

# Functional Analysis of Antigen-Specific T Lymphocytes by Serial Measurement of Gene Expression in Peripheral Blood Mononuclear Cells and Tumor Specimens

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The cloning of cancer Ags recognized by T cells has provided potentially new tools to enhance immunity against metastatic cancer. The biological monitoring of effective immunization has, however, remained a dilemma. We describe here a sensitive molecular quantitation methodology that allows analysis of in vivo immune response to vaccination. Metastatic melanoma patients were immunized with a synthetically modified peptide epitope (209-2M) from the melanoma self-Ag gp100. Using serial gene expression analysis, we report functional evidence of vaccine-induced CTL reactivity in fresh cells obtained directly from the peripheral blood of postimmunized patients. Further, we demonstrate in vivo localization of vaccine-induced immune response within the tumor microenvironment. The results of these molecular assays provide direct evidence that peptide immunization in humans can result in tumor-specific CTL that localize to metastatic sites. *The Journal of Immunology*, 1999, 163: 6867–6875.

The existence of systemic and local CTL that recognize tumor Ags provides strong evidence that natural immune response can exist against metastatic cancer in humans. The ease by which melanoma-specific tumor-infiltrating lymphocytes can be isolated from metastatic deposits illustrates this awareness by the immune system (1). However, the presence of melanoma-specific killer cells does not arrest tumor growth. The recent identification and cloning of melanoma-associated Ags recognized by T cells (2, 3) have provided potentially new tools to enhance natural immunity by the same approach that has been successful in the immunization against foreign pathogens. One such self-Ag, gp100, was identified and shown to be a nonmutated differentiation Ag expressed by cells of melanocytic lineage including melanomas, normal melanocytes, and pigmented retinal cells (4). Previously, we reported our experience with the treatment of metastatic melanoma patients with a synthetically modified (to enhance HLA A-2<sup>+</sup> binding) gp100 peptide (209-2M)<sup>2</sup> that was emulsified in IFA (5). In vitro sensitization (IVS) and expansion assays of PBMC demonstrated that immunization with the modified peptide was superior to the parental (pa) g209-217 peptide in the induction of CTL reactivity against the 209-pa epitope and HLA-A2<sup>+</sup> melanoma cells. Despite measured in vitro PBMC reactivity in 10 of 11 patients who received the 209-2M peptide, no overall clinical responses were observed, although 3 patients exhibited mixed response with complete or partial regression of some lesions. Interestingly, 209-2M immunization combined with the systemic administration of high dose bolus IL-2

resulted in clinical tumor regression in 13 of 31 patients (42%), but only 3 of 19 (16%) patients demonstrated peripheral blood reactivity in the in vitro sensitization assay.

In an attempt to reconcile the dichotomy observed between in vitro generated PBMC reactivity and in vivo tumor regression, we developed a sensitive molecular assay to directly detect specific low precursor CTL reactivity in bulk PBMC and in the local tumor microenvironment. In previous studies involving the immunization of cancer patients, monitoring of immune reactivity has involved single or multiple in vitro restimulation of PBMC to generate cultures with detectable reactivity (5, 6). More direct PBMC analysis with tetrameric HLA/peptide complexes (tHLA) has allowed for measurement of epitope-specific T cell precursor frequency (7) but has been limited by the sensitivity of FACS detection. Further, traditional tetramer analysis does not provide direct assessment of immunological reactivity of the identified cells. The local tumor site, the ultimate target of immunization, has rarely been examined serially in vaccine trials.

In this study, using molecular gene quantitation techniques, we report evidence of specific CTL reactivity in fresh cells obtained directly from the peripheral blood of patients immunized with the 209-2M peptide. We also demonstrate in vivo localization of vaccine-induced immune response by prospectively measuring therapy-specific changes within the tumor microenvironment from sequentially obtained fine needle aspirates of melanoma tumors.

## Materials and Methods

### Peptides

Each of the peptides utilized in this study was prepared according to Good Manufacturing Practice by Multiple Peptide Systems (San Diego, CA). The identity of each of the peptides was confirmed by mass spectral analysis. Peptide sequences are described below with their applications.

### Cultured cell lines

The melanoma cell lines 624.38 Mel (HLA-A2<sup>+</sup>), 624.28 Mel (HLA-A2<sup>-</sup>), 888 (A2<sup>+</sup>) Mel (HLA-A2<sup>+</sup>), and 888 Mel (HLA-A2<sup>-</sup>) were established in the Surgery Branch, National Cancer Institute, and cultured as described (8).

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<sup>2</sup> Abbreviations used in this paper: g209-2 M, gp100:209217 (210 M); IVS, in vitro sensitization; pa, parental; FNA, fine needle aspiration; CM, culture medium; sHLA, soluble HLA.

### Clinical protocols

All patients had histologically confirmed metastatic melanoma and had undergone a complete clinical evaluation including measurement and radiographic imaging of all evaluable tumor sites. HLA typing and subtyping for HLA class I was determined on patients' PBL with sequence-specific primer-PCR. All patients provided informed consent before treatment and were verified to have been free of any treatment in the prior month, nor were they receiving immunosuppressive treatment including steroids. Before treatment, patients underwent leukapheresis. PBMC were isolated by Ficoll-Hypaque (ICN, Aurora, OH) separation and were cryopreserved at  $10^8$  cells/vial and stored at  $-180^\circ\text{C}$ . In addition, patients underwent fine needle aspiration (FNA) of s.c. metastases immediately before treatment; sampling of the same lesion was repeated after 6–7 wk. Thus, because immunizations were administered at 3-wk intervals, PBMC and FNA were obtained simultaneously before treatment and 3–4 wk after the second vaccination. Aspirate material was examined and verified to contain melanoma cells at the bedside by a cytopathologist. The remainder of the aspirate was placed in ice cold culture medium (CM) consisting of RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10 mM HEPES buffer, 100 U/ml penicillin-streptomycin (Biofluids), 0.03% L-glutamine (Biofluids), and 10% heat-inactivated FBS (Biofluids). The sample was immediately brought to the laboratory, centrifuged, placed in RNA lysis buffer (Qiagen, Santa Clarita, CA), and stored at  $-180^\circ\text{C}$  until RNA isolation was performed.

Two sets of experiments were performed. First, a pilot study was done to assess whether gene measurement techniques could provide information comparable to the assessment of T cell reactivity obtainable with *in vitro* sensitization experiments as previously described (5). In this first study, vaccination-specific immune reactivity in pre- and postimmunization PBMC was retrospectively compared in 10 patients who had undergone vaccination with a combination of four peptides including gp100, 209-2M (IMQVPFSV); gp100, 280-9V (YLEPGPVTV); MART-1 27–35 (AAGIGILTV), and tyrosinase 368–370D (YMDGTMSQV). Each peptide (1 mg) was administered s.c. emulsified in IFA (Montanide ISA-51, Seppic, France) at 3-wk intervals. Immune reactivity against the 209-pa peptide (ITQVPFSV) (Table I) was analyzed 3–4 wk after the second immunization. Only 209-pa reactivity elicited by vaccination with 209-2M was assessed given that prior *in vitro* sensitization analysis found no evidence of induction of PBMC reactivity toward the other epitopes.

A second set of experiments was performed after this feasibility study. We evaluated prospectively whether induction of vaccine-specific CTL reactivity could be detected by simultaneous monitoring of tumor and peripheral CTL. With this goal, blood and FNA samples were prospectively collected from patients undergoing simultaneous treatment protocols. Evaluation of response to vaccination was then first tested with the standard *in vitro* sensitization methods previously described (5, 9). According to results obtained with *in vitro* sensitization, the patients were divided, before testing with gene expression methods, into three groups defined by the immunological outcome of the treatment received. The first group included patients who received treatment protocols that had shown evidence of effectiveness in inducing enhancement of vaccine-specific CTL reactivity according to *in vitro* sensitization of pre- and postvaccination PBMC. Nine patients (11 lesions) received "209-2M-based" vaccines: 2 the 209-2M peptide and 7 the modified ES-209-2M peptide (amino-terminal conjugation to adenoviral endoplasmic reticulum signal sequence). Both peptides were emulsified in IFA and administered s.c. at 3-wk intervals as described previously (5). These two treatment categories were combined into one immunological group because previous *in vitro* sensitization experiments had suggested that the two modalities of g209-2M delivery achieved comparable immunological results with detectable enhancement of g209-pa specific reactivity (data not published). A second group of patients (10 patients/11 lesions), "non-209-2M-based," received immunization with naked DNA encoding the full length gp100 protein with the 209-2M modification (8 patients), the tyrosinase (369–370D) peptide in IFA (1 patient), or adoptive transfer of *in vitro* generated autologous 209-pa-reactive CTL (1 patient). This group was considered immunologically unresponsive to the treatment, because comparative *in vitro* sensitization of pre- and postvaccination PBMC had in no case shown evidence of treatment-specific enhancement of T cell reactivity. A final group of patients (8 patients/9 lesions), "IL-2 based," received concomitant IL-2 (Cetus-Oncology Division, Chiron, Emeryville, CA) at a dose of 720,000 IU/kg administered as an i.v. bolus during 15 min starting 1 day after immunization. In this group of patients, immunizations included the ES-209-2M, the gp100 (17–25) (ALLAVGATK), or the tyrosinase (240–244S) DAEKSDICTDEY or (206–214) (AFLPWHLRF) peptides. IL-2 dosing was continued every 8 h until grade 3 or 4 toxicity was reached or to a maximum of 12 doses (10). All peptides were administered s.c. by injecting 1 mg peptide emulsified in

Table I. Quantitative RT-PCR primers and probes

IFN- $\gamma$	(+)-AGCTCTGCATCGTTTTGGGTT <sup>a</sup> (-)-GTTCCATTATCCGCTACATCTGAA FAM-TCTTGGCTGTTACTGCCAGGACCCA-TAMRA
GM-CSF	(+)-CCCGCTGGAGCTGTACA (-)-AATCTGGGTGACAGGAAAGTTT FAM CCCTTGACCATGATGGCCAGCC-TAMRA
TNF- $\alpha$	(+)-CCCCAGGGACCTCTCTAATC (-)-TACAACATGGGCTACAGGCTTG FAM-CAGTCAGATCATCTTCTCGAACCCGAG-TAMRA
IL-2	(+)-ACCAGGATGCTCACATTTAAGTTTT (-)-GAGGTTTGAGTCTTCTTCTAGACACTG FAM-CATGCCCAAGAAGGCCACAGAAGCTG-TAMRA
CD25	(+)-AGCCAGTGGACCAAGCGA (-)-TGTGGCTTCATTTTCCCATG FAM-TCCAGTCACTGCAGGGAACCTCC-TAMRA
CD69	(+)-AACAGCTCTTTCATCCGGA (-)-ACCCTTCATGACGTGTTGAGAA FAM-CAAGAAAATGATGCCACCAGTCCCA-TAMRA
CD8	(+)-CCCTGAGCAACTCCATCATGT (-)-GTGGGCTTCGCTGGCA FAM-TCAGCCACTTCGTGCCGGTCTTC-TAMRA
CD4	(+)-GTCCCTTTTAGGCACTTGCTTCT (-)-TCTTCCCTGAGTGGCTGCT FAM-TGCTGCAACTGGCGTCTCTCC-TAMRA
gp100	(+)-GGTTCCTTTTCCGTCACCTT (-)-CTCACCGGACGGCACAG FAM-ACATTGTCCAGGGATTGAAAGTGCCGAGAT-TAMRA
$\beta$ -Actin	(+)-GGCACCCAGCACAAATGAAG (-)-GCCGATCCACAGGAGTACT FAM-TCAAGATCATTGCTCTCTCTGAGCGC-TAMRA

<sup>a</sup> (+), forward primers; (-), reverse primers; FAM-TAMRA, probe. Concentrations used: 400 nM primers, 150 nM probe.

IFA. As previously noted (5), the concomitant administration of IL-2 to vaccines nullified the ability to detect vaccine-specific T cell reactivity in posttreatment PBMC. Thus, these patients were grouped in a separate immunological group because they had received a potentially immunogenic treatment (peptide vaccination in IFA), yet no evidence of immunization could be detected in standard *in vitro* sensitization assays. Although heterogeneous, these treatments were all administered at 3-wk intervals, and all samples were collected immediately before treatment and 3–4 wk after the second vaccination.

### Epitope-specific T cell staining with HLA-A2 tetramers

PE-tHLA complexes were synthesized as described previously (7). Recombinant HLA-A\*0201 heavy chain containing a biotinylation site and recombinant  $\beta_2$ -microglobulin were synthesized and used for refolding of soluble HLA (sHLA) molecules in the presence of a HLA-A\*0201 binding peptide. sHLA molecules were prepared for the following epitopes: g209-pa; g209-2M and Flu M1:58–66 (GILGFVFTL). All peptides were commercially synthesized and purified by gel filtration (Princeton Biomolecules, Columbus, OH). The refolding reaction was dialyzed and concentrated for purification of correctly refolded sHLA on gel filtration. Monomeric sHLA was biotinylated with BirA (Avidity, Denver, CO) at the heavy chain and separated from free biotin by gel filtration. Biotinylated sHLA was tetramerized by adding avidin-PE (Pierce, Rockford, IL) at a 4:1 molar ratio. The final concentration of tetramer was adjusted to 2  $\mu\text{g}/\text{ml}$  for g209 and g209-2M tHLA and to 1  $\mu\text{g}/\text{ml}$  for Flu tHLA. As examined by gel filtration, all tHLA were without detectable free avidin-PE. After overnight depletion of monocytes, nonadherent PBMC were resuspended at  $10^6$  cells/50  $\mu\text{l}$  ice cold FACS buffer (phosphate buffer plus 5% inactivated FCS, Biofluids), and cells from day 10 CTL cultures were washed and resuspended at  $2 \times 10^5$  cells/50  $\mu\text{l}$  cold FACS buffer. Cells were incubated

on ice with 1  $\mu\text{g}$  tHLA for 15 min, and incubation was then continued for 30 min with 10  $\mu\text{l}$  anti-CD8 mAb (Becton Dickinson, San Jose, CA). Cells were washed twice in 2 ml cold FACS buffer before analysis by FACS (Becton Dickinson). Two hundred thousand events were acquired. tHLA staining specificity was previously established by extensive analysis of T cell clones specific for each of the described epitopes and by comparative analysis of short term CTL cultures also specific for the above epitopes (11).

#### *In vitro sensitization assessment of peptide-specific CTL reactivity*

As previously described (5), cryopreserved PBMC were thawed into CM. Cells were plated at  $3 \times 10^6$  PBMC in 2 ml medium with 1  $\mu\text{M}$  peptide. IL-2 (300 IU/ml) was added on day 2, and cells were harvested between days 11 and 13 after initiation of the culture. The harvested cells were then stimulated with T2 cells pulsed with 1  $\mu\text{M}$  peptide for 18–24 h at 37°C. IFN- $\gamma$  release into the supernatant was measured by a standard ELISA assay. Reactivity was scored as positive (+) if IFN- $\gamma$  release was twice background and  $>100$  pg/ml.

#### *RNA isolation and cDNA synthesis*

RNA isolation from PBMCs or fine needle aspirate biopsies was performed in batches containing patient pre- and posttherapy samples with RNeasy mini kits (Qiagen). The RNA was eluted with water and stored at  $-70^\circ\text{C}$ . For cDNA synthesis,  $\sim 1$   $\mu\text{g}$  total RNA was transcribed with cDNA transcription reagents (Perkin-Elmer, Foster City, CA) with the use of random hexamers. cDNA was stored at  $-30^\circ\text{C}$  until quantitative RT-PCR was performed.

#### *Real time quantitative RT-PCR*

Measurement of gene expression was performed utilizing the ABI prism 7700 Sequence Detection System (Perkin-Elmer) as previously described (12, 13). Primers and TaqMan probes (Custom Oligonucleotide Factory, Foster City, CA) were designed to span exon-intron junctions to prevent amplification of genomic DNA and to result in amplicons  $<150$  bp to enhance efficiency of PCR amplification. TaqMan probes were labeled at the 5'-end with the reporter dye molecule FAM (6-carboxyfluorescein; emission  $\lambda_{\text{max}} = 518$  nm) and at the 3'-end with the quencher dye molecule TAMARA (6-carboxytetramethylrhodamine; emission  $\lambda_{\text{max}} = 582$  nm). cDNA standards were generated by reverse transcriptase, primer-specific amplification of mRNA of the relevant genes by a technique identical with the one used for the preparation of test cDNA. Amplified cDNA was then purified and quantitated by spectrophotometry ( $\text{OD}_{260}$ ). Copies were calculated using the m.w. of each individual gene amplicon. RT-PCR reactions of cDNA specimens and cDNA standards were conducted in a total volume of 25  $\mu\text{l}$  with 1  $\times$  TaqMan Master Mix (Perkin-Elmer) and primers and probes at optimized concentrations (Table I). Thermal cycler parameters included 2 min at 50°C, 10 min at 95°C, and 40 cycles involving denaturation at 95°C for 15 s, annealing/extension at 60°C for 1 min. Real time monitoring of fluorescent emission from cleavage of sequence specific probes by the nuclease activity of *taq* polymerase allowed definition of the threshold cycle during the exponential phase of amplification (12). Standard curves were generated for each gene quantitated and were found to have excellent PCR amplification efficiency (90–100%; 100% indicates that in each cycle the amount of template is doubled) as determined by the slope of the standard curves. Linear regression analysis of all standard curves were  $\geq 0.99$ . Standard curve extrapolation of copy number was performed for the gene of interest as well as an endogenous reference gene for each sample. Normalization of samples was performed by dividing the copies of the gene of interest by copies of the reference gene. All PCR assays were performed in duplicates and reported as the average. A 2-fold difference in gene expression was found to be within the discrimination ability of the assay (data not shown).

#### *Direct molecular assessment of peptide and melanoma-specific CTL reactivity*

Cryopreserved PBMC were thawed into CM as described (5). To determine the optimal conditions for assessing direct PBMC reactivity to the immunizing peptides, experiments were conducted using PBMC obtained from patients after immunization. Peptide concentrations ranging from 0.01 to 10  $\mu\text{M}$  were evaluated, as were harvest times ranging from 2 to 24 h, and optimal recovery time of physiological cell metabolism for thawed PBMC. On the basis of these optimization experiments (data not shown), direct PBMC assays were conducted using  $3 \times 10^6$  PBMC in 2 ml of media, which were allowed to recover by incubation at 37°C in 5%  $\text{CO}_2$  for 10 h. Either 1  $\mu\text{M}$  peptide or  $1 \times 10^6$  melanoma cells were then added to the

PBMC and incubated at 37°C in 5%  $\text{CO}_2$  for 2 h. No exogenous cytokines or other stimulants were added. The cells were then harvested, and RNA isolation and cDNA transcription were performed. Quantitative RT-PCR was performed for IFN- $\gamma$  mRNA expression and normalized to copies of CD8 mRNA from the same sample.

#### *Direct molecular assessment of gene expression within the tumor microenvironment*

Sequential FNA of the same metastatic tumor site were performed before and after therapy. Aspirate material was examined and verified by a cytopathologist to contain melanoma cells. The remainder of the aspirate was placed in RNA lysis buffer (Qiagen) and stored at  $-180^\circ\text{C}$ . RNA isolation and cDNA transcriptions from the aspirated material were performed in batches containing patient pre- and posttherapy samples to minimize variability. Quantitative RT-PCR was performed to assess changes in gene expression within the sequential tumor biopsies. Because the aspirates represent only a portion of any tumor and because the immune infiltrate can vary between individual parts of tumors, apparent changes in gene expression between pre- and postvaccination FNA aspirates could only reflect that different areas of the tumor had been probed over time. Thus, the data presented should be interpreted with caution until a larger patient population can be collected and analyzed.

#### *Direct immunofluorescence of fine needle aspirate biopsies*

Sequential fine needle aspirates obtained from the same metastatic tumor site were performed before and throughout therapy. Aspirated material was examined and verified by a cytopathologist to contain melanoma cells. Cytospins were performed, and the slides were fixed with acetone for 10 min. tHLA (1  $\mu\text{g}$ ) was added to specimens for 2 h at room temperature. Slides were washed vigorously with isotonic saline and visualized with fluorescent microscopy (Olympus, New Hyde Park, NY) at 576 nm.

#### *Statistical analysis*

Result reproducibility was tested performing a set of consecutive experiments in which pre- and postvaccination PBMC obtained from the same plasma pheresis from the same patient were independently thawed, stimulated, and processed for cDNA preparation and qRT-PCR. This set of experiments demonstrated that measurements of cytokine mRNA expression were highly reproducible (Table II). Whereas no significant difference could be noted among pre- and postvaccination PBMC that had not received stimulation, a highly significant difference was noted after stimulation of PBMC with 209-pa peptide (unpaired *t* test,  $p < 0.001$ ). Comparison of pre- vs posttreatment CTL reactivity was assessed by a paired comparison of the fold increment in IFN- $\gamma$  transcript detection in response to stimulation of a given sample over IFN- $\gamma$  transcript detection in the same sample that had not been stimulated. To address the accuracy of this method, 24 PBMC samples obtained from HLA-A\*0201-expressing patients who had never received vaccination with 209-2M or 209-pa were tested. Furthermore, no evidence of reactivity against either epitope could be detected by *in vitro* sensitization assays in these samples. The ratio of IFN- $\gamma$  mRNA transcript detectable in epitope-stimulated PBMC to IFN- $\gamma$  mRNA transcript detectable in nonstimulated PBMC ranged from 0.6 to 1.4 with a median of 0.9 and a mean value of  $0.94 \pm 0.05$  (SEM) at a 95% confidence level of 0.09. To minimize the possibility of falsely considering PBMC immunoreactive, we accepted a 2-fold increase in stimulated-unstimulated IFN- $\gamma$  transcript ratio ( $\geq 2.0$  corresponding to  $>5$  SDs above the median) as evidence of epitope-specific reactivity.

Differences in IFN- $\gamma$  transcript comparing pre- and postvaccination samples were evaluated parametrically by a paired *t* test. Correlation between gp100 transcript expression in FNA and fold increase in IFN- $\gamma$  mRNA detection after vaccination was compared by simple linear regression analysis.

## Results

#### *Direct molecular assessment of peptide- and melanoma-specific CTL reactivity*

To assess direct Ag recognition and reactivity by CTL in peripheral blood, PBMC obtained by leukapheresis from patients before and after two cycles of 209-2M vaccination were directly exposed *ex vivo* to the 209-pa and 209-2M peptides or melanoma tumor cells. No prior *in vitro* sensitization or culturing of lymphocytes was performed, nor were exogenous cytokines added to the cells. Because of the low frequency of 209-pa-reactive CTL in bulk PBMC, changes in cytokine release after peptide elicitation were



Table II. *Interassay variability of quantitative RT-PCR measurement of gene expression<sup>a</sup>*

Expt.		No Stimulation	Stimulation with 209-pa (1 $\mu$ M)
1	PBMC prevaccination	68	76
	PBMC after 2 vaccinations	46	<u>5396</u>
2	PBMC prevaccination	ND	ND
	PBMC after 2 vaccinations	40	<u>5064</u>
3	PBMC prevaccination	83	96
	PBMC after 2 vaccinations	135	<u>6184</u>
4	PBMC prevaccination	65	88
	PBMC after 2 vaccinations	59	<u>5680</u>
Mean $\pm$ SEM		72 $\pm$ 5.4	87 $\pm$ 5.9
		70 $\pm$ 22.0	<u>5581 <math>\pm</math> 237.1</u>

<sup>a</sup> Data of four consecutive experiments are presented for the patient case presented in Fig. 1b. Calculated IFN- $\gamma$  copies of mRNA standardized over CD8 mRNA copy number after 4 h of PBMC stimulation with g209-pa peptide (1  $\mu$ M). Each experiment represent a totally independent thaw and stimulation of PBMC with subsequent analysis of IFN- $\gamma$  mRNA expression. Values represent copies of IFN- $\gamma$  mRNA per  $10^4$  copies of CD8 mRNA. 209-pa reactivities twice control (no peptide stimulation) are underlined.

typically below the sensitivity of standard ELISA assays. Therefore, we used real time quantitative RT-PCR to monitor the PBMC for highly specific and quantitative changes in gene expression. As illustrated in Fig. 1a, PBMC obtained from a patient after two immunizations with the 209-2M peptide demonstrated a phenotypic increase of CD8<sup>+</sup> cells stained with the 209-2M HLA tetramer, as well as the 209-pa HLA tetramer (0.2 and 0.12% of the total PBMC population, respectively). The direct functional analysis of the preimmunized PBMC revealed no detectable reactivity after incubation with either the modified 209-2M or the native 209-pa peptides (Fig. 1b). However, the postimmunized PBMC, within a 2-h peptide exposure, demonstrated significant increases in mRNA for the CD69 CTL activation marker, the IL-2 $\alpha$  receptor (CD25), and the cytokines IFN- $\gamma$ , TNF- $\alpha$ , GM-CSF, and IL-2 (Fig. 1b). Peptide exposure did not result in changes in the gene expression for IL-1 $\alpha$ , IL-1 $\beta$ , IL-4, IL-5, IL-8, IL-12, or IL-15 (data not shown). We consistently noted that postimmunized PBMC demonstrated greater induction of gene expression after exposure to the modified 209-2M peptide when compared with 209-pa peptide (Fig. 1b). Physiologically, the cytokine kinetics of IFN- $\gamma$ , GM-CSF, and IL-2 showed strikingly similar characteristics but the quantitative expression of IFN- $\gamma$  mRNA was severalfold higher than that of the other two genes. The favorable signal-noise ratio for IFN- $\gamma$  mRNA expression made this the most suitable single gene to follow as a highly sensitive and specific marker of immune reactivity in our subsequent studies. Reproducible results further delineated that a 2–3 h elicitation time period with 1  $\mu$ M of peptide to be the optimal parameters for IFN- $\gamma$  mRNA induction (data not shown). To account for potential variability in the number of CD8<sup>+</sup> cells in the samples and reverse transcriptase efficiency during cDNA preparation, normalization of IFN- $\gamma$  transcripts was performed by dividing by CD8 mRNA copies. CD8 mRNA expression was stable during experiments when compared with traditional housekeeping genes such as  $\beta$ -actin, GAPDH, and rRNA (data not shown). To define the approximate sensitivity limit of this molecular assay, in vitro cultured, 209-pa-reactive CTL clones were spiked into nonreactive autologous PBMC. Significant 209-pa reactivity (compared with response to an irrelevant melanoma Ag epitope, MART 27–35) could be seen at a spiked dilution of 1 CTL clone in 50,000 PBMC (Fig. 1c). To determine whether the observed peptide reactivity was associated with tumor reactivity, bulk PBMC were exposed directly to a panel of melanoma cell lines. Response from postimmunized PBMC was found against two HLA-A2+/gp100<sup>+</sup> melanomas (624.38 Mel and 888 Mel (A2<sup>+</sup>)), but not against two HLA-A2-/gp100<sup>+</sup> melanomas (624.28 Mel and 888 Mel) as seen in Fig. 1d. Our cumulative observations

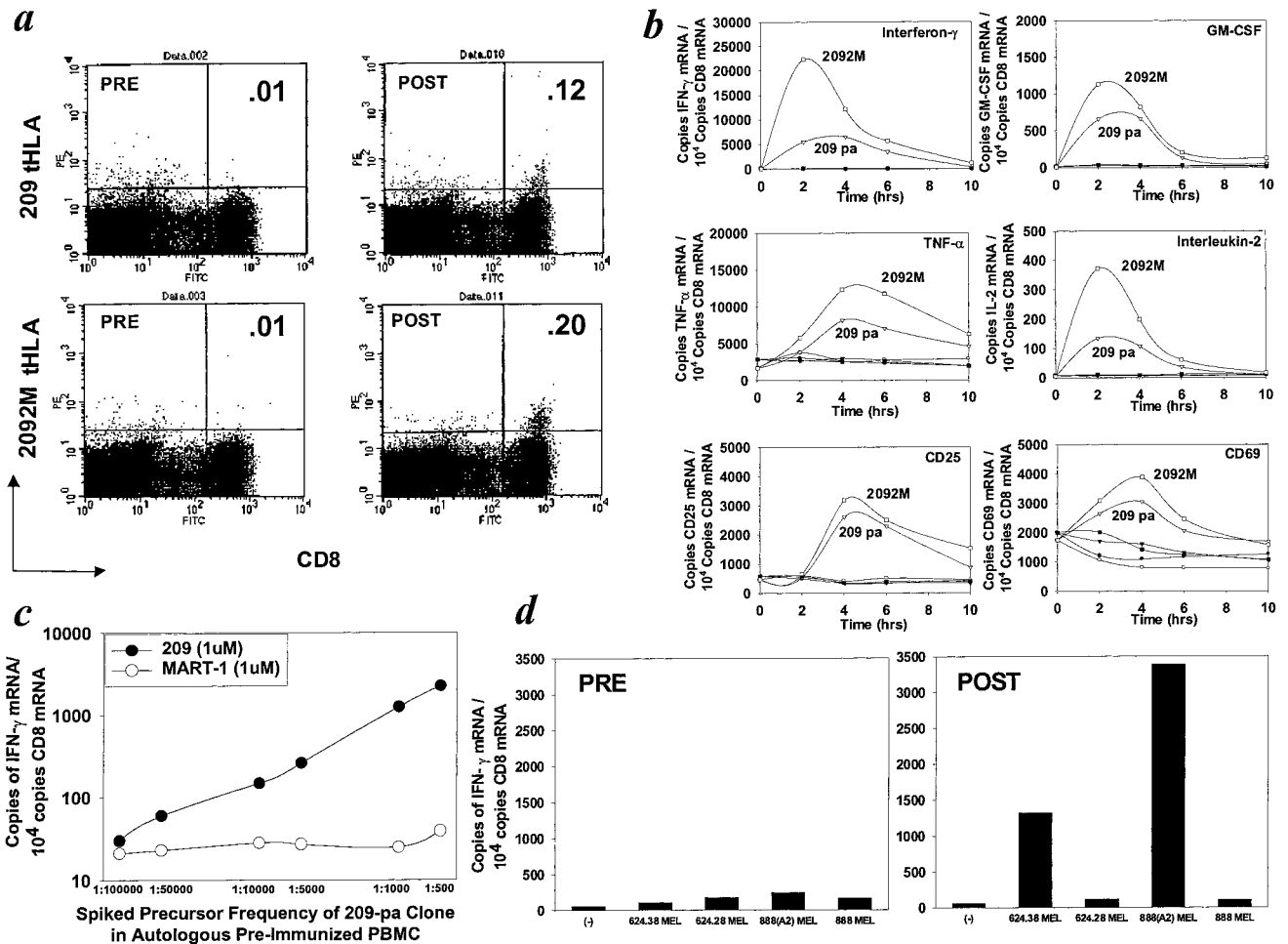
of peripheral lymphocytes demonstrated that 209-2M peptide immunization could result in a significant increase in circulating CTL with highly specific activity directed against a tumor Ag target. Further, these findings were evident in cells, which were obtained directly from patients without any prior in vitro manipulation.

#### *Peptide immunization results in heterogeneous peripheral CTL reactivity*

We next examined the relationship between the level of direct 209-pa peptide reactivity in peripheral blood and clinical tumor response. Pre- and postvaccination PBMC were obtained from 10 consecutive patients who were vaccinated with 4 melanoma-associated peptides that included 209-2M (see *Materials and Methods*). PBMC were analyzed for 209-pa reactivity by direct molecular assay and by standard cytokine release assays performed on in vitro sensitized cultures (Table III). In these experiments, evidence of 209-pa immunization for both assays was defined as 209-pa epitope reactivity in postimmunized PBMC that was measured to be twice the background (i.e., no peptide elicitation) and not demonstrable in the preimmunized samples. Reactivity measured by the IVS assay was qualitatively scored as positive (+) or negative (–) because of the inherent in vitro variability of culture growth that could influence quantitative analysis. We found that the IVS assay could generate PBMC cultures with specific antipeptide reactivity (IFN- $\gamma$  cytokine release) in 7 of 10 patients after immunization, while the direct molecular assay demonstrated immune reactivity (IFN- $\gamma$  mRNA production) in only 5 of the 10 patients. Patients i and j (Table III) demonstrated qualitative evidence of culture reactivity by the IVS assay but had less than a 2-fold increase by direct molecular assay. Further, for the patients who demonstrated evidence of immunization by both assays (patients a–e), direct analysis found significant quantitative heterogeneity among the reactivities, ranging from 2.1- to 76.7-fold over background. Thus, we observed that patients who received identical vaccine therapies mounted very different degrees of peripheral CTL response against the targeted Ag. Despite these findings, there was no correlation between any level of peripheral 209-pa reactivity and objective local tumor response with immunization.

#### *Serial gene expression analysis within the tumor microenvironment demonstrates localization of immune reactivity after 209-2M vaccination*

To determine whether lack of tumor regression was due to lack of localized immune response within the tumor, we examined



**FIGURE 1.** Analysis of presence and function of vaccine-specific T cells in PBMC from a highly reactive patient who had received two vaccinations with the 209-2M peptide. *a*, FACS analysis demonstrated the phenotypic increase of 209-pa/209-2M-specific CD8<sup>+</sup> cells in the postvaccination PBMC. Numbers in the upper right quadrants (URQ) indicate the percentage of tHLA staining CD8<sup>+</sup> T cells calculated according to the formula URQ/(URQ + LRQ)  $\times$  100, in which LRQ is lower right quadrant. PBMC populations were gated only according to forward scatter to include cells of size consistent with lymphocytes. Abscissa, staining with anti-CD8 mAb; ordinate, staining with soluble HLA/epitope tetramers (for details about further controls, see *Materials and Methods*). *b*, Kinetics of immune reactivity after direct elicitation of bulk PBMC with 1  $\mu$ M 209-2M and 209-pa. Real time quantitative RT-PCR performed on PBMC at time points after peptide incubation. Preimmunized PBMC (●), + 209-pa (▼), + 209-2M (■), postimmunized PBMC (○) + 209-pa (▽) + 209-2M (□). *c*, Sensitivity of direct molecular assay; 209-pa-reactive T cell clone was spiked into preimmunized PBMC. Elicitation of 209-pa reactivity (IFN- $\gamma$  mRNA) could be seen at a spiked dilution of 1 clone in 50,000 PBMC. Negative control was performed with exposure to irrelevant peptide, MART 27–35. *d*, Direct reactivity (IFN- $\gamma$  mRNA production) of pre- and postimmunized PBMC to HLA-A2<sup>+</sup>/gp100<sup>+</sup> melanomas, 624.38 Mel and 888 Mel (A2<sup>+</sup>), and HLA-A2<sup>-</sup>/gp100<sup>+</sup> melanomas 624.28 Mel and 888 Mel. These figures represent a complete set of experiments performed in PBMC from the same patient. Each experiment, however, was repeated at least three times on various occasions with similar results with PBMC from the same patient. An exception of Fig. 1c were the spiking of the 209-pa-specific clone performed in prevaccination PBMC from other patients because of lack of sufficient material from the same patient. Thus, the results presented in Fig. 1c are representative of three experiments with characterized by similar results (range of lowest detectable precursor frequency in the three experiments was 1:100,000 in one experiment and 1:50,000 in the other two experiments).

209-2M therapy-related changes within the local tumor microenvironment of subcutaneous melanoma metastases. Sequential fine needle aspirates of individual lesions in situ were performed on 27 patients before and after vaccine-based immunotherapy. These patients were divided into 3 cohorts based on their treatments (see *Materials and Methods*): a 209-2M cohort (9 patients/11 lesions); and 2 control groups, a non-209-2M cohort (10 patients/11 lesions) and an IL-2 cohort (8 patients/9 lesions). The control therapies represented a variety of experimental vaccine protocols that had shown no evidence of immunization by standard in vitro sensitization assays of peripheral blood. Quantitative RT-PCR was performed from RNA isolated directly from aspirated material obtained from the same lesion before and after treatment. No in vitro

culturing or stimulation were performed on the aspirate, so that a true representation of in vivo gene expression and immune reactivity could be ascertained. As demonstrated in Fig. 2a, sequential biopsies from the 209-2M cohort showed statistically significant changes in 8 of 11 lesions having a  $\geq$ 2-fold increase in IFN- $\gamma$  mRNA expression (normalized to CD8) when pretherapy levels were compared with posttherapy levels for the same lesion (paired *t* test; *p* = 0.01). In contrast, there was no significant change in IFN- $\gamma$  mRNA expression for the non-209-2M cohort (2 of 11 lesions, *p* = 0.40) or the IL-2 cohort (3 of 9 lesions, *p* = 0.19). Interestingly, direct quantitation of CD8 and CD4 mRNA (normalized to  $\beta$ -actin mRNA), as a representation of cellular immune infiltration in the biopsies, showed no significant change in any of

Table III. Reactivity of PBMCs from patients immunized with 209-2M peptide in IFA<sup>a</sup>

Patient	Before Immunization				After Immunization				Clinical Response
	IVS <sup>b</sup>	No pep <sup>c</sup>	209-pa	Fold inc <sup>d</sup>	IVS	No pep	209-pa	Fold inc	
a	(-)	118	101	0.9	(+)	131	<u>715</u>	<u>5.5</u>	NR <sup>e</sup>
b	(-)	316	245	0.8	(+)	142	<u>10,893</u>	<u>76.7</u>	NR
c	(-)	103	107	1.0	(+)	67	<u>546</u>	<u>8.1</u>	NR
d	(-)	38	74	1.9	(+)	50	<u>401</u>	<u>8.0</u>	NR
e	(-)	471	302	0.6	(+)	600	<u>1,259</u>	<u>2.1</u>	NR
f	(-)	73	69	0.9	(-)	110	148	1.3	NR
g	(-)	472	323	0.7	(-)	94	177	1.9	NR
h	(-)	65	52	0.8	(-)	58	70	1.2	NR
i	(-)	47	40	0.9	(+)	59	58	1.0	NR
j	(-)	52	61	1.2	(+)	71	77	1.1	NR

<sup>a</sup> Data refer to the first retrospective pilot study in which IVS results were compared with direct reactivity in PBMC using real time quantitative RT-PCR. Preimmunization samples were obtained 1–3 days before administration of the first vaccination. Postimmunization samples were obtained 3–4 wk after the second vaccination.

<sup>b</sup> PBMCs incubated with 209-2M for 11–13 days before assay against T2 cells alone or pulsed with 209-pa peptide (1  $\mu$ g/ml). (+), 209-pa reactivity with IFN- $\gamma$  release (ELISA) that is >100 pg IFN- $\gamma$ /ml and at least twice that of controls. (-), 209-pa reactivity less than twice that of controls.

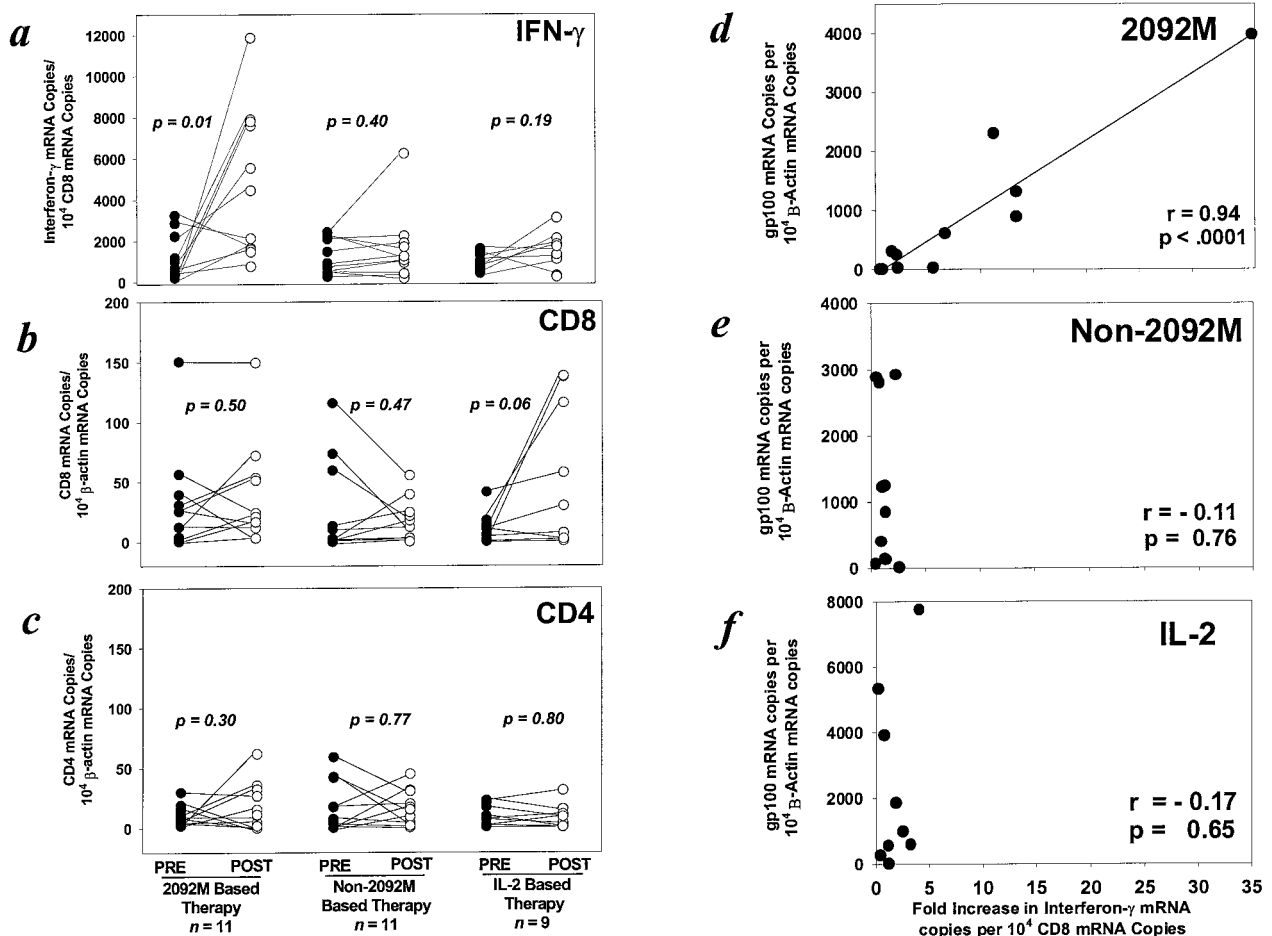
<sup>c</sup> Direct quantitative PCR assay. Fresh PBMC were incubated for 2 h with no peptide (No pep) or 209-pa (1 mg/ml). Values represent copies of IFN- $\gamma$  mRNA per 10<sup>4</sup> copies of CD8 mRNA. 209-pa reactivity twice control (No pep) are underlined.

<sup>d</sup> Fold increase (inc), 209-pa values divided by No pep values. Fold increase  $\geq 2$  are underlined.

<sup>e</sup> NR, no response.

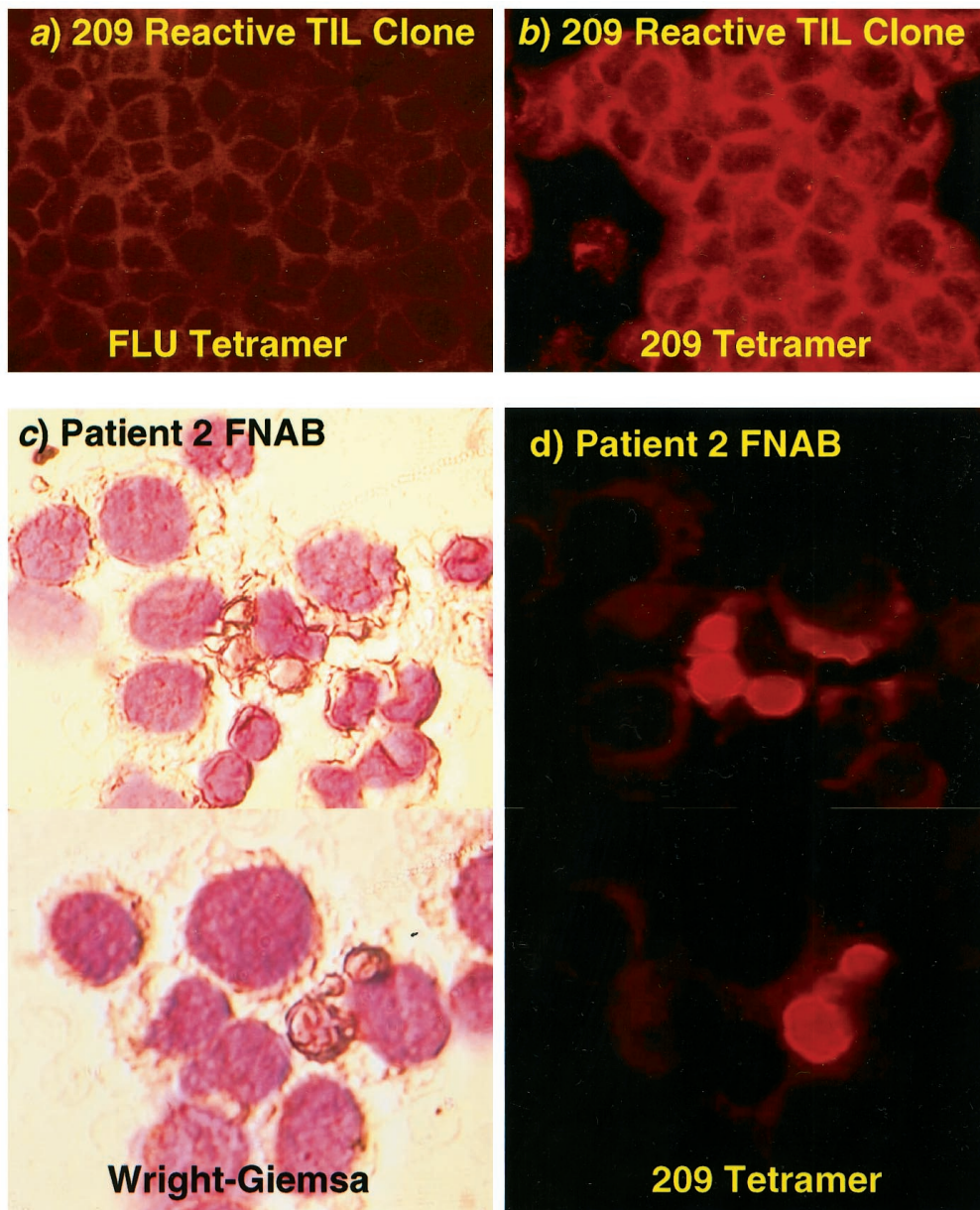
the cohorts (Fig. 2, *b* and *c*). However, there was a slight trend toward significance for an increase in CD8 mRNA in the IL-2 cohort ( $p = 0.06$ ).

Although the postimmunized 209-2M cohort showed an overall increase in IFN- $\gamma$  mRNA expression, the magnitude of this increase was quite variable among the individual lesions, with one



**FIGURE 2.** Quantitative RT-PCR of sequential final needle aspirates of metastatic melanoma lesions. *a*, IFN- $\gamma$  mRNA (normalized to CD8 mRNA); *b*, CD8 mRNA; *c*, CD4 mRNA (normalized to  $\beta$ -actin mRNA). Statistical analysis, paired *t* test. Correlation of fold increase in IFN- $\gamma$  vs gp100 mRNA expression for lesions from 209-2M cohort (*d*), non-209-2M cohort (*e*), and IL-2 cohort (*f*). Correlation between gp100 transcript expression in FNA and fold increase in IFN- $\gamma$  mRNA detection after vaccination were compared by simple linear regression analysis.





**FIGURE 3.** Direct immunofluorescence of CTL using 209-pa tHLA. Control 209-pa reactive tumor-infiltrating lymphocyte (TIL) clones stained with Flu tHLA (background staining) (*a*) and 209-pa tHLA (*b*). Fine needle aspirate of metastasis from patient 1 after 2 cycles of 209-2M immunization; staining with Wright-Giemsa (demonstrating melanoma cells and lymphocytes) (*c*) and 209-pa tHLA (fluorescent orange staining of lymphocytes) (*d*). Control staining included tHLA staining of prevaccination fine needle aspiration (FNA) that did not demonstrate evidence of 209-pa specific T cells at tumor site (not shown). Flu tHLA staining was not performed in this FNA because of lack of sufficient material. Previous studies in other FNA have shown that Flu-specific T cells can be identified in material obtained from melanoma metastases probably in relation to blood contamination; however, they are extremely rare and their frequency does not significantly change in relation to vaccination.

lesion showing a 35-fold increase, whereas others showed marginal or no change. In an effort to explain this heterogeneity, we examined the concurrent mRNA expression for the targeted tumor Ag, gp100, in each of the biopsies. As demonstrated in Fig. 2*d*, quantitative RT-PCR for gp100 mRNA expression (normalized to  $\beta$ -actin mRNA) also demonstrated marked heterogeneity, which correlated very highly with the observed tumor changes in IFN- $\gamma$  mRNA for the 209-2M cohort ( $r = 0.94$ ,  $p < 0.0001$ ). This correlation was not seen in the other two treatment cohorts, whose lesions were obtained and processed in an identical manner and which showed similar variability of gp100 Ag expression (Fig. 2, *e* and *f*). These findings demonstrated that 209-2M immunization (in contrast to other therapies) could result in measurable immu-

nological changes within tumors if appropriate levels of Ag were present. Change in IFN- $\gamma$  mRNA was used as a parameter rather than the absolute levels of IFN- $\gamma$  transcript to better represent the impact of therapy and to account for in vivo baseline variability among lesions.

*Direct immunofluorescence of fine needle aspirate biopsies demonstrates localization of 209-pa reactive CTL*

To visualize the cellular response within the tumor microenvironment after 209-2M vaccination, we examined cytopspins prepared from sequential fine needle aspirates of the lesion which showed the greatest change in IFN- $\gamma$  mRNA levels. As shown in Fig. 3*c*,

Table IV. Comparison of peripheral vs tumor site monitoring after 209-2M-based peptide vaccination<sup>a</sup>

Patient	Therapy	Peripheral Monitoring (PBMC)				Tumor Monitoring (Tumor Aspirates)				Tumor Response Size (cm)
		IVS	No pep	209-pa	Fold inc	Tumor site	No pep <sup>b</sup>	Fold inc <sup>c</sup>	gp100 <sup>d</sup>	
1	Pre	(-)	54	64	1.2	Right axilla	568		1,242	4
	Post	(+)	47	<u>103</u>	<u>2.2</u>		<u>7,586</u>	<u>13.4</u>	1,310	9
2	Pre	(-)	39	45	1.2	Left thigh	331		1,956	1
	Post	(-)	41	43	1.0		<u>11,865</u>	<u>35.8</u>	3,955	1
3	Pre	(-)	22	24	1.1	Right axilla	1,187		6,186	9
	Post	(-)	30	45	1.5		<u>7,891</u>	<u>6.6</u>	611	25
4	Pre	(-)	52	28	0.5	Left thigh	579		2,865	6
	Post	(-)	35	37	1.1		<u>7,788</u>	<u>13.5</u>	894	20.25
5	Pre	(-)	104	92	0.9	Right thigh	2,231		1,226	1
	Post	(+)	50	72	1.4		<u>4,452</u>	<u>2.0</u>	235	4
6	Pre	(-)	95	84	0.9	Left chest	1,013		1	2
	Post	(+)	42	<u>151</u>	<u>3.6</u>		<u>5,532</u>	<u>5.5</u>	18	5
7	Pre	(-)	59	44	0.7	#1 left medial knee	3,247		0	2.25
			29	37	1.3	#2 left lateral knee	1,649	0.5	1	2.5
	Post	(-)					2,865		1	6.25
							2,131	0.7	1	6.25
8	Pre	(-)	73	69	0.9	#1 right lower thigh	140		4,786	6
	Post	(-)	110	148	1.3		<u>1,585</u>	<u>11.3</u>	2,291	4
9	Pre	(-)				#2 right upper thigh	466		532	16
							759	1.6	312	16
							692		60	4
Post	(+)	67	<u>546</u>	<u>8.1</u>	Right groin	<u>1,474</u>	<u>2.1</u>	12	6	

<sup>a</sup> Preimmunization PBMC samples were obtained 1–3 days before administration of the first vaccination. Postimmunization PBMC were obtained 3–4 wk after the second vaccination. FNA samples were obtained within 2 days of the collection of the relative PBMC samples.

<sup>b</sup> Tumor aspirates underwent no peptide exposure (No pep). Quantitative PCR performed directly on fresh sample. Values represent copies of IFN- $\gamma$  mRNA per 10<sup>4</sup> copies of CD8 mRNA. Post values twice pre values are underlined.

<sup>c</sup> Fold increase (Fold inc) for tumor monitoring = Post values divided by Pre values. Fold increases  $\geq 2$  are underlined.

<sup>d</sup> Values represent copies of gp100 mRNA per 10<sup>4</sup> copies of  $\alpha$ -actin mRNA.

Wright-Giemsa staining of the postimmunized sample demonstrated an abundance of intact large nucleated melanoma tumor cells (pink staining). Concurrent staining with the 209-pa HLA tetramer and examination with fluorescent microscopy illustrated the phenotypic presence of g209-pa specific lymphocytes within this lesion (Fig. 3d). In contrast, no 209-pa-specific T cells could be identified in the preimmunized specimen (not shown).

#### Localization of vaccine-induced immune response at the tumor site is not sufficient to cause cancer regression

We next addressed whether peripheral and localized immune response measured in the 209-2M cohort was sufficient to cause tumor regression. Table IV documents individual lesion sizes during 209-2M-based therapy and summarizes the simultaneous analysis of peripheral blood and local tumor reactivity. PBMC reactivity was found in 4 of 9 patients by the IVS assay and in 3 of 9 patients by the direct molecular assay. The magnitude of the direct PBMC reactivity in these patients was low, ranging from 2.2- to 8.1-fold over background. As described in Fig. 2, direct tumor analysis found evidence of immunization in 8 of the 11 lesions. Increases in IFN- $\gamma$  mRNA levels were apparent in 5 of these lesions (patients 2, 3, 4, 5, and 8) in the absence of measured peripheral blood reactivity by direct molecular assay. Despite even these observed localized responses, none of the 11 lesions showed significant regression in size after vaccination.

## Discussion

During the last decade, a major advance in tumor immunology has been the identification of cancer-specific Ags recognized by T cells. These findings have led to strategies to exploit these molecules as cancer vaccines with the purpose of enhancing lymphocyte reactivity. Although clinical tumor regression remains the ultimate goal of immunotherapy, monitoring of in vivo biological

effects of immunization may provide clues to the development of more efficacious regimens. Previous in vivo studies have shown that lack of CTL localization at the tumor site correlated with lack of response to adoptive therapy with melanoma-specific CTL (14). However, rarely have direct functional changes within peripheral blood and the tumor microenvironment been used in analyzing the effects of vaccine-based therapy. Most current monitoring protocols have relied on the analysis of in vitro generated PBMC cultures as an indicator of immunization. In this study, we report results of a methodology that allows the direct molecular analysis of immune responses in vivo. We provide evidence demonstrating vaccine-induced lymphocyte reactivity in fresh cells obtained directly from peripheral blood. Induction of gene expression for the activation marker CD69, the cytokines (IFN- $\gamma$ , GM-CSF, TNF- $\alpha$ , and IL-2), and the proliferation marker CD25 was found directly in postimmunized PBMC after immediate peptide exposure ex vivo. This demonstrated that 209-2M vaccination could result in circulating and functional CTL with activity against the targeted tumor Ag. It is unclear why there was marked quantitative heterogeneity in peripheral blood reactivity among patients receiving identical immunization protocols with the 209-2M peptide. This finding may reflect a biological variable among patients that could play an important role in the optimization of future clinical vaccine regimens.

Our analysis of sequential tumor biopsies during 209-2M-based vaccination (either 209-2M or ES-209-2M; refer to Materials and Methods for details) found that 8 of 11 lesions (73%) demonstrated significant increases in IFN- $\gamma$  mRNA (paired *t* test, *p* = 0.01), whereas the control cohorts demonstrated no significant change. Because 209-2M peptide immunization is highly specific in its ability to generate an epitope-specific CTL response, we believe that the changes in the biopsy IFN- $\gamma$  mRNA levels are indicative of enhanced specific lymphocyte reactivity within the tumor microenvironment. Further, because the tumor-derived IFN- $\gamma$  mRNA



levels were measured without ex vivo stimulation, we believe that these cytokine mRNA levels are representative of the natural in vivo interaction between vaccine-induced CTL and endogenous tumor Ag. This conclusion is supported by the strong correlation between IFN- $\gamma$  and gp100 mRNA expression observed in the 209-2M cohort ( $r = 0.94$ ,  $p < 0.0001$ ). Interestingly, five tumor lesions showed treatment-related changes in the absence of detectable peripheral blood reactivity. Local immune changes without detectable systemic precursor reactivity may imply a greater Ag-specific CTL frequency in the local tumor microenvironment than in bulk PBMC, as suggested previously (15). Because of these findings, we believe that sequential analysis of tumors represents the most sensitive and relevant approach to analyzing in vivo effects of cancer vaccines. Further, this molecular methodology would be ideally suited for the assessment of a variety of novel biological agents in clinical trials, if appropriate target tissue is easily accessible.

Unfortunately, despite our evidence of immune reactivity in peripheral blood and at the local tumor site, there was no significant impact of 209-2M therapy on tumor viability and progression. Given that the increase in IFN- $\gamma$  mRNA in tumors was noted in the absence of significant CD8 and CD4 mRNA increases, we hypothesize that a classically induced response of cellular recruitment and inflammation likely did not occur in these lesions. These results demonstrate the presence but perhaps the limited effectiveness of vaccine-induced T cell response within the target environment. It is possible that the characteristics of Ag presentation exercised by tumors are not optimal to maintain T cells in a state of activation at the tumor site. It has been suggested that tumors induce tolerance by presenting epitope-specific stimulation (signal 1) without costimulation (signal 2) to wandering memory T cells (16). Of interest, our initial evaluation has shown minimal evidence of IL-2 mRNA, a critical growth factor for T cell proliferation, in the tumor microenvironment of vaccine-treated patients (work in progress). Some models predict that in the absence of an ongoing "danger signal," the vaccination response will wane and eventually stop (17). Tumor escape through Ag and/or HLA loss (18) does not fully explain our observed tumor resistance to therapy, given that we had lesions with documented high expression of gp100 and HLA A2 showing no preferential regression. Other possible explanations include the effects of local immunosuppressive factors such as IL-10 and TGF- $\beta$  (19) and expression of apoptotic signals (20). Recently, it has been reported that effector T cells induced against antigenic tumors could be maintained by prolonged or repetitive vaccination (21). Modifications and adjuvants to immunization schemes, such as exogenous IL-2 and modalities to facilitate CTL help, may provide additional stimuli to heighten the immune reactivity that we have observed into effective antitumor response. Direct serial molecular analysis of gene expression in tumors represents an extremely sensitive and powerful tool to monitor these immunological changes in vivo. This methodology may help further guide the development of future biological therapies.

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