of potentially therapeutic CD8<sup>+</sup> T cell mediated antitumor immunity. Other vaccine strategies such as the incorporation of CD4<sup>+</sup> helper T cell coactivation at priming will be required to induce long-term memory CD8<sup>+</sup> T cells that are also functionally competent. Recent reports have identified potential class II-restricted peptides from melanocytic proteins such as tyrosinase, Melan-A/MART-1, and gp100, which bind to dominant HLA-DR haplotypes, and stimulate CD4<sup>+</sup> T cell proliferation and delayed type hypersensitivity responses in immunized melanoma patients (50). The combination of such potential CD4<sup>+</sup> helper T cell-specific antigens with class I-restricted melanoma peptides may result in clinically more effective melanoma vaccines.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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