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Status of Activation of Circulating Vaccine-Elicited CD8$^+$ T Cells

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Selective blunting of the status of activation of circulating tumor-specific T cells was invoked to explain their paradoxical coexistence with unhampered tumor growth. By analogy, lack of tumor regression in the face of observable melanoma vaccine-induced T cell responses might be attributed to their status of activation. We enumerated with HLA-A*0201/peptide tetramers (tHLA) vaccine-elicited T cell precursor frequency directly in PBMC of patients with melanoma undergoing vaccination with the HLA-A*0201-associated gp100:209–217(210 M) epitope (g209-2 M). Furthermore, we tested by intracellular (IC)-FACS analysis and quantitative real-time PCR (qRT-PCR) the ability of postvaccination PBMC to produce cytokine in response to challenge with vaccine-related epitopes or vaccine-matched (HLA-A*0201) melanoma cells. Vaccine-induced enhancement of T cell precursor frequency could be detected with tHLA in PBMC from six of eight patients studied at frequencies ranging between 0.3 and 2.3% of the total CD8$^+$ population. Stimulation with vaccine-related epitopes induced IFN-γ expression detectable by IC-FACS or qRT-PCR, respectively, in five and six of these patients. Furthermore, down-regulation of tHLA staining was noted upon cognate stimulation that could be utilized as an additional marker of T cell responsiveness. Finally, we observed in six patients an enhancement of reactivity against vaccine-matched tumor targets that was partly independent of documented vaccine-specific immune responses. A strong correlation was noted between tHLA staining of postvaccination PBMC and IFN-γ expression by the same samples upon vaccine-relevant stimulation and assessed either by IC-FACS or qRT-PCR. Thus, blunting of the status of T cell activation on itself cannot easily explain the lack of clinical responses observed with vaccination. ***The Journal of Immunology,*** **2000, 165: 2287–2296.**

Results from pivotal melanoma Ag (MA)[2]-specific vaccinations suggest an enigmatic discrepancy between their effectiveness in eliciting circulating T cell responses and clinical efficacy (1, 2). Although many ways to tumor tolerance may be postulated (3), a simple explanation for this paradoxical observation is that the frequency and responsiveness of vaccine-elicited T cells documented by commonly utilized assays may not accurately reflect their number and/or status of activation in vivo. Indeed, circulating MA-specific T cells are detectable in vaccine-naive patients with melanoma and normal nontumor-bearing individuals (4–6). Some suggested that the paradoxical coexistence of unhampered tumor growth with circulating tumor-specific T cells is explainable by selective blunting their status of activation (7). We postulated, therefore that, by analogy, lack of tumor regression might be blamed on poor responsiveness of vaccine-elicited T cells.

In the past, we have judged the extent of the immune response to epitope-specific vaccination by comparative assessment of the sensitivity of pre- and postvaccination PBMC to in vitro stimulation with arbitrary concentrations of vaccine-related epitopes plus exogenous IL-2 (1). This approach may misrepresent and likely exaggerate the extent and quality of vaccine-induced responses occurring in vivo. Recently, direct measurement of circulating vaccine-specific T cells precursor frequency (TCPF) with HLA/epitope tetramers (tHLA) demonstrated a tight correlation between their frequency and susceptibility to in vitro expansion (8). This correlation suggested that historical results obtained with in vitro sensitization were roughly representative of the host's immune competence before and after vaccination. However, tHLA-guided detection of vaccine-specific T cells does not address their functional status (7). Indeed, tHLA binding does not exclude that a promiscuous, low avidity TCR/tHLA interaction has occurred that cannot generate ligand-induced physiological responses (7, 9). In addition, even a high avidity TCR may not generate productive interactions with its natural ligand due to a diminished status of activation of the T cell bearing it (7). In a previous study, serial measurement of cytokine gene expression by quantitative real-time PCR (qRT-PCR) detected functional immune responses to vaccination in circulating lymphocytes and at tumor site (10). However, because qRT-PCR does not identify the cells expressing the transcript of interest, such responses could not be directly attributed to tHLA-determined, CD8$^+$ T cells elicited by the vaccine (7). Therefore, the purpose of this study was to combine methods that directly enumerate and characterize the in vivo status of activation of vaccine-elicited T cells with minimal ex vivo manipulation. The immune response to vaccination of eight patients with metastatic melanoma who received repeated immunization with gp100:209–217(210 M) (g209-2 M) peptide in IFA was monitored using tHLA, qRT-PCR, and intracellular measurement of IFN-γ production by FACS analysis (IC-FACS) (11). A strong correlation was

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[2] Abbreviations used in this paper: MA, melanoma Ag; gp, glycoprotein; IC-FACS, intracellular FACS; qRT-PCR, quantitative real-time PCR; TCPF, T cell precursor frequency; tHLA, HLA/epitope tetrameric complex; TIL, tumor-infiltrating lymphocyte.

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noted between the phenotypic identification by tHLA of vaccine-specific T cells and expression of IFN-γ in response to relevant stimulation, suggesting that blunting of the status of activation of circulating T cells cannot be easily shown to be a significant factor determining failure of vaccination.

Materials and Methods

Patients' selection

Eight HLA-A*0201-expressing patients with metastatic melanoma (P1 to P8) received repeated s.c. injections of the gp100:209–217(210 M) (g209-2 M) peptide in IFA. Vaccinations were administered either at 1-wk (P6, P7) or 3-wk (P1, P2, P3, P4, P5, P8) intervals, and blood samples for PBMC extraction were obtained just before the next vaccination and 3 wk after the prior vaccination. The HLA class I phenotype of patients was determined on PBMC using sequence-specific primer PCR (12). PCR was also used for molecular subtyping of HLA-A*02 (13).

Cells and cultures

Samples were obtained from leukapheresis of patients before and after vaccination with peptides. PBMC were isolated by Ficoll gradient separation and frozen until analysis. Analysis of MA-specific T cells was performed after overnight resting of thawed PBMC in complete medium consisting of RPMI 1640 medium (Biofluids, Rockville, MD) supplemented with 10 mM HEPES buffer, 100 U/ml penicillin-streptomycin (Biofluids), 10 μg/ml Ciprofloxacin (Bayer, West Haven, CT), 0.03% L-glutamine (Biofluids), 0.5 mg/ml amphotericin B (Biofluids), 10% heat-inactivated human AB serum (Gemini Bioproducts, Calabasas, CA), and 300 IU IL-2/ml (Cetus Oncology Division, Chiron, Emeryville, CA). This step allowed depletion of adherent monocytes. The melanoma cell clones 624.38 MEL and 624.28 MEL cells were established and cultured as previously described (14). These clones were derived from the same bulk culture and are identical in expression of MA and HLA alleles with the exception of HLA-A*0201, whose expression is lost in 624.28-MEL due to aberrant splicing of HLA-A*0201 heavy chain mRNA (15). The breast cancer MCF-7 and the lymphoblastoid T2 cell lines were purchased from the American Type Culture Collection (Manassas, VA). The g209/209-2 M-reacting T cell clone C1–35 and 1520 H-3.1 have been previously described (16), while the T cell clones C6, H5, B3, and C11 were expanded and isolated according to a previously described protocol (16, 17) from bulk cultures of earlier patients' PBMC who had received g209-2 M vaccine.

Peptides

The peptides used for vaccination were 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 as relevant. The same peptides were used for stimulation of T cells.

Epitope-specific T cell staining using HLA-A2 tetramer

Tetrameric peptide-HLA-A*0201 complexes were produced as described previously (8, 18). Recombinant HLA-A*0201 heavy chain containing a biotinylation site and recombinant β_2-microglobulin were synthesized and used for refolding of soluble HLA molecules in the presence of a HLA-A*0201-binding peptide. Soluble HLA molecules were prepared for the following epitopes: gp100:209–217 (210 M) (IMDQVPFSV, g209-2 M); MART-1:26–35(27L) (ELAGIGILTV, MART-2L); and Flu-M1:58–66 (GILGFVFTL, Flu). 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 soluble HLA on gel filtration. Monomeric HLA/peptide complexes were biotinylated with BirA (Avidity, Denver, CO) at the heavy chain and separated from free biotin by gel filtration. Biotinylated HLA complexes were tetramerized by adding avidin-PE (Pierce, Rockford, IL) at a 4:1 molar ratio. The final concentration of tetramer was adjusted to 2 mg/ml. As examined by gel filtration, all tHLA were without detectable free avidin-PE. After overnight depletion of monocytes, nonadherent PBMC were resuspended at 1×10^6 cells/50 μl ice-cold FACS buffer (phosphate buffer plus 5% inactivated FCS; Biofluids). Cells were incubated on ice with 1 μg tHLA for 15 min, then cultured for 30 min with 10 μl anti-CD8 mAb (100 μg/ml; Becton Dickinson, San Jose, CA). Cells were washed twice in 2 ml cold FACS buffer before analysis by FACS (Becton Dickinson). Ten thousand events were acquired for analysis of T cell clones and 200,000 for PBMC samples.

FACS analysis for intracellular expression of IFN-γ

Nonadherent PBMC (1×10^6 cells) were stimulated for 6 h by directly adding soluble peptide (1 μg/ml). After 2 h, Brefeldin A (10 μg/ml; Sigma, Deisenhofen, Germany) was added. After additional 4 h, the cells were treated with 4500 U DNase I (Calbiochem, San Diego, CA) for 5 min at 37°C. EDTA (0.1 M) was added to each well before washing with cold PBS. Cells were fixed with 4% paraformaldehyde for 5 min and washed in PBS containing 0.1% BSA/PBS-S. Cells were permabilized and blocked overnight with PBS/Saponin/5% milk at 4°C. After staining with mAb for 30 min on ice, cells were washed in PBS-S. Staining with tHLA was performed before fixation of cells. All samples were analyzed on a Becton Dickinson FACSCalibur flow cytometer using the CellQuest software. The following mAb were used: allophyocyanin-conjugated mouse anti-human CD3 (IgG1), peridinin chlorophyl protein-conjugated mouse anti-human CD8 (IgG1), and FITC-conjugated mouse anti-human CD45RA were purchased from Becton Dickinson (Heidelberg, Germany); FITC-conjugated mouse anti-human IFN-γ (IgG1) and FITC-conjugated mouse anti-human CD45RO were purchased from PharMingen.

Direct molecular assessment of peptide and melanoma-specific CTL reactivity

Cryopreserved PBMC were thawed into complete medium, as described (2). Direct PBMC assays were conducted using 3×10^6 PBMC in 2 ml of media. Thawed PBMC were allowed to recover by incubation at 37°C in 5% CO_2 for 10 h. A total of 1 μM of peptide or 1×10^6 melanoma cells was then added to the PBMC and incubated at 37°C in 5% CO_2 for 2 h. Soluble peptide was added rather than peptide pulsed onto T2 or other APCs, because these cells have high expression of costimulatory and adhesion molecules that could have exaggerated the responsiveness of the PBMC. No exogenous cytokines or other stimulants were added. The cells were then harvested for RNA isolation and cDNA transcription.

Quantitative real-time PCR

RNA isolation from PBMC was performed in batches containing patient pre- and posttherapy samples with RNeasy mini kits (Qiagen, Santa Clarita, CA). The RNA was eluted with water and stored at −70°C. For cDNA synthesis, about 1 μg of total RNA was transcribed with cDNA transcription reagents (Perkin-Elmer, Foster City, CA) using random hexamers and directly tested. Measurement of gene expression was performed utilizing the ABI prism 7700 Sequence Detection System (Perkin-Elmer), as previously described (19, 20). Primers and *Taq* Man 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 (10). *Taq* Man probes were labeled at the 5′ end with the reporter dye molecule FAM (6-carboxy-fluorescein; emission λ_{max} = 518 nm) and at the 3′ end with the quencher dye molecule TAMARA (6-carboxytetramethyl-rhodamine; emission λ_{max} = 582 nm). cDNA standards were generated by reverse-transcriptase, primer-specific amplification of mRNA of the relevant genes using a technique identical to the one used for the preparation of test cDNA. Amplified cDNA was then purified and measured by spectrophotometry (A260 OD). Copies were calculated using the m.w. of each individual gene amplicon. RT-PCR of cDNA specimens and cDNA standards were conducted in a total volume of 25 μl with 1× *Taq* Man Master Mix (Perkin-Elmer). 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 (19). All PCR assays were performed in duplicates and reported as the average. qRT-PCR was performed for IFN-γ and IL-2 mRNA expression and normalized to copies of CD8 mRNA from the same sample. Data were adjusted for CD8 mRNA copies on the basic immunologic assumption that stimulation with an HLA class I-restricted epitope defines $CD8^+$ T cells as the only relevant population. Since the frequency of $CD8^+$ T cells varies in time in individual patients, it is incorrect to present data corrected by expression of housekeeping genes, such as GAPDH or β-actin, expressed by any cell as done in other qRT-PCR applications (19, 20).

Statistical analysis

TCPF was calculated as previously described (8). $CD3^+/CD8^+$ T cells were gated, and the fluorescence intensity of tHLA and IFN-γ staining reported in the *y*- and *x*-axis, respectively. The frequency (*f*) of $tHLA^+$ T cells per 100 $CD8^+$ T cells was calculated according to the formula:

f_{tHLA} = upper left quadrant (ULQ)/(ULQ + lower left quadrant (LLQ)) × 100 with the upper left quadrant containing the tHLA$^+$, CD8$^+$ T cells, and the left lower quadrant the tHLA$^-$, CD8$^+$ T cells. From these frequencies, the background positivity in samples prepared with CD8 mAb but without tHLA was subtracted to correct for nonspecific fluorescence in the ULQ. IFN-γ-TCPF was calculated according to the following formula: $f_{IFN\text{-}\gamma}$ = upper right quadrant (URQ) + lower right quadrant (LRQ)/(URQ + LRQ + LLQ) × 100 with the right quadrants containing the IFN-γ$^+$, CD8$^+$ T cells, and the left quadrants the IFN-γ$^-$, CD8$^+$ T cells. From these frequencies, the background was subtracted as for tHLA fluorescence.

The various methods were compared by adopting as independent parameter the data accrued with tHLA staining of PBMC. Postvaccination samples were divided in positive or negative for enrichment of tHLA staining based on a ≥3-fold increase in frequency of staining cells compared with prevaccination samples. Positive and negative samples were then examined for concordance with other methods by Spearman rank correlation analysis. The following dependent parameters were correlated with tHLA staining. 1) tHLA staining after stimulation of PBMC with 209 and 209-2 M peptide. Positive samples were considered those in which at least a 50% decrease in TCPF was noted compared with unstimulated samples (None column in Table I). 2) tHLA staining after stimulation of PBMC with HLA-A*0201 expressing and not expressing melanoma cells (Table II). Positive samples were considered those in which the difference in TCPF between postvaccination PBMC stimulated with HLA-A2$^+$ or HLA-A2$^-$ negative melanoma cells was at least 3-fold the difference in prevaccination PBMC. 3) IC-FACS staining for IFN-γ in samples stimulated with 209 or 209-2 M peptide. Positive samples were considered those with IFN-γ TCPF at least 3-fold above pretreatment samples (Table I). 4) IC-FACS staining for IFN-γ in samples stimulated with HLA-A*0201 expressing and not expressing melanoma cells. Positive samples were considered postvaccination PBMC in which the difference in frequency of stained cells stimulated with HLA-A2$^+$ and HLA-A$^-$ melanoma was at least 3-fold the difference in prevaccination samples (Table II). 5) qRT-PCR identification of vaccine-induced IFN-γ expression in 209 and 209-2 M stimulated postvaccination PBMC (Table I). Positive samples were postvaccination PBMC with IFN-γ mRNA expression at least 3-fold above prevaccination samples. 6) qRT-PCR identification of vaccine-induced IFN-γ expression in samples stimulated with HLA-A*0201 expressing and not expressing melanoma cells. Positive samples consisted of postvaccination PBMC, in which the ratio of mRNA expression between samples stimulated with HLA-A2$^+$ and HLA-A2$^-$ cells was at least 3-fold the difference in prevaccination samples (Table II). Statistical significance for each pair analyzed is presented as Spearman's ρ correlation coefficient and respective *p* value.

Results

Phenotype of vaccine-elicited CD8$^+$ T cells

Vaccine-specific T cells were enumerated by comparing g209-2 M/tHLA TCPF in pre- and postvaccination PBMC (Table I). In six patients (P3, P4, P5, P6, P7, and P8), a more than 3-fold enhancement of vaccine-elicited TCPF was noted. In one additional patient (P2), a high TCPF of vaccine-specific T cells was also noted. However, in this case, the high TCPF could not be attributed to the vaccine because prevaccination specimens convincingly demonstrated high frequency of circulating T cells that stained with tHLA (Fig. 1). No constitutive expression of IFN-γ was noted in any PBMC population independently of the detection of vaccine-specific T cells by tHLA.

Table I. *Identification of vaccine-specific CD8$^+$ T cells in PBMC of patients with metastatic melanoma by tHLA staining and IFN-γ expression*

					IFN-γ			
		209-2M tHLA$^+$ (% total CD8$^+$)			IC-FACS		qRT-PCR[a]	
Patient	Treatment	None	209[b]	209-2M[c]	209[d]	209-2M[e]	209[f]	209-2M[g]
P1	Pre	0.06	0.06	0.04	0.08	0.09	1	1
	Post 2	0.04	0.07	0.07	0.03	0.05	1	1
	Post 4	0.04	0.02	0.02	0.00	0.00	1	1
P2	Pre	2.46	2.22	2.39	0.03	0.00	1	1
	Post 3	0.43	0.26	0.47	0.00	0.00	1	2
P3	Pre	0.06	0.08	0.08	0.00	0.00	1	1
	Post 2	0.30	0.10	0.09	0.14	0.17	2	4
P4	Pre	0.08	0.09	0.08	0.01	0.00	1	2
	Post 2	0.07	0.10	0.09	0.03	0.02	1	1
	Post 4	0.27	0.18	0.20	0.05	0.10	6	10
P5	Pre	0.00	0.00	0.00	0.00	0.00	1	1
	Post 2	0.05	0.03	0.05	0.00	0.04	1	1
	Post 4	0.60	0.14	0.04	0.22	0.32	4	7
P6	Pre	0.02	0.04	0.04	0.02	0.03	1	1
	Post 4	0.17	0.10	0.07	0.08	0.16	3	6
	Post 8	0.63	0.25	0.20	0.11	0.05	12	12
	Post 12	0.55	0.37	0.23	0.22	0.25	3	9
P7	Pre	0.08	0.03	0.01	0.00	0.00	2	1
	Post 4	0.08	0.07	0.08	0.00	0.00	1	1
	Post 8	0.86	0.07	0.06	0.07	0.00	74	52
P8	Pre	0.11	0.00	0.12	0.20	0.37	1	1
	Post 4	2.35	0.17	0.36	1.00	1.32	77	155

[a] Increase in IFN-γ mRNA per 10^6 CD8 mRNA copies in stimulated vs unstimulated PBMC.
[b] tHLA-209 Spearman's $\rho = 0.746$, $p = 0.007$.
[c] tHLA-209-2M Spearman's $\rho = 0.900$, $p = 0.001$.
[d] IC-FACS 209 Spearman's $\rho = 0.785$, $p = 0.005$.
[e] IC-FACS 209-2M Spearman's $\rho = 0.785$, $p = 0.005$.
[f] qRT-PCR 209 Spearman's $\rho = 0.815$, $p = 0.003$.
[g] q-RT-PCR 209-2M Spearman's $\rho = 0.900$, $p = 0.001$.

Table II. *Tumor recognition of vaccine-specific CD8$^+$ T cells in PBMC of patients with metastatic melanoma by tHLA staining and IFN-γ expression*

					IFN-γ		
		209-2M tHLA$^+$ (% total CD8$^+$)			IC-FACS		qRT-PCR[a]
Patient	Treatment	None	A2^{+b}	A2$^-$	A2^{+c}	A2$^-$	A2$^+$/A2^{-d}
P1	Pre	0.06	0.00	0.01	0.00	0.00	1
	Post 2	0.04	0.01	0.03	0.13		6
	Post 4	0.04	0.02	0.02	0.10	0.01	100
P2	Pre	2.46	0.77	0.66	0.24	0.01	1
	Post 3	0.43	0.00	0.00	0.36	0.00	6
P3	Pre	0.06	0.06	0.08	0.05	0.09	2
	Post 2	0.30	0.13	0.24	0.13	0.05	2
P4	Pre	0.08	0.07	0.08	0.00	0.00	5
	Post 2	0.07	0.10	0.10	0.07	0.05	1
	Post 4	0.27	0.20	0.36	0.10	0.02	86
P5	Pre	0.00	0.00	0.00	0.00	0.00	1
	Post 2	0.05	0.03	0.04	0.01	0.03	7
	Post 4	0.60	0.19	0.55	0.37	0.13	6
P6	Pre	0.02	0.06	0.05	0.12	0.12	1
	Post 4	0.17	0.11	0.11	0.33	0.33	5
	Post 8	0.63	0.50	0.59	0.65	0.59	11
	Post 12	0.55	0.48	0.61	1.44	0.16	10
P7	Pre	0.08	0.02	0.03	0.05	0.07	1
	Post 4	0.08	0.09	0.09	0.25	0.09	2
	Post 8	0.86	0.30	0.71	0.45	0.07	83
P8	Pre	0.11	ND[e]	ND	ND	ND	1
	Post 4	2.35	ND	ND	ND	ND	46

[a] IFN-γ mRNA per 10^6 CD8 mRNA copies in PBMC stimulated with the HLA-A2$^+$ melanoma cell line 624.38-MEL. All patients with the exception of P3, P4, and P6 matched 624.38 only at HLA-A*0201. P3, P4, and P6 matched also A*0301. Reactivity associated with this allele was excluded by presenting data as fold IFN-γ expression by 624.38-MEL-stimulated PBMC over expression after stimulation with the HLA-A*0201, otherwise identical melanoma cell line 624.28-MEL. No HLA-B or Cw alleles matched 624-MEL lines.

[b] tHLA-A2+ Spearman's $\rho = 0.900$, $p = 0.001$.

[c] IC-FACS-A2$^+$ Spearman's $\rho = 0.785$, $p = 0.01$.

[d] qRT-PCR-A2$^+$ Spearman's $\rho = 0.531$, $p = 0.055$.

[e] In this patient no sufficient material was available for this experiment.

Functional response of vaccine-elicited T cells to vaccine-related epitopes

tHLA-based TCPF were compared with the ability of PBMC to produce IFN-γ upon stimulation with epitopes relevant to the vaccination such as g209-2 M (vaccine) or the parental g209. This functional component of the immune response to vaccination was addressed using IC-FACS analysis and qRT-PCR (Table I). We, however, consistently noted that stimulation caused a down-regulation of tHLA staining that accompanied IFN-γ expression (Fig. 2). To better characterize the down-regulation of tHLA staining in response to stimulation, we examined the behavior of a clone (1520 H.3-1) from a tumor-infiltrating lymphocyte (TIL) that naturally recognizes the g209 epitope and cross-reacts with g209-2 M. This CD8$^+$TIL was expanded from a melanoma metastasis by exogenous administration of IL-2 and without ex vivo exposure to Ag/epitope-specific stimulation (16). Down-regulation and IFN-γ expression occurred upon exposure of 1520 H.3-1 to tHLA/209 (or tHLA-209-2 M) at 37°C, but not 4°C, and correlated with CD69 up-regulation (Fig. 3). To avoid the direct effect of tHLA on the intensity of staining of epitope-specific T cells at 37°C, all stainings in this study were performed, therefore, at 4°C. Interestingly, CD3 detection decreased in response to incubation of 1520 H.3-1 with tHLA at 37°C; however, it was never of the extent characterizing tHLA-staining down-regulation (data not shown). This persistence of CD3 had the important practical advantage of allowing sorting of CD3$^+$ cells in the four-color system utilized for FACS analysis in this study.

The down-regulation of staining caused by tHLA required an active metabolic state. Stimulation of 1520 H.3-1 at 37°C with T2 cells exogenously pulsed with 1 μM g209, but not Flu peptide induced contemporarily down-regulation of tHLA staining and production of IFN-γ (Fig. 4*a*). Stimulating at 4°C reversed both phenomena (data not shown). Furthermore, coculture with an HLA-A*0201/gp100 expressing melanoma cells (624.38 MEL), but not an HLA-A*0201-negative melanoma (624.28 MEL) nor a breast cancer (MCF-7) cell line caused dramatic down-regulation of tHLA staining and production of IFN-γ (Fig. 4*b*). Also in this case, stimulation of 1520 H-3.1 at 4°C reversed down-regulation and ability to produce IFN-γ (not shown). Thus, an inverse correlation exists between tHLA staining of CD8$^+$ T cells and their activation that could be considered an additional, though indirect, sign of productive TCR engagements (Table I). As a consequence of staining down-regulation, however, stimulation concealed vaccine-specific T cells, not allowing direct attribution of IFN-γ expression to tHLA-stained T cells.

IC-FACS demonstrated that PBMC from five of the six patients who experienced increased TCPF in response to vaccination could definitively produce IFN-γ in response to g209-2 M and g209

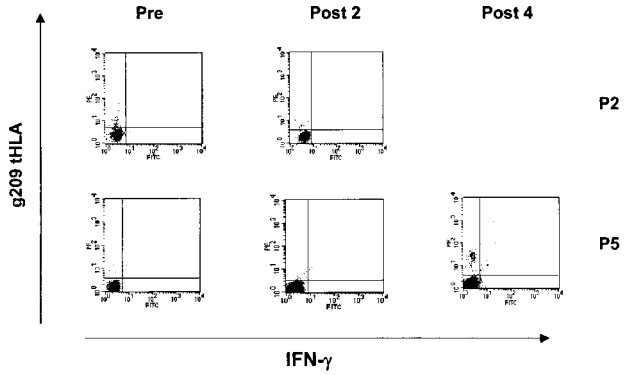

FIGURE 1. Identification of vaccine-specific TCPF by g209-2 M/tHLA in PBMC from two patients (P2 and P5) before and after vaccination (Post 2 and Post 4 = after two and four vaccinations). Four-color FACS was used. $CD3^+/CD8^+$ cells were gated during acquisition and analyzed for tHLA staining (PE, *y*-axis) and intracellular IFN-γ expression (FITC, *x*-axis). PBMC were cultured for 2 h at 37°C. Brefeldin A (10 μg/ml) was added and the culture was continued at 37°C for an additional 4 h. Staining with tHLA was performed at 4°C. Shown here is one experiment representative of two yielding similar results. TCPF from this and the following figure are reported in Table I for the first experiment performed in each patient.

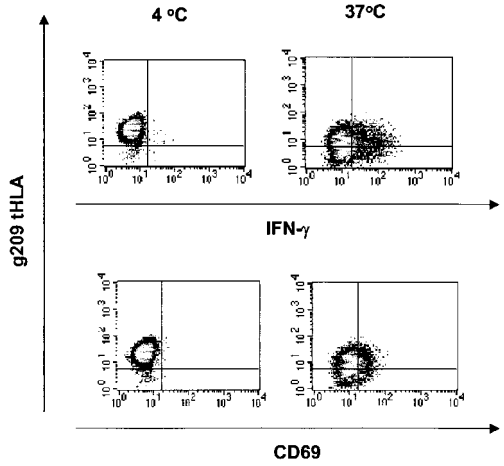

FIGURE 3. *A*, Intensity of staining with tHLA of relevant T cells can be down-regulated by incubation at 37°C with tHLA itself, and this down-regulation correlates with IFN-γ production and CD69 expression. The HLA-A*0201-restricted/g209-specific T cell clone 1520 H.3-1 TIL derived from a melanoma metastasis was incubated for 2 h at 37°C or 4°C with g209-2 M/tHLA. Brefeldin A (10 μg/ml) was then added, and the culture was continued for an additional 4 h at 37°C and 4°C, respectively. Intensity of staining is down-regulated only at 37°C, but not 4°C. For this reason, all experiments in this study were performed incubating samples with tHLA at 4°C. Shown is one experiment representative of three yielding similar results.

peptides (P3, P4, P5, P6, and P8). Another patient (P7) demonstrated equivocal results. In the remaining patients, no vaccine-responsive T cells could be detected. Furthermore, PBMC from the patient with high TCPF in the vaccine-naive status (P2) were unresponsive to vaccine-related stimuli. Spearman rank correlation analysis demonstrated a strong correlation between detection of vaccine-enhanced TCPF and IFN-γ expression by IC-FACS (ρ = 0.78 for both g209 and g209-2 M-stimulated PBMC; p = 0.005). Measurement of IFN-γ transcript by qRT-PCR matched the results observed by IC-FACS and detected vaccine responsiveness in one additional patient (P7). The discrepancy between the results obtained by IC-FACS and qRT-PCR was consistent upon various retesting and is not easily explainable, as higher sensitivity of qRT-PCR over other methods as not been, to this point, demonstrated. Also, in the case of qRT-PCR, a strong correlation was noted between tHLA-based TCPF and IFN-γ expression (Spearman's ρ = 0.81 and 0.90 for g209 and g209-2 M-stimulated PBMC, respectively; p = 0.003 and 0.001). Expression of IL-2 mRNA was also tested by qRT-PCR. In five patients (P4, P5, P6, P7, and P8), it was possible to identify a more than 3-fold increase in IL-2 transcript compared with prevaccination PBMC (data not shown). This evaluation of vaccine-specific T cells, therefore, suggested that repeated administration of minimal epitope sequences in IFA could enhance the frequency of functional $CD8^+$ T cells in patients with metastatic melanoma. Interestingly, vaccine-naive PBMC from P2 that demonstrated high tHLA-based TCPF did not demonstrate significant levels of IFN-γ production upon stimulation, suggesting that promiscuous TCR/tHLA interactions at 4°C might have been responsible for these TCPF (9). Attempts to stain this PBMC population at higher temperatures revealed that at room temperature is was still possible to identify a tHLA-staining population. Staining carried at 37°C demonstrated almost a total loss of tHLA staining. However, it could not be totally excluded that this loss of staining was due to tHLA-induced down-regulation that can occur at this temperature, as shown in Fig. 3. Indeed, marked reduction

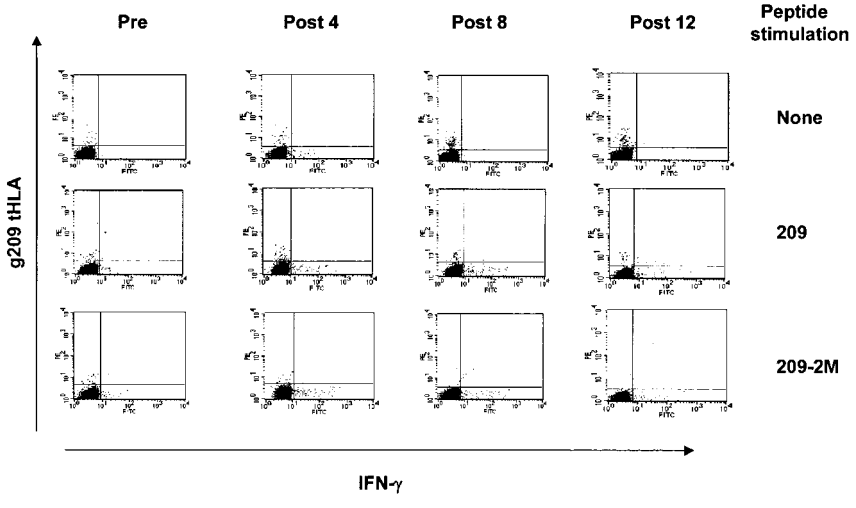

FIGURE 2. FACS analysis for tHLA staining and intracellular IFN-γ expression in pre- and postvaccination PBMC from patient 6. $CD3^+/CD8^+$ cells were gated during acquisition and analyzed for tHLA staining (*y*-axis) and intracellular IFN-γ expression (*x*-axis). PBMC obtained before and after 4, 8, and 12 vaccinations were cultured for 2 h at 37°C alone (*top row*) or in the presence of g209 (*middle row*) or g209-2 M (*bottom row*, 1 μM). Brefeldin A (10 μg/ml) was then added and the culture was continued for an additional 4 h at 37°C. Staining with tHLA was performed at 4°C. Down-regulation is seen in all samples receiving stimulation compared with unstimulated specimens. TCPF are reported in Table I. Shown is one experiment representative of three yielding similar results.

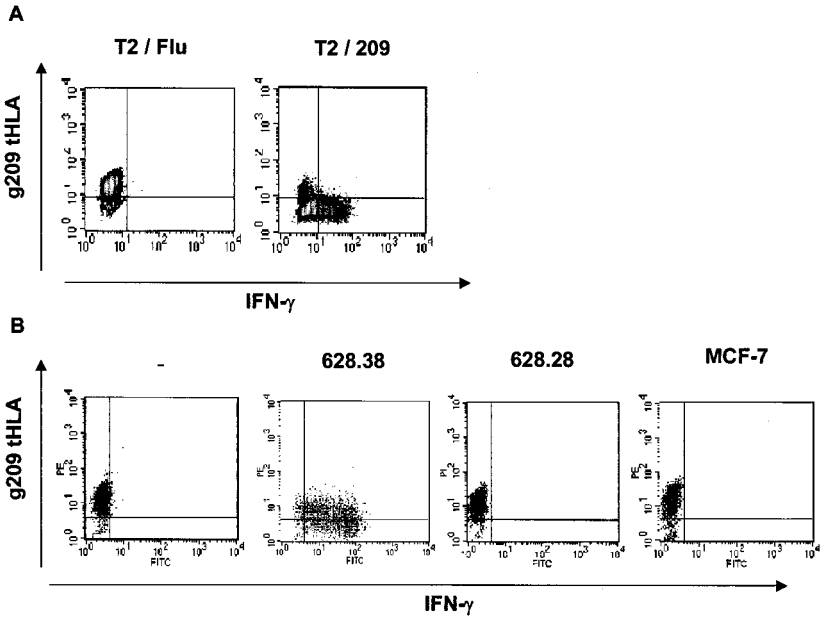

FIGURE 4. *A*, Down-regulation of tHLA staining induced by TCR-ligand interaction with cognate epitope. The HLA-A*0201-restricted/g209-specific T cell clone 1520 H.3-1 TIL derived from a melanoma metastasis was stimulated in the presence of T2 cells exogenously pulsed with 1 μM Flu M1: 58–66 or g209-2 M peptide. Stimulation and incubation in the presence of Brefeldin A (10 μg/ml) were performed at 37°C. Staining with tHLA was performed at 4°C. Coincidentally to production of IFN-γ, a strong reduction of tHLA staining is noted. *B*, The same down-regulation of tHLA staining and IFN-γ production are noted when 1520 H.3-1 is stimulated with an HLA-A*0201-expressing melanoma cell line (624.38-MEL), but not an HLA-A*0201-negative melanoma (624.28 MEL) nor an HLA-A*0201-expressing breast cancer cell line (MCF-7) not expressing gp100. One experiment representative of three yielding similar results is shown.

in intensity of tHLA staining was noted in all PBMC populations when staining was carried at 37°C.

Affinity of tHLA for vaccine-elicited T lymphocytes

Affinity of vaccine-elicited T cells for cognate epitope, rather than their status of activation, might modulate their sensitivity to stimulation. In fact, tHLA have been used to identify high avidity tumor-reactive CTL (21). Therefore, intensity of tHLA staining of vaccine-elicited T cells was compared with that of T cell clones whose requirements for activation could be characterized (Fig. 5*A*). Epitope-driven CTL cultures of postvaccination PBMC from earlier patients vaccinated with g209-2 M were cloned according to previously described methods (16, 17). The clones were then tested for ability to produce IFN-γ in response to stimulation with T2 cells pulsed with g209 (and 209-2 M, not shown) peptide. Among them, a clone was selected for its poor avidity for the HLA-A*0201/g209 complex (C1–35), two for intermediate avidity (C11 and C6), and two for increasingly high avidity (B3 and H5). In addition, the TIL clone 1520 H-3.1 naturally recognizing g209 and cross-reacting with g209-2 M was analyzed. Staining for CD3 and tHLA was then compared among these clones and tHLA$^+$ PBMC from patients in this study (Fig. 5*B*). Most tHLA-positive PBMC stained for tHLA with intensity comparable with that of intermediate affinity CTL and TIL (C6, C11, and 1520 H-3.1). Although the expression of CD3 and CD8 coreceptors can play an important role in T cell activation/avidity (22, 23), we did not notice large differences in expression of these two markers in tHLA-staining PBMC in these patients. Thus, differences and similarities of tHLA staining in these patients could probably be attributed directly to avidity of the TCR for the cognate ligand. Interestingly, in two patients

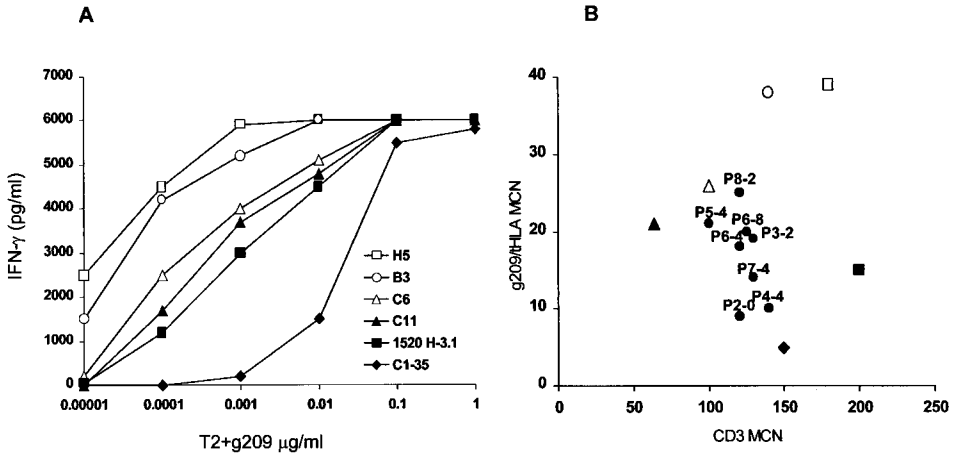

FIGURE 5. Intensity of g209-2 M/tHLA staining of vaccine-elicited lymphocytes. *A*, CD8$^+$ T cell clones obtained after in vitro stimulation of postvaccination PBMC were ranked according to their sensitivity to stimulation with T2 cells exogenously pulsed with limiting dilutions of g209 peptide. In addition, the HLA-A*0201-restricted/g209-specific T cell clone 1520 H.3-1 TIL derived from a melanoma metastasis was included. *B*, Intensity of staining for CD3 and tHLA/209 of the same T cells was compared with staining of tHLA-positive PBMC in vaccinated patients (P3-2, P4-4, P5-4, P6-4, P6-8, P7-4, and P8-2) and in prevaccination PBMC of patient 2 (P2-0) that also demonstrated enhanced 209-2 M/tHLA TCPF. The second digit for each patient refers to the number of vaccinations received from the patient before the sample was collected (see Table II). Data are presented as mean channel of fluorescence within the same experiment. Since no significant differences in T cell size were noted among different PBMC populations, no correction for cell size was introduced.

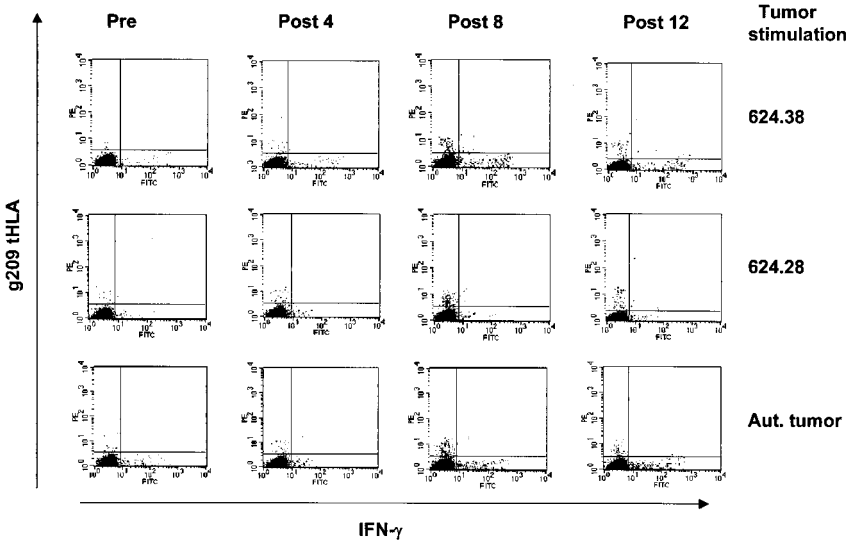

FIGURE 6. MA-specific reactivity of vaccine-specific $CD8^+$ T cells. PBMC from P6 were cocultured for 2 h at 37°C with melanoma cells expressing HLA-A*0201 (624.38-MEL) or a clone from the same cell line that had lost expression of this allele (624.28-MEL). In addition, PBMC could be tested against autologous tumor cells. Brefeldin A (10 μg/ml) was added and PBMC were cultured for an additional 4 h at 37°C. Staining with tHLA was performed at 4°C. PBMC were obtained before vaccination and 3 wk after 4, 8, and 12 vaccinations. $CD3^+/CD8^+$ cells were gated during acquisition and analyzed for 209-2 M/tHLA and IFN-γ staining. TCPF are reported in Table II. Shown is one experiment representative of three yielding similar results.

(P4 and P7), relatively lower intensity of staining was noted that correlated with a reduced ability to detect IFN-γ upon stimulation by IC-FACS. In both patients, however, qRT-PCR could demonstrate evidence of vaccination-induced enhancement of IFN-γ expression upon cognate stimulation. It is possible that this might reflect higher sensitivity of this method compared with IC-FACS. Fluorescence of individual T cells producing low amounts of IFN-γ might not be discriminated from background fluorescence by FACS. At the same time, qRT-PCR might detect low abundance transcript if a sufficient number of cells express, although at low levels, IFN-γ. Vaccine-naive PBMC from P2 (P2-0) were also included in this analysis. These PBMC demonstrated the lowest intensity of staining that correlated with total unresponsiveness to stimulation, strengthening the hypothesis that these PBMC promiscuously bound g209-2 M/tHLA at the temperatures used in this study.

Recognition of tumor cells by vaccine-elicited $CD8^+$ T cells

PBMC from the same patients were assessed for ability to express IFN-γ upon exposure to melanoma cells expressing the HLA allele targeted by the vaccination (A*0201). In general, a good correlation was noted between vaccine-epitope and tumor cell recognition whether assessed by IC-FACS (Fig. 6) or by qRT-PCR (Table II). However, surprisingly, tumor cells appeared to be recognized more frequently after vaccination and independently of documented vaccine-elicited T cell responses. This finding suggested that vaccines (either the MA epitope or the IFA) might induce broader immune effects than those strictly expected by the specificity of the vaccination.

Development of vaccine-specific T cells does not correlate with a specific CD45 RO/RA phenotype

In all patients (P3, P4, P5, P6, P7, and P8) in which enhancement of TCPF was noted after vaccination, we tested the CD45 RO/RA phenotype of vaccine-specific T cells. We found an unpredictable pattern of CD45 expression with a patient characterized by an almost totally CD45 RO^{high} tHLA-staining population (P7) (Fig. 7*A*). A second patient (P8) demonstrated a high frequency of CD45 RA/RO double-negative tHLA staining T cells (Fig. 7*B*). Interestingly, in this patient, the double-negative T cells appeared to be extremely sensitive to cognate stimulation, as judged by the level of tHLA down-regulation upon epitope stimulation. The other patients (P3, P4, P5, and P6) demonstrated a pattern in between the other two patients with a small proportion of tHLA-staining T cells included in all CD45 categories. Thus, the correlation between reactivity of tumor-specific T cells and their CD45 RO/RA phenotype noted by others (6, 7) was not identified in this study, indicating that subsets of memory lymphocytes with various effector function might be better resolved by other markers (24).

Discussion

Others and we have been addressing the paradoxical coexistence in nature of tumors and circulating tumor Ag-specific CTL (4, 5). In addition, by following tumor Ag/epitope-specific vaccinations, we have identified clear immune responses that do not lead to tumor regression (1). Obviously, by inducing epitope-specific T cell responses, the primary goal of vaccination is, at least in principle, achieved by these treatments. The missing secondary impact on tumor growth might be explained by a chain of downstream events that include lack of localization at tumor site and/or unfavorable circumstances within the tumor microenvironment, as recently summarized (3).

Alternatively, vaccine-elicited responses identified by current methods might have not achieved in qualitative and/or quantitative terms the richness required for clinical effectiveness. Vaccine-elicited T cell responses have been predominantly characterized by indirect methods, including parallel in vitro sensitization of pre- and postvaccination PBMC with vaccine-related epitopes (1, 2). Although in vitro expansion of epitope-specific T cells indisputably demonstrated vaccine-related effects on vaccine recipients, it cannot enumerate and characterize the in vivo function of vaccine-elicited T cells because of the arbitrary amount of Ag and cytokine applied in culture.

Adopting tHLA technology, we directly documented and quantified the immune response in PBMC of patients receiving the gp100-related epitope: g209-2 M (8). We noted a correlation between increased vaccine-specific TCPF and susceptibility to in vitro stimulation. However, it was generally felt that such demonstration did not adequately address the in vivo status of T cell activation (7). In fact, the ability of tumor-specific circulating T cells identified by tHLA to respond to physiologic levels of cognate stimulation has been questioned, and selective anergy has been invoked to explain T cell and tumor coexistence (7). Others have attributed discrepancies between tHLA staining and T cell responsiveness to promiscuity of TCR/tHLA interactions since the enhanced affinity induced by multimerization of peptide/HLA complexes may allow binding to TCRs with avidity too low to ever

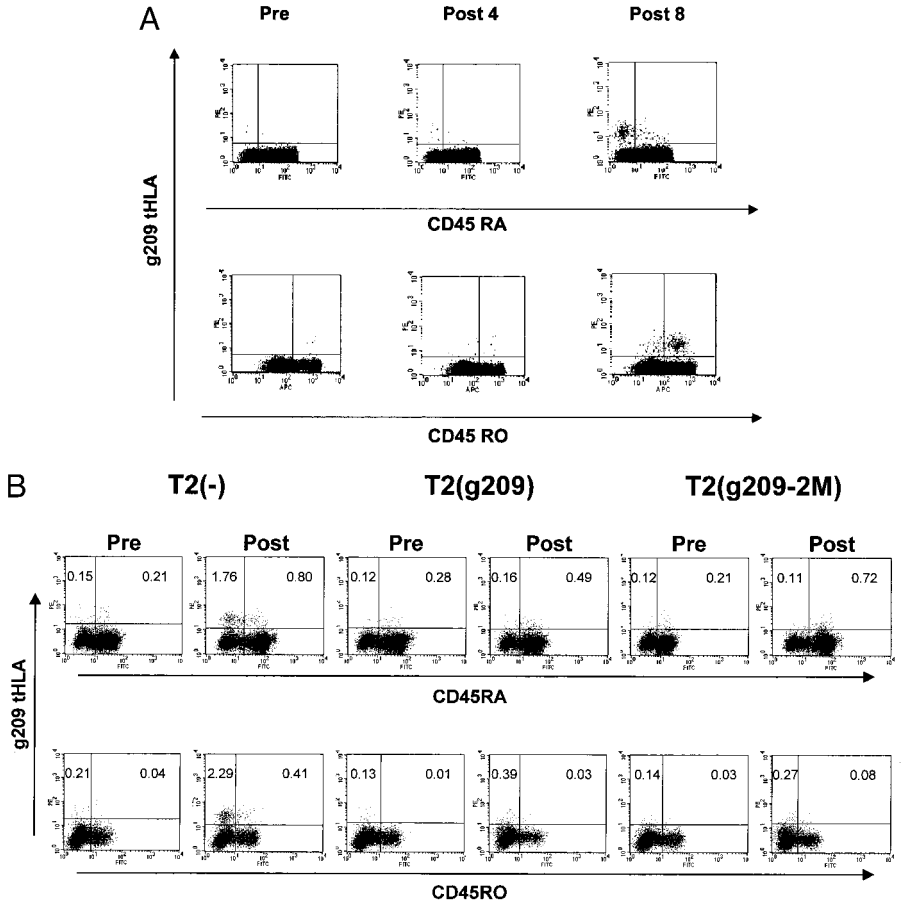

FIGURE 7. CD45 RA and CD45 RO expression of vaccine-specific T cells. *A*, High frequency of vaccine-specific T cells is associated with a high percentage of CD45 RO^{high} T cells in P7. *B*, A different pattern of CD45 expression is noted in P8: a high proportion of vaccine-specific T cells in this patient did not express CD45 RA or RO. Still, these double-negative T cells were responsive to stimulation, as judged by tHLA-staining down-regulation when cocultured in the presence of T2 cells pulsed with 1 μM g209 or g209-2 M peptide.

generate ligand-induced physiological responses (9). We, therefore, studied the level of activation of vaccine-elicited circulating CD8$^+$ T cells by combining a series of methods that allow direct assessment of T cell function in PBMC with minimal ex vivo manipulation and comparing these results with tHLA-based phenotyping. These methods included measurement upon vaccine-specific stimulation of IFN-γ transcript and protein by qRT-PCR or IC-FACS (8, 10).

Originally, we hoped to identify tHLA/IFN-γ double-positive T cells in vaccinated patients, but rapidly learned that such events cannot occur. Staining of epitope-specific T cells was disproportionately down-regulated when incubation with tHLA was performed at 37°C, but not at 4°C. Lack of detection of tHLA$^+$/IFN-γ-producing T cells was due to a direct effect of TCR engagement in metabolically active cells than tHLA internalization since it occurred upon cognate stimulation at 37°C, followed by tHLA staining at 4°C, a temperature at which internalization of tHLA does not occur (9). tHLA down-regulation could be observed in a g209 and g209-2 M-reacting T cell clone characterized by good responsiveness to limiting concentrations of epitope and naturally recognizing melanoma cells (16). This down-regulation followed incubation with tHLA at 4°C, following stimulation at 37°C with epitope-loaded T2 cells or melanoma cells. Furthermore, down-regulation correlated with CD69 expression, suggesting that it is more likely related to the metabolic and functional state of the cell rather than to artifactual tHLA/TCR interactions at various temperatures. Thus, we concluded that down-regulation of HLA staining is associated with T cell activation and parallels TCR down-regulation (25). We, therefore, considered down-regulation of tHLA staining additional evidence of productive TCR/epitope engagements detectable in vaccine-specific PBMC.

Because of tHLA-staining down-regulation, direct evidence of vaccine-elicited T cell responsiveness to vaccine-related stimuli could not be sought by identifying tHLA/IFN-γ double-positive T cells. Thus, we evaluated responsiveness of PBMC by correlating tHLA down-regulation with staining for IFN-γ protein and measurement of IFN-γ transcript by qRT-PCR in pre- and postvaccination PBMC. IC-FACS staining suggested responsiveness to stimulation with peptide in five of the six patients who demonstrated vaccine-elicited increase in TCPF by tHLA. qRT-PCR appeared more sensitive, as it could detect IFN-γ transcript in all of them. Upon stimulation with tumor cells, enhancement of IFN-γ production could be detected in all patients independently of the method (IC-FACS or qRT-PCR), suggesting that stimulation with tumor cells represented a stronger stimulus than the exogenous administration of soluble peptide. Indeed, the direct administration of soluble peptide to PBMC preparations may represent a suboptimal stimulus that was purposefully chosen to approximate the responsiveness of vaccine-elicited T cells to nonspecialized Ag presentation. It was felt that stimulation of PBMC with T2 cell line or other professional APCs that express abundant amounts of costimulatory and adhesion molecules might have arbitrarily exaggerated the responsiveness of vaccine-elicited T cells.

It has been shown that suboptimal activation of melanoma Ag-specific lymphocytes could be related to low avidity of TCR/MHC tumor peptide interaction (26). Such low avidity TCR may be included in the repertoire of postvaccination PBMC. A limited g209-specific TCR repertoire might be naturally available in vaccine-naive patients, since gp100 is a self protein and elimination of high affinity TCRs has recently been shown to play a role in these circumstances in another tumor model (27). Furthermore, the vaccination itself

might have eliminated high avidity T cell clones (28–31), although such phenomenon is, at the moment, controversial (32). In this study, tHLA-guided identification of vaccine-specific T cells did not always correlate with IFN-γ expression upon stimulation. Since it has been shown that the avidity of TCR/MHC epitope interaction can be graded by measuring the intensity of tHLA staining of tumor-specific T cells (21), we evaluated this parameter in vaccine-elicited PBMC. Assessment of tHLA staining of vaccine-elicited T cells in those cases in which no IFN-γ induction was noted upon stimulation suggested a lower avidity of their TCR compared with that of PBMC that could clearly produce IFN-γ upon stimulation. Thus, lack of correlation between tHLA-guided identification of vaccine-elicited T cells and IFN-γ production appeared to be explainable by lower avidity of the TCR repertoire induced by the vaccination in some patients.

An additional finding of this study was the consistently larger number of T cells that could be identified with g209-2 M/tHLA compared with those that could be identified by IC-FACS upon stimulation. This finding emphasizes that tHLA staining may overestimate the frequency of functional Ag-specific CTL especially when, like in our case, tetramer-low cells are counted as tetramer positive (for instance, in patient P2-0) (9). Alternatively, a significant proportion of vaccine-specific T cells identified by tHLA could display blunted reactivity to antigenic stimulation. Whether the glass is half empty or half full remains an open question; however, the identification of a significant percentage of stimulation-responsive T cells in postvaccination PBMC suggests that anergy is not a global occurrence among vaccine-elicited T cells.

It has been noted that tHLA staining calculates CTL precursor frequencies considerably higher than those suggested by enzyme-linked immunospot or limiting dilution assays (33). Several studies have measured the response of T cells to acute or chronic viral infections or during ongoing autoimmune episodes (33–38). Studies on tumor reactivity have shown that, in melanoma patients with vitiligo, MART-1-specific CTL have been identified at a frequency up to 0.67% of $CD8^+$ T cells (39). Furthermore, MART-1-specific T cells could be identified in melanoma cell-infiltrated lymph nodes, with a frequency ranging from 0.22 to 1.8% of $CD8^+$ T cells, and correlated with MA expression (40). Characterization of circulating T cells demonstrated identifiable MA-specific T cells in approximately one-half of patients affected with metastatic melanoma (7). In this study, vaccine-specific T cell frequency increased up to 2.3% of $CD8^+$ cells. This is a relatively low number of CTL precursors after vaccination compared with viral and autoimmune models. An inverse correlation has been reported between HIV-specific CTL frequency and viral RNA load in HIV-infected individuals (34). Furthermore, TCPF as high as 2% of $CD8^+$ T cells have been reported in HIV-infected patients, who remained asymptomatic (36). Thus, it is possible that the immune response elicited by the vaccination regimen used in this study did not reach the quantitative capacity necessary for tumor regression rather that not being qualitatively adequate in terms of individual T cell responsiveness.

In this study, we could not easily attribute lack of tumor regression after vaccination to anergy of circulating vaccine-elicited T cells. Although possible that anergic tumor Ag-specific T cells may coexist with growing tumor masses in naive patients, the same phenomenon does not apply in vaccine settings. Considering our recent identification of IFN-γ-producing vaccine-elicited T cell responses at tumor site (10) and the enhanced ability to induce vaccine-specific T cells from identical lesions after vaccination (41), we hypothesize that vaccine-elicited T cells coexist with tumor cells in the tumor microenvironment. Thus, we blame the lack of clinical effectiveness of MA-specific vaccines on still unknown quantitative and/or qualitative limits of the T cell response within the tumor microenvironment.

References

1. Cormier, J. N., M. L. Salgaller, T. Prevette, K. C. Barracchini, L. Rivoltini, N. P. Restifo, S. A. Rosenberg, and F. M. Marincola. 1997. Enhancement of cellular immunity in melanoma patients immunized with a peptide from MART-1/Melan A. *Can. J. Sci. Am. 3:37.*
2. Rosenberg, S. A., J. C. Yang, D. Schwartzentruber, P. Hwu, F. M. Marincola, S. L. Topalian, N. P. Restifo, E. Dufour, L. Schwartzberg, P. Spiess, et al. 1998. Immunologic and therapeutic evaluation of a synthetic tumor associated peptide vaccine for the treatment of patients with metastatic melanoma. *Nat. Med. 4:321.*
3. Marincola, F. M., E. M. Jaffe, D. J. Hicklin, and S. Ferrone. 2000. Escape of human solid tumors from T cell recognition: molecular mechanisms and functional significance. *Adv. Immunol. 74:181.*
4. Marincola, F. M., L. Rivoltini, M. L. Salgaller, M. Player, and S. A. Rosenberg. 1996. Differential anti-MART-1/MelanA CTL activity in peripheral blood of HLA-A2 melanoma patients in comparison to healthy donors: evidence for in vivo priming by tumor cells. *J. Immunother. 19:266.*
5. D'Souza, S., D. Rimoldi, D. Lienard, F. Lejeune, J. C. Cerottini, and P. Romero. 1998. Circulating Melan-A/Mart-1 specific cytolytic T lymphocyte precursors in $HLA\text{-}A2^+$ melanoma patients have a memory phenotype. *Int. J. Cancer 78:699.*
6. Pittet, M. J., D. Valmori, P. R. Dumbar, D. E. Speiser, D. Lienard, F. Lejeune, K. Fleischhauer, V. Cerundolo, J.-C. Cerottini, and P. Romero. 1999. High frequencies of naive Melan-A/MART-1-specific $CD8^+$ T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals. *J. Exp. Med. 190:705.*
7. Lee, P. P., C. Yee, P. A. Savage, L. Fong, D. Brockstedt, J. S. Weber, D. Johnson, S. Swetter, J. Thompson, P. D. Greenberg, et al. 1999. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat. Med. 5:677.*
8. Lee, K.-H., E. Wang, M.-B. Nielsen, J. Wunderlich, S. Migueles, M. Connors, S. M. Steinberg, S. A. Rosenberg, and F. M. Marincola. 1999. Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to in vitro stimulation but does not lead to tumor regression. *J. Immunol. 163:6292.*
9. Whelan, J. A., P. R. Dunbar, D. A. Price, M. A. Purbhoo, F. Lechner, G. S. Ogg, G. Griffiths, R. E. Phillips, V. Cerundolo, and A. K. Sewell. 1999. Specificity of CTL interactions with peptide-MHC class I tetrameric complexes is temperature dependent. *J. Immunol. 163:4342.*
10. Kammula, U. S., K.-H. Lee, A. Riker, E. Wang, G. A. Ohnmacht, S. A. Rosenberg, and F. M. Marincola. 1999. Functional analysis of antigen-specific T lymphocytes by serial measurement of gene expression in peripheral blood mononuclear cells and tumor specimens. *J. Immunol. 163:6867.*
11. Kern, F., I. P. Surel, C. Brock, B. Freistedt, H. Radtke, A. Scheffold, R. Blasczyk, P. Reinke, J. Schneider-Mergener, A. Radbruch, et al. 1998. T-cell epitope mapping by flow cytometry. *Nat. Med. 4:975.*
12. Bunce, M., C. M. O'Neill, M. C. Barnardo, P. Krausa, M. J. Browning, P. J. Morris, and K. I. Welsh. 1995. Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens 46:355.*
13. Player, M. A., K. C. Barracchini, T. B. Simonis, L. Rivoltini, F. Arienti, C. Castelli, A. Mazzocchi, F. Belli, and F. M. Marincola. 1996. Differences in frequency distribution of HLA-A2 sub-types between American and Italian Caucasian melanoma patients: relevance for epitope specific vaccination protocols. *J. Immunother. 19:357.*
14. Rivoltini, L., K. C. Baracchini, V. Viggiano, Y. Kawakami, A. Smith, A. Mixon, N. P. Restifo, S. L. Topalian, T. B. Simonis, S. A. Rosenberg, and F. M. Marincola. 1995. Quantitative correlation between HLA class I allele expression and recognition of melanoma cells by antigen specific cytotoxic T lymphocytes. *Cancer Res. 55:3149.*
15. Wang, Z., F. M. Marincola, L. Rivoltini, G. Parmiani, and S. Ferrone. 1999. Selective human leukocyte antigen (HLA)-A2 loss caused by aberrant pre-mRNA splicing in 624MEL28 melanoma cells. *J. Exp. Med. 190:205.*
16. Lee, K.-H., M. C. Panelli, C. J. Kim, A. Riker, M. Roden, P. A. Fetsch, A. Abati, M. P. Bettinotti, S. A. Rosenberg, and F. M. Marincola. 1998. Functional dissociation between local and systemic immune response following peptide vaccination. *J. Immunol. 161:4183.*
17. Riddell, S. R., B. A. Walter, M. J. Gilbert, and P. D. Greenberg. 1994. Selective reconstitution of $CD8^+$ cytotoxic T lymphocyte responses in immunodeficient bone marrow transplant recipients by the adoptive transfer of T cell clones. *Bone Marrow Transplant. 14(Suppl. 4):S78.*
18. Altman, J. D., P. H. Moss, P. R. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science 274:94.*
19. Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams. 1996. Real time quantitative PCR. *Genome Res. 6:986.*
20. Kruse, N., M. Pette, K. Toyka, and P. Rieckmann. 1997. Quantification of cytokine mRNA expression by RT PCR in samples of previously frozen blood. *J. Immunol. Methods 210:195.*
21. Yee, C., P. A. Savage, P. P. Lee, M. M. Davis, and P. D. Greenberg. 1999. Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramers. *J. Immunol. 162:2227.*
22. Girgis, L. M., M. M. Davis, and B. Fazekas de St. Groth. 1999. The avidity spectrum of T cell receptor interactions accounts for T cell anergy in a double transgenic model. *J. Exp. Med. 189:265.*

23. Sherman, L. A., S. V. Hesse, M. J. Irwin, D. L. Face, and P. Peterson. 1992. Selecting T cell receptors with high affinity for self-MHC by decreasing the contribution of CD8. *Science 258:815.*
24. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature 401:659.*
25. Lanzavecchia, A., G. Iezzi, and A. Viola. 1999. From TCR engagement to T cell activation: a kinetic view of T cell behavior. *Cell 96:1.*
26. Gervois, N., Y. Guilloux, E. Diez, and F. Jotereau. 1996. Suboptimal activation of melanoma infiltrating lymphocytes (TIL) due to low avidity of TCR/MHC-tumor peptide interactions. *J. Exp. Med. 183:2403.*
27. Hernandez, J., P. P. Lee, M. M. Davis, and L. A. Sherman. 2000. The use of HLA A2.1/p53 peptide tetramers to visualize the impact of self tolerance on the TCR repertoire. *J. Immunol. 164:602.*
28. Alexander-Miller, M. A., G. R. Leggatt, A. Sarin, and J. A. Berzofsky. 1996. Role of antigen, CD8, and cytotoxic T lymphocyte (CTL) avidity in high dose antigen induction of apoptosis of effector CTL. *J. Exp. Med. 184:485.*
29. Toes, R. E., R. Offringa, R. J. Blom, C. J. Melief, and W. M. Kast. 1996. Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction. *Proc. Natl. Acad. Sci. USA 93:7855.*
30. Toes, R. E., R. J. Blom, R. Offringa, W. M. Kast, and C. J. Melief. 1996. Enhanced tumor outgrowth after peptide vaccination: functional deletion of tumor-specific CTL induced by peptide vaccination can lead to the inability to reject tumors. *J. Immunol. 156:3911.*
31. Toes, R. E., S. P. Schoenberger, E. I. van der Voort, W. M. Kast, R. C. Hoeben, C. J. Melief, and R. Offringa. 1997. Activation or frustration of anti-tumor responses by T-cell-based immune modulation. *Semin. Immunol. 9:323.*
32. Savage, P. A., J. J. Boniface, and M. M. Davis. 1999. A kinetic basis for T cell receptor repertoire selection during an immune response. *Immunity 10:485.*
33. Tan, L. C., N. Gudgeon, N. E. Annels, P. Hansasuta, C. A. O'Callaghan, S. Rowland-Jones, A. J. McMichael, A. B. Rickinson, and M. C. F. Callan. 1999. A re-evaluation of the frequency of $CD8^+$ T cells specific for EBV in healthy virus carriers. *J. Immunol. 162:1827.*
34. Ogg, G. S., X. Jin, S. Bonhoeffer, P. R. Dunbar, M. A. Nowak, S. Monard, J. P. Segal, Y. Cao, S. L. Rowland-Jones, V. Cerundolo, et al. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science 279:2103.*
35. Kuroda, M. J., J. E. Schmitz, D. H. Barouch, A. Craiu, T. M. Allen, A. Sette, D. I. Watkins, M. A. Forman, and N. L. Letvin. 1998. Analysis of Gag-specific cytotoxic T lymphocytes in simian immunodeficiency virus-infected rhesus monkeys by cell staining with a tetrameric major histocompatibility complex class I-peptide complex. *J. Exp. Med. 187:1373.*
36. Gray, C. M., J. Lawrence, J. M. Schapiro, J. D. Altman, M. A. Winters, M. Crompton, M. Loi, S. K. Kundu, M. M. Davis, and T. C. Merigan. 1999. Frequency of class I HLA-restricted anti-HIV $CD8^+$ T cells in individuals receiving highly active antiretroviral therapy (HAART). *J. Immunol. 162:1780.*
37. Bieganowska, K., P. Hollsberg, G. J. Buckle, D. G. Lim, T. F. Greten, J. Schneck, J. D. Altman, S. Jacobson, S. L. Ledis, B. Hanchard, et al. 1999. Direct analysis of viral-specific $CD8^+$ T cells with soluble HLA-A2/Tax 11–19 tetramer complexes in patients with human T cell lymphotropic virus-associated myelopathy. *J. Immunol. 162:1765.*
38. Callan, M. F., L. Tan, N. Annels, G. S. Ogg, J. D. Wilson, C. A. O'Callaghan, N. Steven, A. J. McMichael, and A. B. Rickinson. 1998. Direct visualization of antigen-specific $CD8^+$ T cells during the primary immune response to Epstein-Barr virus in vivo. *J. Exp. Med. 187:1395.*
39. Ogg, G. S., D. P. Rod, P. Romero, J. L. Chen, and V. Cerundolo. 1998. High frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in autoimmune vitiligo. *J. Exp. Med. 188:1203.*
40. Romero, P., P. R. Dunbar, D. Valmori, M. Pittet, G. S. Ogg, D. Rimoldi, J. L. Chen, D. Lienard, J. C. Cerottini, and V. Cerundolo. 1998. Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes. *J. Exp. Med. 188:1641.*
41. Panelli, M. C., A. Riker, U. S. Kammula, K.-H. Lee, E. Wang, S. A. Rosenberg, and F. M. Marincola. 2000. Expansion of tumor/T cell pairs from fine needle aspirates (FNA) of melanoma metastases. *J. Immunol. 164:495.*